Fusion of Sendai virions with phosphatidylcholine-cholesterol liposomes reflects the viral activity required for fusion with biological membranes

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Sendai virus envelopes were reconstituted after solubilization of intact virions with either Triton X-100 or octylglucoside. Envelopes obtained from Triton X-100, but not from octylglucoside solubilized virions, were hemolytic and promoted cell-cell fusion. Fluorescence dequenching studies [using *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine-bearing viral envelopes] revealed that both preparations fused with negatively charged phospholipids. Fusion with phosphatidylcholine (PC)/cholesterol (chol) liposomes was promoted only by the hemolytic viral envelopes. Fluorescence dequenching studies, using intact virions bearing octadecylrhodamine B chloride, revealed that intact virions fused with PC/chol as well as with negatively charged phospholipids. Only fusion with PC/chol liposomes was inhibited by phenylmethylsulfonyl fluoride and dithiothreitol, reagents which are known to block the viral ability to fuse with biological membranes.

Liposome *Membrane fusion* *Sendai virus* *Reconstitution*

1. INTRODUCTION

Infection of animal cells by enveloped viruses belonging to the paramyxovirus group, such as Sendai virus, involves 2 successive steps: (i) virions are first attached to cell surface receptors composed mainly of sialoglycoproteins and sialoglycolipids, and (ii) the virus envelope is fused with the cell plasma membrane. Binding is mediated by the viral hemagglutinin/neuraminidase polypeptide (HN glycoprotein), while for fusion the presence of the viral fusion (F) polypeptide is required [1]. In spite of extensive research [1–3] performed during recent years, the detailed mechanism of the process of virus-membrane fusion is still obscure.

It is possible to reconstitute empty virus envelopes, containing only the 2 viral glycoproteins, i.e. the HN and F polypeptides, following solubilization of intact Sendai virions by various detergents [3]. Such reconstituted viral envelopes offer an excellent experimental tool for the elucidation of some of the as yet unknown steps in the process of virus-cell fusion and infection [2,3]. In our work [3] as well as in that of Uchida et al. [4], fusogenic viral envelopes were obtained only after solubilization of intact Sendai virions with Triton X-100 or Nonidet P-40. Solubilization of intact Sendai virions by other detergents such as deoxycholate or octylglucoside led to the formation of empty virus envelopes which although structurally resembling envelopes of intact virions, were inactive, namely, unable to promote hemolysis or cell-cell fusion [3].

We have recently established a new system to study and follow, on a quantitative basis, the fusogenic ability of reconstituted Sendai virus envelopes (RSVE) [5,6]. Using fluorescently labeled RSVE and fluorescence dequenching measurements [7], we have shown that RSVE are

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able to fuse with liposomes composed of only phosphatidylcholine (PC) and cholesterol (chol). To prove that the ability of viral envelopes to fuse with PC/chol liposomes indeed reflects the viral fusogenic activity, it was essential to show that intact virions behaved similarly to RSVE in this experimental system. Here, we show that (i) intact virions are able to fuse with liposomes composed of PC and chol. This has been demonstrated through the use of fluorescently labeled intact virions; (ii) only fusion with PC/chol liposomes and not with negatively charged phospholipids reflects the viral fusogenic activity needed for infection of animal cells; and (iii) fusion with PC/chol liposomes was obtained with envelopes reconstituted from Triton X-100 solubilized intact virions but not with envelopes obtained after solubilization of intact virions with octylglucoside.

2. EXPERIMENTAL

2.1. *Chemicals*

PC, chol, phosphatidylserine (PS), dicetyl phosphate (dihexadecyl phosphate, DCP), octylglucoside (octyl-β-D-glucopyranoside), phenylmethylsulfonyl fluoride (PMSF), trypsin (type III) and dithiothreitol (DTT) were all obtained from Sigma (USA). *N*-4-Nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (N-NBD-PE) was purchased from Avanti (USA), and octadecylrhodamine B chloride (R_{18}) was obtained from Molecular Probes (USA).

