JOURNAL OF VIROLOGY, July 1987, p. 2245–2252
0022-538X/87/072245-08$02.00/0

Vol. 61, No. 7

Reconstitution of Functional Influenza Virus Envelopes and Fusion with Membranes and Liposomes Lacking Virus Receptors

OFER NUSSBAUM, MOSHE LAPIDOT, AND ABRAHAM LOYTER*

Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Received 18 November 1986/Accepted 30 March 1987

Reconstituted influenza virus envelopes were obtained following solubilization of intact virions with Triton X-100. Quantitative determination revealed that the hemolytic and fusogenic activities of the envelopes prepared by the present method were close or identical to those expressed by intact virions. Hemolysis as well as virus-membrane fusion occurred only at low pH values, while both activities were negligible at neutral pH values. Fusion of intact virions as well as reconstituted envelopes with erythrocyte membranes—and also with liposomes—was determined by the use of fluorescently labeled viral envelopes and fluorescence dequenching measurements. Fusion with liposomes did not require the presence of specific virus receptors, namely sialoglycolipids. Under hypotonic conditions, influenza virions or their reconstituted envelopes were able to fuse with erythrocyte membranes from which virus receptors had been removed by treatment with neuraminidase and pronase. Inactivated intact virions or reconstituted envelopes, namely, envelopes treated with hydroxylamine or glutaraldehyde or incubated at low pH or 85°C, neither caused hemolysis nor possessed fusogenic activity. Fluorescence dequenching measurements showed that only fusion with liposomes composed of neutral phospholipids and containing cholesterol reflected the viral fusogenic activity needed for infection.

Reconstituted envelopes of animal virions are a convenient tool for studying the relationships between the structure of the viral envelope glycoproteins and their biological function (19). Indeed, reconstituted Sendai virus envelopes (RSVE) have been shown to be as fusogenic as intact virus particles and to be able to effectively fuse with biological membranes as well as with phospholipid vesicles (4, 19). The availability of fusogenic Sendai virus envelopes made it possible to demonstrate that, in addition to the viral fusion (F) glycoprotein, the viral binding protein, namely, the hemagglutinin-neuraminidase (HN) glycoprotein, actively participates in the virus-membrane fusion process (8). Reconstituted viral envelopes bearing only the F glycoprotein failed to fuse with liposomes or biological membranes even when the binding of the viral envelopes was mediated by a nonviral binding protein (4, 8). These studies have clearly demonstrated that virus-membrane fusion can be promoted by viral glycoproteins possessing a certain three-dimensional structure which is preserved only in envelopes possessing both the HN and F glycoproteins (5). RSVE have been also used as efficient biological carriers. Viral envelopes loaded with either polypeptides or polynucleotides have been shown to be able to fuse with the plasma membranes of cells and consequently to microinject their contents into the cytoplasm of the recipient cells (19).

Influenza virions resemble Sendai virus particles in their morphology, capability to specifically interact with sialic acid residues of membrane components, and ability to fuse with biological membranes (30). However, while Sendai virions fuse at neutral pHs, fusion of influenza virions occurs at low pHs and is promoted by the viral envelope HA glycoprotein (30). From previous studies, it appears that the binding of influenza virions to membrane receptors is mediated by the viral HA_1 polypeptide, while fusion is promoted by the viral HA_2 polypeptide, both of which are connected by disulfide bonds (30). The neuraminidase activity of this virus particle is localized on a separate polypeptide designated as the neuraminidase (NA) glycoprotein (30).

It is still not clear whether virus-membrane fusion is promoted only by the HA_2 polypeptide or whether the HA_1 polypeptide also participates in the fusion step itself. Furthermore, it is still disputable whether the NA glycoprotein is also required for virus-membrane fusion (13). Evidently, fusogenic reconstituted influenza virus envelopes (RIVE) should be of invaluable importance for the elucidation of the detailed mechanism of virus-membrane fusion and may open the possibility of using such envelopes as biological carriers.

RIVE have been previously obtained following solubilization of intact virions with octylglucoside (14). Based on virus-induced hemolysis measurments and electron-microscopic observations, it appeared that the envelopes obtained possessed some, although low, fusogenic activity (14). In our laboratory, we used Triton X-100 to solubilize intact Sendai virions. The reconstituted envelopes obtained following removal of the detergent were found to be as fusogenic as intact virions (27).

In the present study, a rapid and efficient method for the preparation of highly fusogenic influenza virus envelopes is described; it uses Triton X-100 as a detergent and, essentially, the method used for the preparation of Sendai virus envelopes (27). The fusogenic activity of the reconstituted envelopes obtained was identical to that of intact virus particles, as demonstrated by the use of fluorescence dequenching measurements (3, 10). Using fluorescently labeled intact virions and RIVE, we also showed here that both viral preparations were able to fuse with virus-receptor-depleted liposomes or, under hypotonic conditions, with erythrocyte membranes from which virus receptors had been removed by treatment with neuraminidase and pronase.

MATERIALS AND METHODS

Chemicals. Triton X-100 (scintillation grade) was obtained from Koch Light Laboratory Ltd. Neuraminidase (*Vibro*

* Corresponding author.

cholera) was obtained from Boehringwerke. Octadecylrhodamine B, chloride (R_{18}) was obtained from Molecular Probes. Pronase (*Streptomyces griseus*, type XIV), trypsin (bovine pancrease, type III), phosphatidylcholine (PC) (type V-E), cholesterol (chol), gangliosides (gang) (bovine brain, type II), and octylglucoside were obtained from Sigma Chemical Co. SM-2 Bio-beads (20/50 mesh) were obtained from Bio-Rad Laboratories.

Virus. Influenza virus (A/PR/8 strain) was isolated from the allantoic fluid of fertilized chicken eggs (15). Influenza A virus, possessing uncleaved HA (HA_0) (N virus H10N7), was a generous gift from R. Rott, Institute of Virology, Giessen, West Germany. Trypsinization of the HA_0 virus was performed by incubating 200 μg of virus with 3 μg of trypsin in a final volume of 200 μl of phosphate-buffered saline (PBS) (pH 7.4) for 20 min at 37°C essentially as described before (15). Viral hemagglutinating units and hemolytic activity were determined essentially as described previously (12). If not otherwise stated, strain A/PR/8 was used.

