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Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2

(liposomes/phospholipid transfer/lysis/envelope fusion/spin label)

TOYOZO MAEDA*, KAZUNORI KAWASAKI, AND SHUN-ICHI OHNISHI

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

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ABSTRACT **The molecular mechanism of hemolysis and fusion by influenza virus in acidic media was studied. First, the effect of trypsin treatment on the activity of fibroblast-grown influenza virus was studied. The results showed that the split form of viral hemagglutinin, HA_1 and HA_2, but not the precursor, is responsible for the activity. Second, the interaction of egg-grown influenza virus, which contains the split hemagglutinin, with lipid liposomes was studied by spin labeling and electron microscopy. Phospholipid transfer from the viral envelope to the lipid bilayer membrane occurred within 30 s at pH 4.5–5.4. The transfer is largely independent of the lipid composition and the crystalline vs. liquid/crystalline state of the membrane. Virus-induced lysis of liposomes also took place rapidly in the same pH range. Envelope fusion with liposomes occurred at pH 5.2 but not at pH 7.0. These characteristic interactions were similar to those between influenza virus and erythrocytes reported previously. On the other hand, hemagglutinating virus of Japan did not interact with liposomes at neutral pH. These results suggest that protonation of the NH_2-terminal segment of the HA_2 form causes interaction of the segment with the lipid core of the target cell membrane, leading to hemolysis and fusion.**

We recently found that influenza virus shows a markedly high enhancement of hemolysis and fusion of human erythrocytes in acidic media (pH 5.0–5.4) (1). This is closely related to the observation of hemolysis and fusion in acid (pH $\leq$6.0) by togaviruses such as Semliki forest, Sindbis, and rubella (2, 3). Fusion of Semliki forest virus with lipid liposomes in acid has also been observed (4, 5). These observations have opened up a new aspect in the infection mechanism of these viruses (1, 4, 5). Virus particles are adsorbed onto the target cell membrane and phagocytized via viropexis. The particles then reach intracellular secondary lysosomes, and the low pH inside the organella triggers fusion of the viral envelope with the lysosomal membranes. The viral core materials are thus transferred into cytoplasm. On the other hand, hemagglutinating virus of Japan (HVJ, a synonym for Sendai virus) causes hemolysis and fusion at neutral pH. This virus can therefore transfer its genetic material by envelope fusion with target cell plasma membranes.

The present study was undertaken to obtain further details on the molecular mechanism of the acid-induced hemolysis and fusion by influenza virus. First, involvement of the viral hemagglutinin glycoprotein was studied by using virus grown in chicken embryo fibroblasts. It has been shown that the virus has a precursor form of hemagglutinin (HA_0) and that trypsin treatment splits the precursor into two fragments, HA_1 and HA_2, to activate infectivity 10-fold (6, 7). In influenza virus grown in eggs, a post translational modification has been performed by protease in host cells or chorioallantoic fluid. We therefore studied the hemolysis and fusion activities of influenza virus containing the precursor or the split form of hemagglutinin. Second, the interaction of the viral hemagglutinin with the lipid bilayer was studied by using liposomes. The results suggest that the split form of hemagglutinin is responsible for the hemolysis and fusion activities in acid and that lipids are the probable target site for the split hemagglutinin-induced lysis and fusion.

MATERIALS AND METHODS

Materials. Influenza virus A_0PR8 and HVJ Z strain grown in chicken eggs and their spin-labeled and radiolabeled derivatives were prepared as described (8). Influenza virus A_0PR8 was also grown in monolayer culture of chicken embryo fibroblasts. Influenza virus was sonicated to enhance hemolysis activity by a tip-type sonifier under N_2 for 90 s at 0°C (1). Virus quantity was expressed as total protein weight. The hemagglutination activities of influenza virus and HVJ were 1.3×10^5 and 6.3×10^3 hemagglutinating units per mg of protein at pH 7.0, respectively. Phosphatidylcholine (PtdCho) from egg yolk (9) and phosphatidylserine (PtdSer) from bovine brain (10) were prepared as described. Phosphatidylethanolamine (PtdEtn) and dimyristoylphosphatidylserine were synthesized by enzymatic conversion of egg yolk phosphatidylcholine and dimyristoylphosphatidylcholine, respectively, according to Comfurius and Zwaal (11). Tempocholine was synthesized as described (12). Buffers were 0.85% NaCl/20 mM NaOAc, pH 4.5–5.8; 0.85% NaCl/10 mM sodium phosphate, pH 6.0–8.0; and 0.85% NaCl/20 mM glycine·NaOH, pH 9.0–10.0.