2.2. *Virus*

Sendai virus was isolated from the allantoic fluid of fertilized chicken eggs, and its hemagglutinating units and hemolytic activity were determined as described [8].

2.3. *Cells and preparation of membranes*

Human erythrocytes, type O, freshly drawn from a healthy donor, were washed 3 times with solution A (160 mM NaCl, 20 mM Tricine, pH 7.4). The washed erythrocytes were either resuspended in solution A, to give 2–3% (v/v), or processed as in [9] for the preparation of white human erythrocyte ghosts (HEG). Hepatoma tissue culture cells (HTC), clone GM22, were grown in suspension in modified Swim's 77 medium, supplemented with 10% newborn calf serum [10].

2.4. *Preparation of liposomes*

Large unilamellar vesicles of the following compositions: PC, PC/chol (1:0.5 molar ratio), PC/chol/DCP (1:0.25:0.3 molar ratio) and PS were prepared by the removal of the detergent from the octylglucoside solution of the appropriate lipids (10:1 detergent to lipid molar ratio) as described [6].

2.5. *Reconstitution of fluorescent Sendai virus envelopes*

N-NBD-PE-bearing RSVE were prepared either after solubilization of intact virions with Triton X-100 or with octylglucoside, following removal of the detergent by the direct addition of SM-2 Bio-Beads or by slow dialysis, respectively, exactly as described in [3,11]. N-NBD-PE was dissolved in the detergent solution of the viral envelopes as in [5,6], to give a viral phospholipid:N-NBD (mol/mol) ratio of 94:6. Under such conditions, the N-NBD fluorescence was self-quenched and its dequenching was shown to take place in a linear fashion [7]. The fluorescent RSVE were washed in 20 vols solution A (100000 × *g*, 30 min at 4°C) and resuspended in the same buffer, to give 0.1 mg/ml protein concentration. All RSVE preparations contained equal amounts of F and HN glycoproteins, as revealed by SDS-polyacrylamide gel electrophoresis [12] (not shown).

2.6. *Preparation of fluorescent whole Sendai virus particles*

Sendai virions were labeled with R_{18} as described [13], with slight modifications. Briefly, 7 μl of 6 mg/ml ethanolic solution of R_{18} were rapidly injected into 700 μl solution A containing 1.7 mg Sendai virus. The mixture was incubated for 15 min at room temperature in the dark. After incubation, the virions were washed in 20 vols solution A (100000 × *g* for 30 min at 4°C) and resuspended in the same buffer, to give a final protein concentration of 0.2 mg/ml. Under such conditions, R_{18} surface density in viral membranes was 3 mol% of total viral phospholipids, and its decrease was shown to be proportional to the fluorescence dequenching [13].

2.7. *Inhibition of viral fusogenic activity*

To inhibit their fusogenic activity, RSVE or Sendai virions were incubated for 30 min at 37°C with either trypsin (60 μg/mg viral protein) [14], 3 mM DTT [3], or 7 mM PMSF [14]. After incubation, the viral preparations were washed in 20 vols solution A (100000 × *g*, 30 min at 4°C) and resuspended in the same buffer.

2.8. *Fluorescence measurements*

Fluorescence RSVE or Sendai virions (0.5–1.0 μg) were incubated with liposomes or cells in a final volume of 500 μl. After incubation, the degree of fluorescence was estimated (N-NBD excitation at 469 nm, emission in 543–546 nm range; R_{18} excitation at 560 nm, emission at 590 nm). The fluorescence degree in the presence of 0.1% Ammonyx-LO (Onyx) and Triton X-100 for RSVE bearing N-NBD-PE and R_{18}, respectively, was considered to represent 100% dequenching. All fluorescence measurements were carried out with a Perkin-Elmer MPF-4 spectrofluorometer, with 520 nm high-pass filter and narrow excitation slits to reduce light scattering.

2.9. *Protein and lipid determinations*

Protein was determined by the method of Lowry et al. [15], with bovine serum albumin as a standard. Lipid concentrations were estimated by the method of Stewart [16], using PC as a standard.