Preparation of RIVE. RIVE were obtained essentially as described before for the preparation of RSVE (27). Briefly, 10 mg of intact, pelleted influenza virions was solubilized with 20 mg of Triton X-100 in a final volume of 400 μl of PBS (pH 7.4). The detergent was removed from the clear supernatant obtained after centrifugation by the direct addition of SM-2 Bio-beads (27). The turbid suspension obtained was centrifuged, and the pellet containing RIVE was suspended in PBS (pH 7.4) to yield about 1.5 mg of protein per ml.

Cells. Human blood (type O, Rh^+), recently outdated, was washed three times in PBS (pH 7.4), and the final pellet obtained was suspended in PBS to 40% (vol/vol). The washed erythrocytes were desialized by treatment with neuraminidase as described before (22). Human erythrocyte ghosts (HEG) were obtained following hemolysis of the human erythrocytes with 40 volumes of 5 mM phosphate buffer (pH 8.0) (7). After three washings with the same buffer, the final pellet of white HEG was suspended in PBS (pH 7.4) to yield 4 mg of protein per ml.

Preparation of ROV. Sealed human erythrocyte right-side-out membrane vesicles (ROV) were prepared from freshly drawn human blood and treated either with neuraminidase or with neuraminidase and pronase exactly as described before (4).

Preparation of fluorescently labeled intact influenza virions and RIVE. Intact virions, HA_0 virus, and RIVE were labeled with R_{18} essentially as described before for Sendai virus (3, 10). Briefly 2 to 3 μl of a 2.5-mg/ml ethanolic solution of R_{18} was rapidly injected into 250 μl of PBS (pH 7.4) containing 1.5 mg of viral protein. After 15 min of incubation at room temperature in the dark, the viral preparations were washed with 60 volumes of PBS (Eppendorf centrifuge, 15 min). Under such conditions, R_{18} was inserted into the viral membranes at a self-quenching surface density (about 3 mol% of total viral phospholipids), and its decrease was shown to be proportional to the fluorescence dequenching.

Preparation of liposomes. Large, unilamellar vesicles of the following compositions (PC; PC-chol, 1:0.5 [mol/mol]; and PC-chol-gang, 1:0.5:0.3 [mol/mol]) were prepared by removal of the detergent from the octylglucoside solutions of the appropriate lipids (detergent-lipid, 10:1 [mol/mol]) as described before (4).

Fluorescence measurements. Fluorescent intact influenza virions or RIVE (5 μg of each) were incubated with HEG, ROV, or liposomes in a final volume of 200 μl of PBS (pH 7.4). Following 10 min of incubation at 4°C, the pH of the medium was adjusted to the desired pH by the addition of 50 μl of sodium acetate (0.5 M), and the suspension obtained was then incubated at 37°C. At the end of the incubation period, a 1-ml volume of PBS (pH 7.4) was added to the reaction mixture, and the degree of fluorescence (excitation at 560 nm, emission at 590 nm) of each sample was estimated before and after solubilization with 0.1% Triton X-100. The extent of fluorescence obtained in the presence of the detergent was considered to represent 100% dequenching, i.e., infinite dilution of the probe (3, 10). All fluorescence measurements were carried out with a Perkin-Elmer MPF-4 spectrofluorometer. Viral preparations were also incubated under the same experimental conditions in the absence of recipient membranes.

The percentage of fluorescence dequenching was calculated from the following equation (1, 2): percent of fluorescence dequenching = $\{[F - (F_t \cdot I)]/[F_t - (F_t \cdot I)]\} \times 100$, where F is the fluorescence obtained from the virus-membrane reaction mixture at the end of the incubation period, F_t is the fluorescence obtained from the same samples after solubilization with Triton X-100, and I is the fluorescence intensity of the fluorescent RIVE before incubation with the cells (zero time of incubation) divided by the fluorescence intensity of the fluorescent RIVE after solubilization with Triton X-100.

RESULTS

Structure and hemolytic activity of RIVE. The gel electrophoresis pattern seen in Fig. 1A shows that the RIVE obtained, as described in Material and Methods, contained only the viral envelope glycoproteins, namely, the NA, HA_1, and HA_2 polypeptides. This is especially clear when the polypeptide pattern of the intact virus (lanes a and d, under reducing and nonreducing conditions, respectively) is compared with that of the viral envelopes (lanes b and c). Incubation of influenza virions with Triton X-100 resulted in solubilization of only the viral glycoproteins, while the viral M and NP proteins remained insoluble and, therefore, were absent from the viral envelopes. Observations by electron microscopy (Fig. 1B and 1C) revealed that the envelopes obtained resembled in their size and appearance the envelopes of intact virions. In many of the reconstituted envelopes, a high density of spikes (Fig. 1C, arrows) was clearly visualized on their surfaces.

It appears that during the reconstitution process some of the viral envelope phospholipids were absorbed by the Bio-beads used to remove the Triton X-100 from the system. The lipid/protein (wt/wt) ratio in the reconstituted envelopes was about twofold lower than that found in the intact virions (Table 1). Since the viral M and NP proteins were missing from the reconstituted envelopes, it was expected that the lipid/protein ratio in such viral envelopes would be higher than that observed in the intact virions. However (Table 1), the addition of external lipids, up to a certain degree, did not have a significant effect on the viral hemolytic activity. Almost the same neuraminidase and hemolytic activities were obtained by the use of envelopes with lipid/protein ratios of 0.1 to 0.7. In the presence of relatively high amounts of external lipids (2,000 μg of phospholipid per system), almost complete inhibition of the hemolytic activity, without a change in the neuraminidase activity, was observed (Table 1).

The hemolytic activity of the RIVE was slightly higher than that of the intact virions (Fig. 2A) and was manifested only at low pH values, namely, pH 5.0 to 5.5 (Fig. 2B).