Methods. Hemolysis of human erythrocytes was assayed spectrophotometrically at 540 mμ. Hemagglutination was assayed by using Salk's pattern method (13). Phospholipid transfer from the viral envelope to the target membranes was assayed by using spin-labeled phosphatidylcholine as described (14). Liposomes were prepared as follows. Lipids, usually PtdCho/PtdEtn/cholesterol, 2:1:1 (wt/wt), were dissolved in a small amount of benzene and dried on a glass tube wall by using a rotary evaporator and then at reduced pressure. A few glass beads and isotonic NaCl at 0.2 ml/mg of lipid were added and the mixture was spun in a Vortex. Virus-induced lysis of liposomes was assayed by using tempocholine-loaded liposomes. For the loading, liposomes were prepared in 140 mM tempocholine, in place of isotonic NaCl, and washed three times with isotonic NaCl by centrifugation for 20 min at 10,000 $\times$ *g* at 4°C. Leakage of tempocholine out of liposomes was measured by the increase in electron spin resonance peak height. The peak-height increase on complete lysis was estimated by addition of

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Abbreviations: HVJ, hemagglutinating virus of Japan; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

*Deceased January 8, 1981.

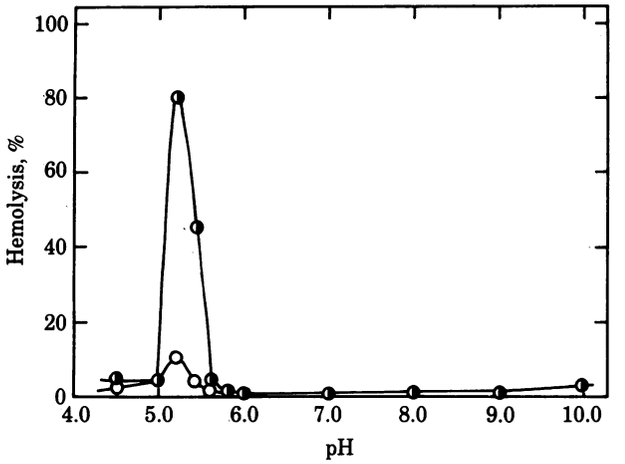

FIG. 1. Enhancement of hemolysis activity of fibroblast-grown influenza virus by trypsin treatment. Virus (200 μg/ml) was treated with trypsin (20 μg/ml; Miles–Seravac, Indiana, IN) for 15 min at 37°C in 1 mM Tris·HCl, pH 7.2, and soybean trypsin inhibitor (40 μg/ml; Miles–Seravac) was added. Treated (◑) or untreated (○) virus (15 μg/ml) was mixed with human erythrocytes, 2.5% (vol/vol), in isotonic buffer at various pH values and incubated for 30 min at 37°C. Hemolysis was determined spectrophotometrically.

2% Triton to the liposomes. Virus binding to liposomes was determined by a stepwise sucrose gradient centrifugation consisting of 1.2 ml of 10%, 2 ml of 20%, and 1.5 ml of 60% (wt/wt) sucrose in 10 mM Tris·HCl, pH 7.4. A mixture of radiolabeled virus (10 μg, 5×10^5 cps) and liposomes (0.35 μmol) was incubated for 10 min at 4°C in 0.2 ml of the appropriate buffer. Sucrose was added to the mixture to 30% (wt/wt), and it was put between the 20% and 60% sucrose layers. After centrifugation for 12 hr at 100,000 × *g* at 4°C, the top layer was assayed for radioactivity. Envelope fusion of influenza virus with liposomes was observed by electron microscopy (JEOLCO Model 100B). A mixture of egg-grown virus and liposomes was incubated for 5 min at 37°C at pH 5.2 or 7.0 and negatively stained with 2% phosphotungstate.

RESULTS

Split Hemagglutinin Is Responsible for Hemolysis and Fusion. Influenza virus harvested from chicken embryo fibroblasts caused only slight hemolysis over a wide pH range, while trypsin treatment of the virus dramatically enhanced hemolysis activity in acidic media. Fig. 1 compares hemolysis by untreated and trypsin-treated viruses. The treated virus showed marked hemolysis activity at pH 5.2–5.4. This pH profile is the same as that for egg-grown influenza virus (1). Hemagglutination activity of the virus was not influenced by trypsin treatment. Both intact and treated viruses caused hemagglutination at pH 5.0–9.0.

Fig. 2 shows the dose dependence of hemolysis at pH 5.2. The marked enhancement by trypsin treatment is again evident. Untreated virus also caused some hemolysis at higher doses. This could be due to the presence of a small fraction of activated virus in the culture preparations.