3. RESULTS

The results in table 1 confirm previous observations [3,4] showing that viral envelopes reconstituted from Triton X-100 solubilized virions were hemolytic and able to promote cell-cell fusion, while those obtained using octylglucoside neither hemolysed human erythrocytes nor promoted their fusion. Trypsinization of the RSVE greatly inhibited its hemolytic activity (table 1).

To follow the processes of virus-membrane fusion using fluorescence dequenching methods [7], we have inserted the fluorescent probe N-NBD-PE into the 2 preparations of the RSVE according to previous methods [5,6]. The results in table 1 show that a relatively high degree of fluorescence dequenching was obtained only when the hemolytic reconstituted envelopes were incubated with either human erythrocyte ghosts (HEG) or HTC. It has been demonstrated [5,6] that the degree of fluorescence dequenching can be considered as a direct and quantitative measurement of the extent

Table 1

Fusogenic activity of RSVE obtained after solubilization of Sendai virions with Triton X-100 and octylglucoside: fusion with biological membranes and phospholipid vesicles

Sendai virions solubilized with	Hemolysis (%)	Degree of cell-cell fusion	Fluorescent RSVE incubated with			
			HEG	HTC	PC/chol (N-NBD-PE dequenching %)	PC/chol/DCP
Triton X-100:						
RSVE	95	+ + +	60	52	35	45
Trypsinized RSVE	8	–	3	4	5	6
Octylglucoside:						
RSVE	4	–	7	10	7	36
Trypsinized RSVE	3	–	3	4	N.D	8

N-NBD-PE-labeled RSVE (0.5 μg) from Triton X-100 or octylglucoside solubilized virions, were incubated for 45 min at 37°C with human erythrocytes (150 μl of 2–3%, v/v), HEG (200 μg), HTC (10^6 cells) and liposomes composed of PC/chol or PC/chol/DCP (250 μM in PC each). At the end of the incubation period, the degree of N-NBD-PE fluorescence dequenching was estimated as described in section 2. Hemolysis and the degree of cell-cell fusion were estimated as described [8]. (+ + +) Most (70–80%) of the cells in the population fused to form polyerythrocytes. N.D., not determined

of virus-membrane fusion. Therefore, it should be concluded that about 60 and 52% of the viral envelope present in the incubation system fuse with the HEG and HTC, respectively. On the other hand, only 7 and 10% of the non-hemolytic RSVE, namely, viral envelopes reconstituted by removal of octylglucoside, fused with HEG and HTC, respectively. It is noteworthy that both envelope preparations were able to induce a massive cell agglutination, indicating a high degree of virus-cell binding (not shown).

The results in table 1 also show that a relatively high degree (35%) of fluorescence dequenching was obtained when the hemolytic RSVE were incubated with liposomes composed of only PC and chol or of PC/chol containing 30 mol% DCP. In contrast, very little fluorescence dequenching (7%) was observed when the non-hemolytic RSVE were incubated with the PC/chol liposomes (table 1). Incubation of these non-hemolytic RSVE with negatively charged phospholipid vesicles (PC/chol/DCP), however, resulted in fluorescence dequenching (36%). Proteolytic digestion with trypsin greatly inhibited the fluorescence dequenching observed with the 2 preparations of the RSVE (table 1).

The results in fig.1 show, as demonstrated [11,13], that fluorescence dequenching methods can be used to follow fusion of intact virions, bearing R_{18}, with biological membranes. Incubation of fluorescent, intact virions with saturating amounts of HEG resulted in 40% fluorescence dequenching (fig.1A) vs only 10% fluorescence dequenching obtained in [11,14]. Furthermore, from the results in fig.1B it appears that fluorescence dequenching methods can be used to follow the fusion of intact virions with living cultured cells such as HTC. From the degree of fluorescence dequenching observed, it seems that about 40% of the virus particles in the preparation fused with either HEG or HTC. This percentage is very close to the extent of virus-membrane fusion obtained by the use of indirect methods [17] and is somewhat lower to that obtained with the hemolytic RSVE (cf. also results in tables 1 and 2).