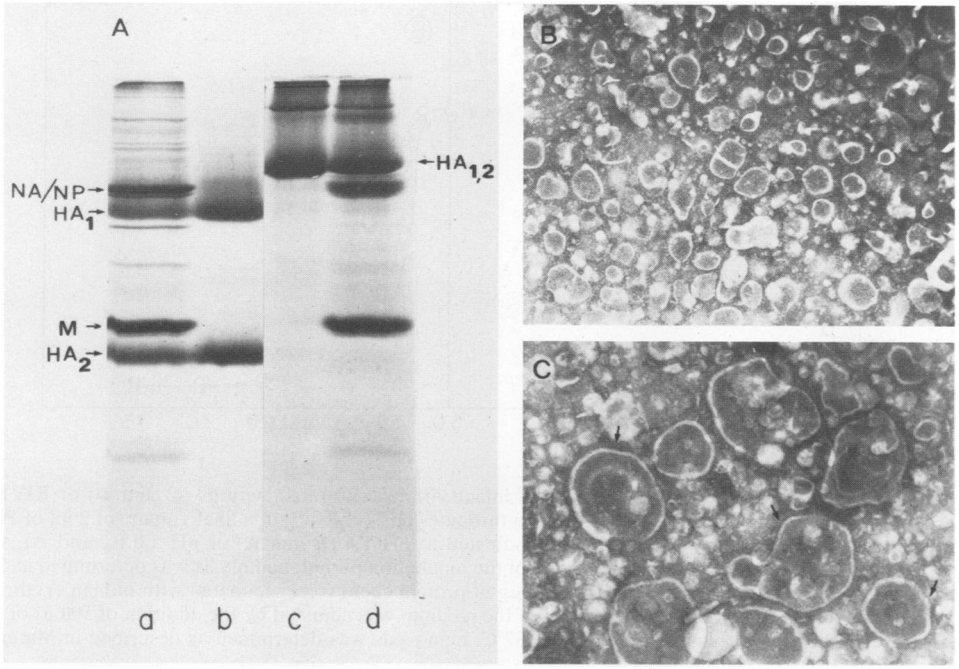

FIG. 1. Gel electrophoresis pattern and electron-microscopic observations of RIVE. Influenza virions were solubilized with Triton X-100, and empty envelopes were reconstituted following removal of Triton X-100 by the direct addition of SM-2 Bio-beads as described in Materials and Methods. (A) Intact influenza virions (lanes a and d) or RIVE (lanes b and c), 80 μg of protein each, were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% acrylamide) as described by Laemmli (16). In lanes c and d, the pattern of nonreduced viral glycoproteins can be seen, since β-mercaptoethanol was omitted from the solubilizing buffer (16). (B and C) RIVE were negatively stained and prepared for electron-microscopic observations as described before (27). Magnifications, ×16,500 in panel B and ×42,000 in panel C.

Practically no hemolysis was induced by RIVE or intact virions at pH values above 6.0 (Fig. 2B), indicating that it was induced by the activity of the viral envelope glycoproteins and not by the residual amount of Triton X-100 left in the RIVE preparations.

Fusion of intact influenza virions or RIVE with human erythrocyte membranes. The results in Figure 3 show that incubation of fluorescently labeled RIVE or intact influenza virions with HEG resulted in fluorescence dequenching. The extent of the fluorescence dequenching was dependent on the amount of the erythrocyte membranes present (Fig. 3A) as well as on the pH of the incubation medium (Fig. 3B). As for the viral hemolytic activity, the increase in the degree of fluorescence was also observed only at low pH values (Fig. 3A and B). The change in the degree of fluorescence observed following incubation of fluorescently labeled RIVE with HEG was almost identical to that obtained with intact influenza virions. It has been well established that under the conditions used, the increase in fluorescence reflects the process of virus-membrane fusion (3, 10). It appears, therefore, that the fusogenic activity of the RIVE is very similar to that expressed by intact virions.

TABLE 1. Characterization and hemolytic activity of RIVE[a]

System	Lipid added (μg)	Lipid/protein ratio (wt/wt)	Neuraminidase activity (mU/mg of viral protein)	Hemolysis (% of total) at pH:	
				7.4	5.0
Intact virions	0	0.25	150	3	50
RIVE	0	0.1	125	5	67
RIVE	400	0.6	110	7	75
RIVE	800	0.7	122	6	73
RIVE	2,000	0.85	112	5	12

[a] RIVE were prepared exactly as described in Materials and Methods. Phospholipids (PC-chol, 1:0.5 [mol/mol]; 2 mg/ml in 4% Triton X-100) at the amounts indicated were added to the detergent-solubilized virus envelopes. Triton X-100 was removed from the above-described mixture as described in Materials and Methods, keeping the ratio between the sm-2 Bio-beads added and the detergent at 1:7 (wt/wt). The hemolytic activity of the various viral preparations was estimated as described in Materials and Methods. The amount of Triton X-100 left in the RIVE was found to be 0.035% (wt/vol) by use of ^{3}H-Triton X-100. For viral neuraminidase activity, 1 U of enzyme is the amount of enzyme which hydrolyzes 1 μmol of *N*-acetylneuramin lactose to *N*-acetylneuraminic acid in 1 min at 37°C, and the activity determined exactly as described before (17).

The increase in the degree of fluorescence observed at pH 5.0 was highly dependent upon the incubation temperature, reaching a maximum at 37°C (data not shown). A much lower effect of the incubation temperature was noted upon incubation of RIVE with HEG at pH 7.4 or incubation of HA_0 with HEG at pH 5.0 and pH 7.4.

Preincubation of influenza virions at 55°C and higher caused inactivation of the viral hemolytic activity (Fig. 4A) as well as of the ability of the viruses to undergo fluorescence dequenching (Fig. 4B). The effect of the preincubation temperature on the viral hemolytic activity was almost identical to its effect on the ability of the viruses to undergo fluorescence dequenching.