A light microscope observation showed that trypsin treatment of influenza virus activated fusion activity in acid concomitant with the appearance of the hemolytic activity. The fusion index was as high as unity (Fig. 3).

Enhancement of hemolysis and fusion activities studied as a function of trypsin concentration (Fig. 3) showed similar concentration dependences, suggesting a close connection between them. Full activation was attained with trypsin at 3–10 μg/ml;

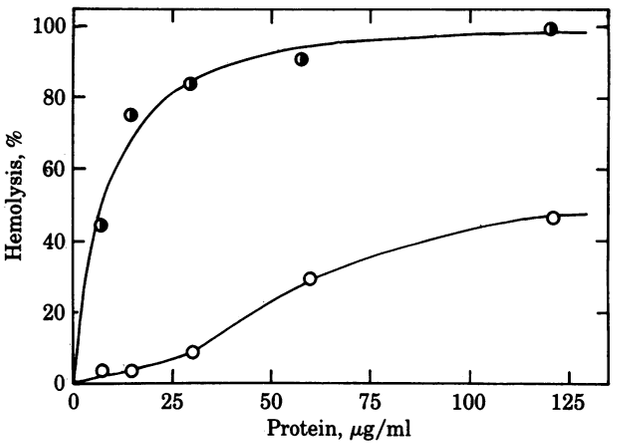

FIG. 2. Dose dependence of hemolysis by fibroblast-grown influenza virus at pH 5.2. ◑, Trypsin-treated virus; ○, untreated virus. Experimental conditions were as in Fig. 1.

the activity level remained the same at higher concentrations. Virus particles digested by various concentrations of trypsin were examined for protein composition by NaDodSO$_4$/polyacrylamide gel electrophoresis (15). The gel pattern (Fig. 4) shows that the activation is tightly coupled to the splitting of precursor HA_0 (M_r 72,000) into fragments HA_1 (M_r 50,000) and HA_2 (M_r 30,000) as it occurred at trypsin concentrations of 1–3 μg/ml.

Interaction of Influenza Virus with Liposomes. *Phospholipid transfer.* Phospholipid transfer from the viral envelope to the lipid bilayer membrane was studied by using egg-grown influenza virus labeled with spin-labeled phosphatidylcholine. When the labeled virus was mixed with liposomes at pH 5.2 and the temperature was increased to 37°C, the electron spin resonance spectrum changed almost instantaneously (<30 s). The peak height was increased by a factor of ≈3. The spectral change clearly indicates transfer of spin-labeled phosphatidylcholine from the viral envelope to the liposome membrane (14). The phospholipid transfer was not complete; complete dilution of the spin-labeled phospholipid should increase the peak height by a factor of ≈7. The rapid phospholipid transfer is similar to that from influenza virus to erythrocytes at pH 5.2. The spectral

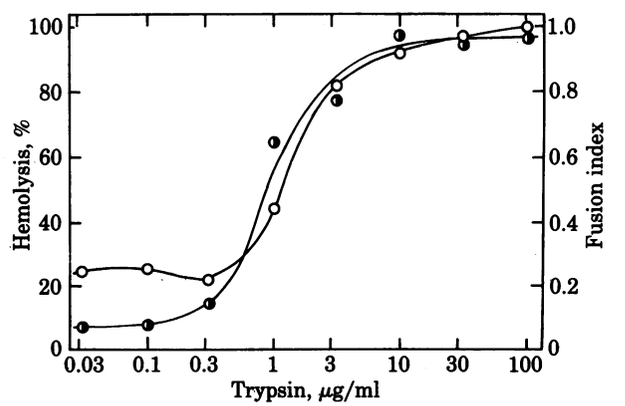

FIG. 3. Hemolysis (○) and fusion (◑) of human erythrocytes at pH 5.2 induced by fibroblast-grown influenza virus treated with various concentrations of trypsin. Virus (200 μg/ml) was treated with trypsin for 15 min at 37°C, and soybean trypsin inhibitor was added to terminate the reaction. Hemolysis was assayed as in Fig. 1. Fusion was determined by phase-contrast light microscopy after incubation of erythrocytes, 2.5% (vol/vol), with virus (60 μg/ml) for 30 min at 37°C in isotonic acetate buffer. Fusion index is defined as (total cell number before incubation/total cell number after incubation) − 1.