As can be inferred from the results in table 2, very little fluorescence dequenching was observed when trypsinized, fluorescent intact virions were incubated with HEG or HTC, indicating, as expected, that the presence of intact viral glycopro-

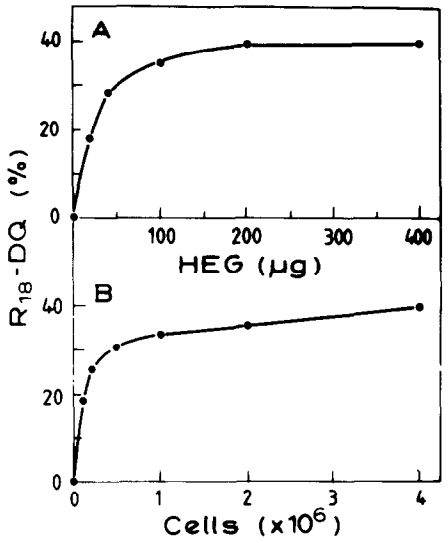

Fig.1. Fusion of Sendai virus with biological membranes: dependency on HEG and HTC concentrations. R_{18}-labeled Sendai virions (1.0 μg) were incubated for 30 min at 37°C with increasing concentrations of either HEG (A) or HTC (B). After the incubation period, the degree of R_{18} fluorescence dequenching (R_{18}-DQ) was estimated as described in section 2.

Table 2

Fusion of intact Sendai virions with biological membranes as monitored by fluorescence dequenching

Fluorescent Sendai virions treated with	Incubation with		Hemolysis (%)
	HEG (% of R_{18} dequenching)	HTC	
None	42	35	85
Trypsin	8	3	9
DTT	10	6	4
PMSF	3	6	11

Sendai virions were labeled with R_{18} and treated with trypsin, DTT and PMSF, as described in section 2. Untreated and treated fluorescently labeled Sendai virions (1.0 μg) were incubated with either HEG (200 μg), HTC (10^6 cells) or human erythrocytes (150 μl, 2–3%, v/v) for 30 min at 37°C. After the end of the incubation period, the degree of fluorescence dequenching and hemolysis was estimated as in section 2 and according to Peretz et al. [8], respectively

teins is required for the promotion of virus-membrane fusion. The results in table 2 further emphasize the view that the fluorescence dequenching observed indeed reflects the fusogenic activity of the virus, which is needed for its infectivity and promotion of cell-cell fusion. As can be seen in table 2, incubation of DTT or PMSF-treated virions with HEG or HTC resulted in a very low degree of fluorescence dequenching. It has been shown before that both treatments greatly inhibit the fusogenic activity of the virus, as DTT- or PMSF-treated virus is able neither to lyse nor infect or fuse animal cells [3,14].

Similar to RSVE (see results in table 1 and [6]), intact virions were also able to fuse with PC/chol or negatively charged liposomes, as can be inferred from the results shown in fig.2. Fusion with liposomes composed of neutral phospholipids necessitates the presence of cholesterol. Very little or no fluorescence dequenching was observed when intact virions were incubated with liposomes composed of only PC (arrow in fig.2A and B).

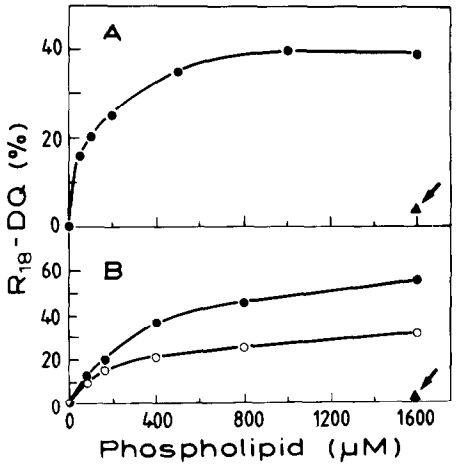

Fig.2. Effect of liposome concentration on the extent of fusion between Sendai virions and phospholipid vesicles. R_{18}-labeled Sendai virus (1.0 μg) was incubated for 45 min at 37°C with increasing concentrations of PC/chol (A, ●), PC/chol/DCP (B, ●—●) and PS (B, ○—○) liposomes. At the end of the incubation period, the degree of R_{18} fluorescence dequenching (R_{18}-DQ) was determined. Arrows indicate the degree of fluorescence dequenching obtained following incubation of fluorescent virions (1.0 μg) with the indicated concentrations of liposomes composed of only PC.