The results in Table 2 show that a very low degree of hemolysis as well as of fluorescence dequenching was observed upon incubation of intact virions or RIVE with neuraminidase-treated HEG. These results clearly show that as for virus-induced hemolysis, induction of fluorescence dequenching also was mediated by virus receptors, i.e., membrane sialoglycolipids and sialoglycoproteins. A very

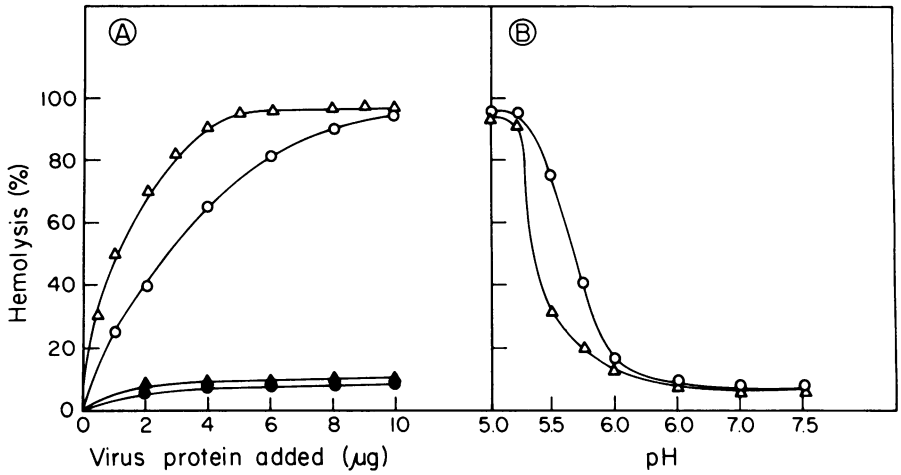

FIG. 2. Hemolytic activity of RIVE as compared with that of intact virions. (A) Intact virions (○ and ●) or RIVE (△ and ▲) at the indicated amounts were incubated for 10 min at 4°C with human erythrocytes (1% [vol/vol]) in a final volume of 2 ml of PBS (pH 7.4). At the end of the incubation period, 500 μl of sodium acetate (0.5 M), adjusted to pH 7.4 (● and ▲) or pH 5.0 (○ and △), was added, and the suspension obtained was incubated for 10 min at 37°C. At the end of the incubation period, hemolysis was determined at 540 nm as described in Materials and Methods. (B) Intact virions (○) or RIVE (△) (10 μg of protein each) were incubated with human erythrocytes as described for panel A. At the end of the incubation period (at 4°C), the pH of the medium was adjusted by the addition of 500 μl of sodium acetate (0.5 M) to the indicated pH value. Following 10 min of incubation at 37°C, hemolysis was determined as described in Materials and Methods.

low increase in the degree of fluorescence was also observed upon incubation with glutaraldehyde-treated HEG (Table 2). Treatment with glutaraldehyde has been shown to block virus-membrane fusion but not lipid-lipid exchange processes (20). It should be emphasized that such treated HEG were still able to bind influenza virions and to be agglutinated by them (data not shown). Thus, only 10 to 13% of the extent of the fluorescence dequenching observed may be due to a process of lipid-lipid exchange. The possibility that, under the conditions used, glutaraldehyde did not completely block the ability of the erthrocyte membranes to undergo fusion processes cannot be excluded.

Treatment of influenza viruses with hydroxylamine (25) or incubation of influenza viruses in a low-pH medium (24) has been shown to block the viral hemolytic and fusogenic activities. Indeed, the results in Table 3 show that such treated virions or treated RIVE were nonhemolytic. Also, virions or RIVE treated with glutaraldehyde or heated at 85°C were nonhemolytic (Table 3). A relatively low degree of fluorescence dequenching was observed upon incubation of fluorescently labeled, treated virions or RIVE with HEG (Table 3). This low degree of fluorescence dequenching may be attributable to a process of lipid-lipid exchange or to a residual fusogenic activity remaining in the treated viral preparations. It should be noted that the extent of fluorescence dequenching observed following incubation of untreated hemolytic influenza virions or RIVE at pH 7.4 was very close to that observed with treated virions at pH 5.0

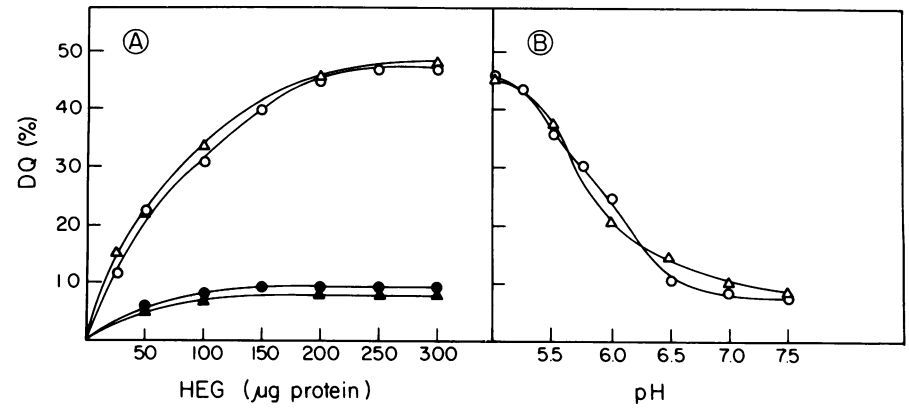

FIG. 3. Interaction of fluorescently labeled intact virions or RIVE with HEG. Labeling of intact virions or RIVE with R_{18} and preparation of HEG were as described in Materials and Methods. (A) Intact influenza virions (○ and ●) or RIVE (▲ and △) (5 μg of viral protein each) were incubated with the indicated amounts of HEG suspended in a final volume of 200 μl of PBS (pH 7.4) for 10 min at 4°C. At the end of the incubation period, the pH of the medium was either retained at 7.4 (● and ▲) or adjusted to 5.0 (○ and △) by the addition of 50 μl of sodium acetate (0.5 M) as described in the legend to Fig. 2. Following 30 min of incubation at 37°C, the extent of fluorescence dequenching (DQ) was monitored as described in Materials and Methods. (B) Intact virions (○) or RIVE (△) (5 μg of viral protein each) were incubated with 200 μg of HEG. The pH of the incubation medium was adjusted to the indicated pH values by the addition of 50 μl of 0.5 M sodium acetate. After 30 min of incubation at 37°C, the extent of fluorescence dequenching was estimated as described in Materials and Methods.

(Tables 2 and 3). It is also likely that this low degree of fluorescence dequenching may be due to a low degree of lipid-lipid exchange.