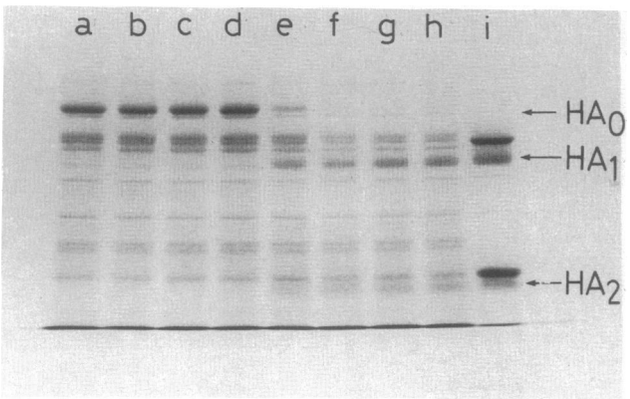

FIG. 4. Conversion of precursor hemagglutinin HA_0 into fragments HA_1 and HA_2 by treatment of fibroblast-grown influenza virus with various concentrations of trypsin. After incubation of virus (200 μg/ml) with trypsin for 15 min at 37°C and addition of trypsin inhibitor, the mixture was analyzed by 12.5% $NaDodSO_4$/polyacrylamide gel electrophoresis (15). Lanes: a–h, trypsin at 0, 0.033, 0.1, 0.33, 1, 3.3, 10, and 33 μg/ml, respectively; i, egg-grown influenza virus.

change occurred within a few minutes, and the peak height increased by a factor of ≈5 (1), indicating somewhat slower but more extensive transfer in the latter system.

The transfer from influenza virus to liposomes is activated only in acidic media (pH 4.5–5.4) (Fig. 5A). This pH dependence is the same as that of transfer to erythrocytes (1) and also of hemolysis (Fig. 1).

The phospholipid transfer was rather insensitive to liposome composition. Table 1 compares the electron spin resonance peak-height increase (factors of 2.0–2.7) after incubation of spin-labeled influenza virus with liposomes of various compositions. Cholesterol did not influence the transfer. Transfer to phosphatidylcholine liposomes was somewhat less efficient. The transfer was also rather insensitive to the physical state of the target lipid (Fig. 6). Transfer to dimyristoylphosphatidylserine at pH 5.2 was only slightly dependent on temperature in the region of the phase transition from crystalline state to liquid/crystalline state. The peak-height increase did not show any abrupt change at the transition temperature (37°C).

Binding of influenza virus to liposomes [PtdCho/PtdEtn/PtdSer/cholesterol, 2:1:1:1 (wt/wt)] was not affected by pH. The fractions of virus bound were 24% at pH 5.0, 23% at pH 5.2, 26% at pH 5.6, 27% at pH 6.0, 26% at pH 7.0, and 28% at pH 9.0.

Phospholipid transfer from the HVJ envelope to liposomes (the same composition as above) at neutral pH was negligible. Binding of HVJ to the liposomes was also negligible (3% at pH 7.0).

Lysis of liposomes. Liposomes were prepared in the presence of 140 mM tempocholine, and the external spin probe was washed out by centrifugation. Liposomes thus prepared contained ≈20 mM tempocholine inside and, because of the high concentration, showed an exchange-broadened electron spin resonance spectrum. Addition of egg-grown influenza virus to liposomes and incubation at 37°C at pH 5.2 caused a rapid change in the electron spin resonance spectrum. The peak height increased by a factor of ≈3 within 30 s and then ceased to change. This change indicates leakage of tempocholine out of the liposomes, corresponding to lysis. Lysis was not complete; complete lysis should give a 10-fold increase in peak height. The extent of lysis was dependent on virus dose, increasing with increasing amounts of virus. It became saturated at a level of 33% at a virus/lipid ratio of 33 μg/μmol. The pH dependence of lysis (Fig. 5B) was the same as that of phospholipid transfer. HVJ did not cause lysis of liposomes [PtdCho/PtdEtn/cholesterol, 2:1:1 (wt/wt)].