However, chol was not required to allow the fusion between Sendai virions and negatively charged liposomes such as those composed of only PS (fig.2B).

The results in table 3 show that trypsinization of fluorescent, intact virions greatly affected their ability to fuse with either liposomes composed of neutral (PC/chol) or negatively charged phospholipids. A very low degree of fusion was also observed when DTT- or PMSF-treated virions were incubated with PC/chol liposomes. As can be seen (table 3), 48% fluorescence dequenching was obtained upon incubation of non-treated virions with PC/chol liposomes vs 9% fluorescence dequenching with DTT- or PMSF-treated virions. Conversely, no decrease in the degree of fluorescence dequenching was observed when DTT- or PMSF-treated fluorescent virions were incubated with negatively charged liposomes (table 3). As can be seen from the results summarized in table 3, almost the same degree of fluorescence dequenching was obtained when untreated or DTT- and PMSF-treted virions were incubated with liposomes composed of PC/chol/DCP or PS. Thus, it seems that non-hemolytic virions are able to fuse with liposomes composed of negatively charged phospholipids but not of PC/chol.

Table 3

The ability of DTT- and PMSF-treated Sendai virions to fuse with negatively charged but not with PC/chol liposomes

Fluorescent Sendai virions treated with	Incubation with		
	PC/chol	PC/chol/DCP	PS
	(% of R_{18} dequenching)		
None	48	51	29
Trypsin	9	7	9
DTT	9	52	24
PMSF	9	51	28

R_{18}-labeled Sendai virions (1.0 μg) were incubated with liposomes (400 μM phospholipids) of the indicated compositions, for 45 min at 37°C. The degree of fluorescence dequenching was estimated as described in section 2. All other experimental conditions were as in table 2

4. DISCUSSION

Our results clearly show that intact Sendai virions are able to fuse with phospholipid vesicles lacking virus receptors, i.e. lacking sialic acid-containing components. This is deduced from the results showing that incubation of fluorescent intact virions with liposomes composed of either neutral or negatively charged phospholipids resulted in a relatively high degree of fluorescence dequenching. However, it appears that only fusion with liposomes composed of PC/chol, but not with liposomes containing negatively charged phospholipids, reflects the fusogenic activity of the virus required for allowing its penetration into animal cells. This is inferred from the results showing that non-hemolytic, namely, DTT- or PMSF-treated, virions readily fused with liposomes composed of PS or of PC/chol/DCP but not of PC/chol. This view is strengthened by the results showing that reconstituted viral envelopes obtained from octylglucoside-solubilized virions were unfusogenic and non-hemolytic, although able to fuse with liposomes containing negatively charged phospholipid molecules. On the other hand, these inactive RSVE exhibited very little, if any, fusogenic activity when incubated with PC/chol liposomes.

Fusion of Sendai virions with negatively charged phospholipids may be due to an unspecific electrostatic interaction between the viral glycoproteins and the phospholipid molecules. Instead, recent experiments in our laboratory have shown that, as opposed to fusion with PC/chol, fusion with negatively charged phospholipids does not require the involvement of the viral fusion (F) protein [18]. Fusion with PS or PC/chol/DCP occurs with membrane vesicles bearing only the viral HN glycoprotein [18]. Our present results also show that concerning the interaction with phospholipid vesicles as well as biological membranes, only RSVE obtained by extraction with Triton X-100 behaved exactly as intact virions, and therefore they can be used as an appropriate experimental system to study the mechanism of virus-membrane fusion.

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