Ability of influenza virions and RIVE to fuse with liposomes or erythrocyte membranes lacking virus receptors. The results in Table 4 show that incubation of fluorescently labeled influenza virions at pH 5.0 with liposomes composed of PC-chol and bearing virus receptors, namely, sialoglycolipids (gang), resulted in a relatively high degree of fluorescence dequenching. The extent of fluorescence dequenching (36%) obtained following incubation with PC-chol-gang liposomes was only slightly lower than that observed under optimal conditions with human erythrocyte membranes (Tables 2 and 3). A relatively high degree of fluorescence dequenching (29%) was also observed following incubation at pH 5.0 with liposomes composed of PC-chol and lacking sialoglycolipids (Table 4). On the other hand, a relatively low degrees of fluorescence dequenching (9%) was obtained upon incubation with liposomes composed of only PC, indicating a requirement for chol to allow virus-liposomes fusion. A low degree of fluorescence dequenching was observed when influenza virions or RIVE were incubated with the above-described liposomes at pH 7.4 (Table 4).

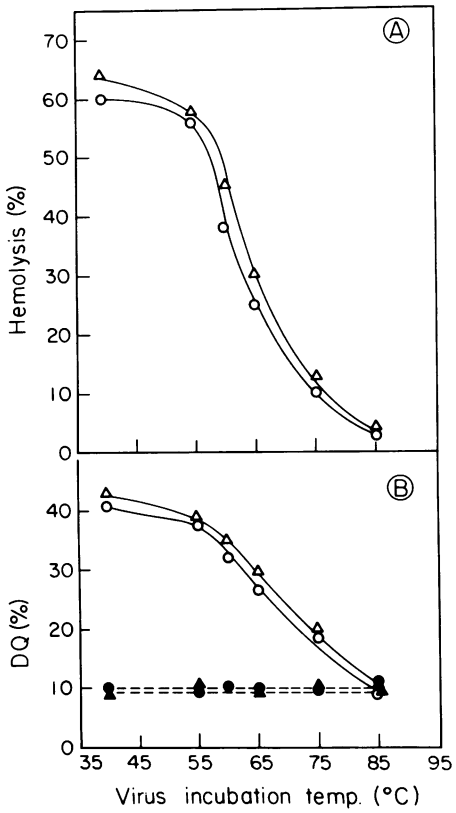

FIG. 4. Heat inactivation of the viral hemolytic and fusogenic activities. Intact virions (○ and ●) or RIVE (△ and ▲) (150 μg of protein suspended in 100 μl of PBS [pH 7.4]) were labeled with R_{18} as described in Materials and Methods and then incubated at the indicated temperatures for 30 min with gentle shaking. At the end of the incubation period, 5 μg of the treated viral preparations was incubated with human erythrocytes (1% [vol/vol]) (A) or HEG (B) at pH 7.4 (● and ▲) or pH 5.0 (○ and △). Estimation of the degree of hemolysis (A) and extent of fluorescence dequenching (DQ) (B) together with all other experimental conditions were as described in the legends to Fig. 2 and 3 and in Materials and Methods.

TABLE 2. Interaction of fluorescently labeled intact influenza virions and RIVE with neuraminidase- and glutaraldehyde-treated human erythrocytes[a]

Treatment of human erythrocytes or HEG	pH	% Hemolysis		% Fluoresence dequenching with:	
		Intact virions	RIVE	Intact virions	RIVE
None	7.4	8	6	10	9
	5.0	95	97	45	42
Neuraminidase	7.4	5	3	5	4
	5.0	9	8	14	13
Glutaraldehyde	7.4	ND[b]	ND	6	5
	5.0	ND	ND	13	12

[a] Intact human erythrocytes or HEG were treated with neuraminidase or glutaraldehyde (0.1% for 30 min at 37°C) as described in Materials and Methods and before (2). For induction of hemolysis, fluorescently labeled intact virions or RIVE (5 μg of each) were incubated with untreated or neuraminidase-treated human erythrocytes (1% [vol/vol] in 2 ml of PBS [pH 7.4]) for 10 min at pH 7.4 and then, following adjustment of the pH to 5.0, for 10 min at 37°C as described in Materials and Methods and in the legend to Fig. 2. The extent of fluorescence dequenching was monitored following incubation of 5 μg of the viral preparations with 200 μg of untreated or treated HEG for 10 min at 4°C and pH 7.4 and then for 30 min at 37°C and either pH 5.0 or pH 7.4. All other experimental conditions and estimation of the extent of fluorescence dequenching were as described in Materials and Methods and in the legend to Fig. 3.

[b] ND, Not determined.

Similarly, a low degree of fluorescence dequenching was also observed when HA_0 virions were incubated at either pH 5.0 or 7.4 with the above-described liposome preparations (Table 4). Trypsin treatment of HA_0 virions is known to activate their fusogenic activity (15). Indeed, an increase in the degree of fluorescence was observed upon incubation at pH 5.0 but not at pH 7.4 of trypsinized HA_0 virions with liposomes composed of PC-chol or PC-chol-gang. These results prove that the fluorescence dequenching observed reflects a process of virus-liposome fusion. Essentially the same results were obtained when RIVE were used (Table 4). An increase in the degree of fluorescence was observed only at pH 5.0 upon incubation of RIVE with liposomes composed of PC-chol or PC-chol-gang.

TABLE 3. Inactivation of hemolytic and fusogenic activities of intact influenza virions or RIVE[a]

Treatment of viral preparations	% Hemolysis		% Fluorescence dequenching with:	
	Intact virions	RIVE	Intact virions	RIVE
None	85	98	42	45
NH_2OH	6	8	17	13
pH 5.0	5	6	4	6
Glutaraldehyde	3	5	15	12
85°C	7	8	15	17

[a] For inactivation of the hemolytic and fusogenic activities of intact virions or RIVE, 400 μg of viral protein in a volume of 200 μl was treated as follows. For heat and glutaraldehyde inactivation, a viral suspension in PBS (pH 7.4) was incubated at 85°C for 30 min or with 0.1% glutaraldehyde for 30 min at 37°C, respectively. Inactivation by low pH was performed essentially as described before (24) by incubating a viral suspension in PBS-sodium acetate (pH 5.0) for 30 min at 37°C. For treatment with NH_2OH, a viral suspension in 1 M NH_2OH (pH 6.5) was incubated for 30 min at 37°C as previously described (25). At the end of the incubations, the viruses in the various systems were washed twice with 10 volumes of PBS (pH 7.4), resuspended in 200 μl of PBS, and labeled with R_{18} as described in Materials and Methods. The induction of hemolysis and determination of the extent of fluorescence dequenching were as described in Table 2, footnote *a*.

TABLE 4. Interaction of influenza virus preparations with phospholipid vesicles[a]

System	pH	% Fluorescence dequenching with liposomes composed of:		
		PC-chol-gang	PC-chol	PC
Intact virions	7.4	9	5	6
	5.0	36	29	9
HA_0 virus	7.4	12	11	9
	5.0	12	13	8
Trypsinized HA_0 virus	7.4	12	11	8
	5.0	35	25	9
RIVE	7.4	6	3	3
	5.0	33	27	8

[a] Fluorescently labeled viral preparations (5 μg of each) were incubated with liposomes composed of PC-chol-gang (1:0.5:0.3 [mol/mol]), PC-chol (1:0.5 [mol/mol]), or PC (200 μg) in 200 μl of PBS (pH 7.4) for 10 min at 4°C, after which 50 μl of 0.5 M sodium acetate (pH 7.4 or 5.0) was added, and the extent of fluorescence dequenching was monitored following 30 min of incubation at 37°C.

Inactivated, nonhemolytic influenza virions or RIVE failed to fuse with phospholipid vesicles. Incubation of treated, nonhemolytic (Table 3) influenza virions or RIVE with liposomes composed of either PC-chol or PC-chol-gang resulted in a relatively low degree of fluorescence dequenching (Fig. 5). Thus, it appears that influenza virions and RIVE are able to fuse almost to the same extent with liposomes and that such virus-liposome fusions do not require the presence of specific virus receptors.

The results in Fig. 6A show that incubation of RIVE under isotonic conditions with ROV resulted in a relatively high degree (35%) of fluorescence dequenching. As expected, much lower degrees of fluorescence dequenching (7 to 9%) were obtained when RIVE were incubated with ROV from which the virus receptors had been removed by treatment either with neuraminidase or with neuraminidase and pronase (Fig. 6A). On the other hand, under hypotonic conditions, an increase in the degree of fluorescence was observed following incubation with neuraminidase-pronase-treated ROV (Fig. 6A). As can be seen, about 30% fluorescence dequenching was oserved when RIVE were incubated with treated ROV in a medium of 240 mosM (Fig. 6A). A slight increase in the degree of fluorescence was also observed upon incubation with untreated ROV in a medium of low osmolarity, reaching a maximum degree at 240 mosM. A lower degree of fluorescence dequenching was noted in media of 210 to 120 mosM (Fig. 6A). This result may have been due to disruption of the ROV in media containing such low amounts of salts. Alteration of the osmolarity of the media did not have any significant effect on the degree of

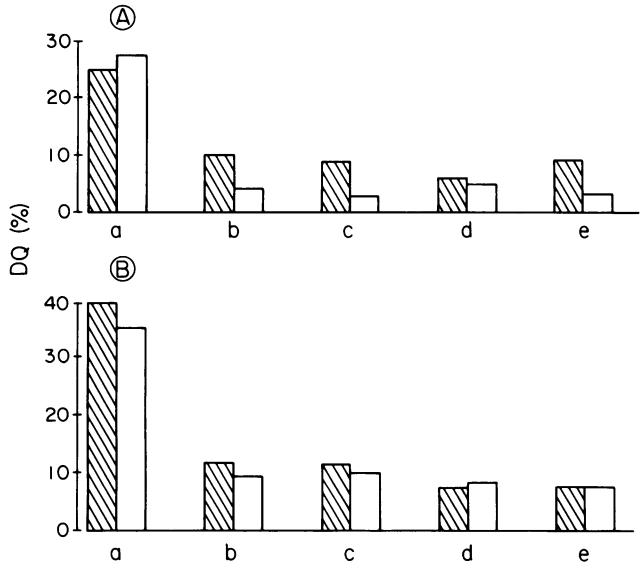

FIG. 5. Inactivation of the ability of intact influenza virus and RIVE to fuse with phospholipid vesicles. Fluorescently labeled intact influenza virions (□) or RIVE (▨) were incubated at low pH (pH 5.0) (b), incubated at 85°C (c), treated with NH_2OH (d), or treated with glutaraldehyde (e) as described in Table 3, footnote *a*. (a) Untreated intact influenza virions or RIVE. The viral preparations (5 μg of viral protein each) were then incubated with liposomes composed of PC-chol (1:0.5 [mol/mol]) (A) or PC-chol-gang (1:0.5:0.3 [mol/mol]) (B) as described in Table 4, footnote *a*. All other experimental conditions were as described in Materials and Methods. DQ, Fluorescence dequenching.

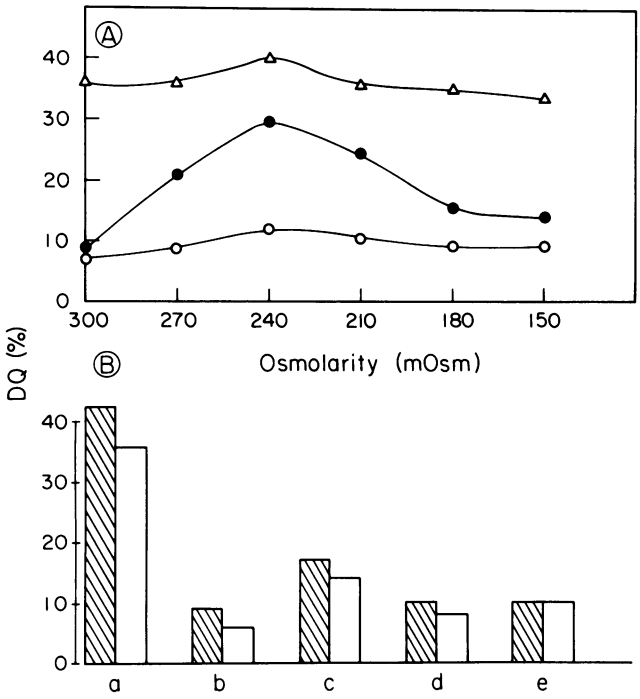

FIG. 6. Fusion of RIVE with neuraminidase-pronase-treated ROV. ROV were prepared and treated with neuraminidase or with neuraminidase and pronase as described before (4). All other experimental conditions were as described in Materials and Methods. (A) Fluorescently labeled RIVE (5 μg of protein) were added to ROV (△), neuraminidase-treated ROV (○), or neuraminidase-pronase-treated ROV (●) (200 μg of ROV protein each) suspended in 250 μl of PBS-sodium acetate (pH 5.0), and the mixtures were adjusted by the addition of water to the indicated osmolarities. Following 30 min of incubation at 37°C, the extent of fluorescence dequenching (DQ) was estimated. (B) Fluorescently labeled intact influenza virions (□) or RIVE (▧), untreated (a), treated with glutaraldehyde (b) or NH_2OH (c), or incubated at 85°C (d) or pH 5.0 (e), were incubated with 200 μg of neuraminidase-pronase-treated ROV in PBS-sodium acetate (pH 5.0), and the mixtures were adjusted to 240 mosM as described for panel A.

fluorescence obtained following incubation of RIVE with ROV treated only with neuraminidase. The degree of fluorescence dequenching remained relatively low under isotonic or hypotonic conditions (Fig. 6A). Our results showed that the increase in the degree of fluorescence obtained under hypotonic conditions (240 mosM) was observed only at pH 5.0 and was dependent upon the amount of treated ROV (data not shown), clearly indicating that the fusion observed under hypotonic conditions with virus-receptor-depleted ROV is promoted by the viral HA glycoprotein.

This view is further strengthened by results showing that incubation of inactivated, nonhemolytic RIVE under hypotonic conditions with neuraminidase-pronase-treated ROV did not result in fluorescence dequenching (Fig. 6B). Essentially the same results were obtained upon incubation of inactivated, nonhemolytic intact influenza virions with pronase-neuraminidase-treated ROV (Fig. 6B).

DISCUSSION

The results of the present study unequivocally show that fluorescence dequenching measurements can be used to follow—on a quantitative basis—fusion processes occurring between influenza virus preparations and biological membranes or liposomes. Our results showed that an increase in the degree of fluorescence was observed only upon incubation of hemolytic virions with erythrocyte membranes or liposomes at low pH values. A low degree of fluorescence dequenching (8 to 11%) was observed upon incubation of virus particles with glutaraldehyde-treated erythrocyte membranes. Glutaraldehyde-treated membranes have been shown previously (20) to be resistant to virus-membrane fusion but to allow lipid-lipid exchange processes. Based on these results, it appears that about 30 to 35% of the added virions actually fused with the erythrocyte membranes under the present experimental conditions.

RIVE have been prepared previously (11, 14, 30) mainly by the use of octylglucoside as a detergent of choice for the solubilization of intact influenza virions. However, in previous experiments in our laboratory with Sendai virus (3), it was observed that this detergent caused partial inactivation of the viral fusogenic activity. Recent results also showed that RIVE obtained by the use of octylglucoside possessed very low hemolytic and fusogenic activities (data not shown). The use of Triton X-100 for the solubilization of enveloped virions was avoided by other groups since, because of its low critical micellar concentration, it is difficult to remove from reconstitution systems (9). However, it appears that by use of the method described previously (27), namely, by the direct addition of SM-2 Bio-beads to a detergent solution of virus envelopes, most of the Triton X-100 can be removed. Furthermore, the results of the present work showed that very small amounts of Triton X-100 were left in the RIVE preparations.

The results of the present work show that RIVE obtained by use of Triton X-100 are as fusogenic as intact influenza virions. Experiments in which various inhibitors were used clearly demonstrated that the hemolytic as well as the fusogenic activities of the reconstituted envelopes were promoted by the viral glycoproteins whose presence is necessary for viral infectivity and fusion with biological membranes.

Previous studies (21, 26, 28, 29) have demonstrated that influenza virions are able to interact and fuse with liposomes composed of various phospholipids but lacking virus receptors. No specificity was observed in these studies regarding the interaction between influenza virions and phospholipid vesicles. It appeared that influenza virions were able to interact and fuse almost to the same extent with liposomes composed of either neutral or negatively charged phospholipids (21, 26). The results of the present work show that chol is required to allow fusion between influenza virions or RIVE and liposomes composed of PC. Apparently, the presence of chol is not essential for fusion of influenza virions and liposomes containing negatively charged phospholipids such as phosphatidylserine or cardiolipin (21, 26). In addition, Sendai virions were shown to fuse with liposomes composed of PC-chol but not with those composed of PC only (3, 8). It is our view that fusion with liposomes composed of neutral lipids such as PC and possessing chol, but not with negatively charged liposomes is due to the activity of the viral glycoproteins and reflects the fusogenic activity needed for fusion with biological membranes (3, 4, 6). This conclusion is supported by recent results (26), published during the preparation of the present manuscript, which demonstrated, by the use of fluorescence dequenching measurements, fusion of intact virions with HEG and with negatively charged liposomes. These authors (26) clearly showed that fusion of intact influenza virions with liposomes composed of cardiolipin does not exhibit the same features as fusion with HEG. Our results also showed that neither HA_0 virions nor inactivated, treated virions or RIVE fused with liposomes composed of PC-chol.

Fusion of intact influenza viruses or RIVE with biological membranes—as opposed to fusion with liposomes composed of PC-chol—is absolutely dependent on the presence of virus receptors, i.e., sialic acid-containing components such as sialoglycolipids or sialoglycoproteins (30). In biological membranes whose lipid bilayer is masked and unavailable to external ligands (18), a direct interaction between virus envelopes and membrane phospholipid molecules is probably impossible. It has been shown that osmotic swelling of human erythrocytes promotes exposure of their membrane lipid bilayer (18). Only phospholipid molecules of erythrocytes incubated in hypotonic media can be hydrolyzed by added phospholipases or are able to interact with external cross-linking reagents (18).

The results of the present work demonstrate that influenza virions or RIVE could fuse with virus-receptor-depleted erythrocyte membranes, provided that fusion occurred in hypotonic medium. Under such conditions, the erythrocyte lipid bilayer is probably exposed and available to interact with the influenza virus HA glycoprotein. However, it appears that even under hypotonic conditions, a large part of the membrane external glycoprotein must be removed by treatment with proteolytic enzymes such as pronase to allow interaction between the lipid bilayer and viral glycoproteins.

Influenza virions are known to fuse readily and effectively at pH 5.0 and under isotonic conditions with biological membranes or living cells bearing virus receptors. Under such conditions, the interaction of influenza virions with cultured cells promotes an increase in cell membrane permeability, cell swelling and, eventually, induction of hemolysis (23). It is conceivable that the interaction between the viral glycoproteins and their membrane receptors promotes the increase in cell membrane permeability, entry of water and, consequently, cell swelling and hemolysis. Virus-membrane fusion is suggested to occur only with membranes of such swollen cells. Hence, an active role for virus receptors is suggested in the process of virus-membrane fusion and penetration of living cells by animal virions.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Council for Research and Development, Jerusalem, Israel, and by a grant from the GSF, Munich, Federal Republic of Germany.

LITERATURE CITED

1. **Chejanovsky, N., Y. I. Henis, and A. Loyter.** 1986. Fusion of fluorescently labeled Sendai virus envelopes with living cultured cells as monitored by fluorescence dequenching. Exp. Cell Res. **164**:353–365.
2. **Chejanovsky, N., and A. Loyter.** 1985. Fusion between envelopes and biological membranes. J. Biol. Chem. **260**:7911–7918.
3. **Citovsky, V., R. Blumenthal, and A. Loyter.** 1985. Fusion of Sendai virions with phosphatidylcholine-cholesterol liposomes reflects the viral activity required for fusion with biological membranes. FEBS Lett. **193**:135–140.
4. **Citovsky, V., and A. Loyter.** 1985. Fusion of Sendai virions or reconstituted Sendai virus envelopes with liposomes or erythrocyte membranes lacking virus receptors. J. Biol. Chem. **260**:12072–12077.
5. **Citovsky, V., P. Yanai, and A. Loyter.** 1986. The use of circular dichroism to study conformational changes induced in Sendai virus envelope glycoproteins. J. Biol. Chem. **261**:2235–2239.
6. **Citovsky, V., N. Zakai, and A. Loyter.** 1986. Specific requirement for liposome-associated sialoglycolipids, but not sialoglycoproteins, to allow lysis of phospholipid vesicles by Sendai virions. Exp. Cell Res. **166**:279–294.
7. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606–2617.
8. **Gitman, A. G., I. Kahane, and A. Loyter.** 1985. Use of virus-attached antibodies or insulin molecules to mediate fusion between Sendai virus envelopes and neuraminidase-treated cells. Biochemistry **24**:2762–2768.
9. **Helenius, A., and K. Simons.** 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta **415**-29–79.
10. **Hoekstra, D., T. de Boer, K. Klappe, and J. Wilschut.** 1984. Fluorescence method for measuring the kinetics of fusion between biological membrances. Biochemistry **23**:5675–5681.
11. **Huang, R. T., K. Wahn, H. D. Klenk, and R. Rott.** 1979. Association of the envelope glycoproteins of influenza virus with liposomes: a model study on viral envelope assembly. Virology **97**:212–217.
12. **Huang, R. T. C., R. Rott, and H. D. Klenk.** 1981. Influenza viruses cause hemolysis and fusion of cells. Virology **110**: 243–247.
13. **Huang, R. T. C., R. Rott, K. Wahn, H. D. Klenk, and T. Kahana.** 1980. The function of neuraminidase in membrane fusion induced by myxoviruses. Virology **107**:313–319.
14. **Huang, R. T. C., K. Wahn, H. D. Klenk, and R. Rott.** 1980. Fusion between cell membranes and liposomes containing the glycoproteins of influenza virus. Virology **104**:294–306.
15. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza viruses by trypsin treatment. Virology **68**:426–439.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lalazar, A., D. Michaeli, and A. Loyter.** 1977. Restoration of the fusion capacity of human erythrocyte ghosts by SH-blocking reagents. Exp. Cell Res. 107:79–88.
18. **Laster, Y., E. Sabban, and A. Loyter.** 1972. Susceptibility of membrane phospholipids in erythrocyte ghosts to phospholipase C and their refractiveness in the intact cell. FEBS Lett. **20**:307–310.
19. **Loyter, A., and D. J. Volsky.** 1981. Reconstituted Sendai virus envelopes as carriers for the introduction of biological material into animal cells. Cell Surf. Rev. **8**:215–266.
20. **Maeda, T., A. Asano, Y. Okada, and S.-I. Ohnishi.** 1977. Transmembrane phospholipid motions induced by F glycoprotein in hemagglutinating virus of Japan. J. Virol. **21**:232–241.
21. **Maeda, T., K. Kawasaki, and S. Ohnishi.** 1981. Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus induced hemolysis and fusion at pH 5.2. Proc. Natl. Acad. Sci. USA **78**:4132–4137.
22. **Nussbaum, O., N. Zakai, and A. Loyter.** 1984. Membrane-bound antiviral antibodies as receptors for Sendai virions in receptor-depleted erythrocytes. Virology **138**:185–197.
23. **Patel, K., and C. A. Pasternak.** 1985. Permeability changes elicited by influenza and Sendai viruses: separation of fusion and leakage by pH-jump experiments. J. Gen. Virol. **66**: 767–775.
24. **Sato, S. B., K. Kawasaki, and S. Ohnishi.** 1983. Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments. Proc. Natl. Acad. Sci. USA **80**:3153–3157.
25. **Schmidt, M. F. G., and B. Lambrecht.** 1985. On the structure of the acyl linkage and the function of fatty acyl chains in the influenza virus hemagglutinin and the glycoproteins of Semliki Forest virus. J. Gen. Virol. **66**:2635–2647.
26. **Stegman, T., D. Hoekstra, G. Scherphof, and J. Wilschut.** 1986. Fusion activity of influenza virus. J. Biol. Chem. **261**:10966–10969.
27. **Vainstein, A., M. Hershkovitz, S. Israel, S. Rabin, and A. Loyter.** 1984. A new method for reconstitution of highly fusogenic Sendai virus envelopes. Biochim. Biophys. Acta **773**:181–188.
28. **Van Meer, G., J. Davoust, and K. Simons.** 1985. Parameters affecting low-pH-mediated fusion of liposomes with the plasma membrane of cells infected with influenza virus. Biochemistry **24**:3593–3602.
29. **White, J., J. Kartenbelk, and A. Helenius.** 1982. Membrane fusion activity of influenza virus. EMBO J. **1**:217–222.
30. **White, J., M. Kielian, and A. Helenius.** 1983. Membrane fusion proteins of enveloped animal viruses. Q. Rev. Biophys. **16**:157–195.