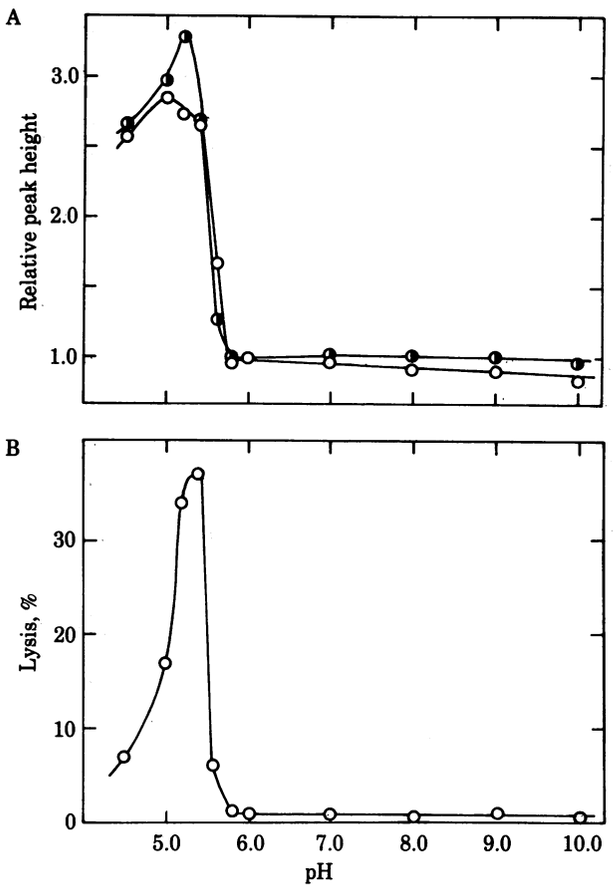

FIG. 5. (A) Phospholipid transfer from egg-grown influenza virus to liposome membranes. Virus labeled with spin-labeled PtdCho (30 μg/ml) was mixed with 1 mM liposomes at 4°C in isotonic buffer at various pH values. Electron spin resonance spectra of the mixture were recorded repeatedly at 37°C; the ratio of the peak height after 2 min to that at 0 time is plotted. (B) Lysis of liposomes induced by egg-grown influenza virus. Virus (50 μg/ml) was mixed with 1 mM tempocholine-loaded liposome at 4°C in isotonic buffer at various pH values. Electron spin resonance spectra of the mixture were recorded at 37°C repeatedly; the ratio of the peak height after 2 min to that at 0 time was measured. The extent of lysis was determined from the peak-height ratio. ○, PtdCho/PtdEtn/cholesterol, 2:1:1 (wt/wt); ●, PtdCho/PtdEtn/PtdSer/cholesterol, 2:1:1:1 (wt/wt).

Envelope fusion. In the electron micrograph of liposomes incubated with egg-grown influenza virus at pH 5.2, spike structure can be seen on the liposome surface (Fig. 7B). On the other hand, when the incubation was carried out at pH 7.0, the virus particles were observed outside the liposomes (Fig. 7A). This result indicates that envelope fusion with liposomes occurs at pH 5.2 but not at neutral pH.

DISCUSSION

Conversion of precursor hemagglutinin HA_0 into HA_1 and HA_2 activates hemolysis and fusion activity of influenza virus in acid. As the same conversion markedly increases the viral infectivity (6, 7), it can be concluded that hemolysis and fusion activity in acid are causally related to infection, supporting the infection mechanism proposed previously (1).

F protein of HVJ is synthesized as a precursor form F_0, and its splitting into F_1 and F_2 is required for hemolysis, fusion, and infectivity (16). The NH_2-terminal sequence of F_1 and those of HA_2 from various strains are similar to each other, and many

Table 1. Phospholipid transfer from influenza virus envelope to liposome membranes at pH 5.2

Liposome composition*	Electron spin resonance peak-height increase†
PtdCho	1.5
PtdCho/cholesterol (2:1)	1.9
PtdCho/PtdEtn (2:1)	1.8
PtdCho/PtdEtn/cholesterol (2:1:1)	2.5
PtdCho/PtdSer/cholesterol (2:1:1)	2.0
PtdSer	2.5
PtdSer/PtdEtn (2:1)	2.6
PtdSer/cholesterol (2:1)	2.3
PtdSer/PtdEtn/cholesterol (2:1:1)	2.7
None	1.0
PtdSer (pH 7.0)	1.0
PtdCho/PtdEtn/cholesterol (2:1:1) (pH 7.0)	1.1

Spin-labeled egg-grown influenza virus (30 μg/ml) was mixed with 1 mM liposomes at 4°C in isotonic acetate buffer and incubated at 37°C.
* Wt/wt.
† Ratio of peak height after 5 min to that at 0 time; the higher the ratio, the greater the extent of transfer; unity represents no transfer.

amino acid residues are conserved (17). A distinguished common feature of the sequence is its hydrophobicity. The terminus may therefore be an essential segment for interaction with the hydrophobic core of the target cell membrane. The F_1 terminus consists of 15 hydrophobic residues. The HA_2 terminus contains 21 hydrophobic residues interrupted by a few acidic residues; glutamate-11 and -13 (glutaminate for strain A/Japan/305/57) and aspartate-19 (18). The presence of the acidic residues may explain the markedly different pH profiles between influenza virus and HVJ (1). Protonation of the acidic residue(s) is required for hemolysis and fusion activity. HVJ, lacking acidic residues in the terminus, has these activities in a broad pH range (5.0–10.0).

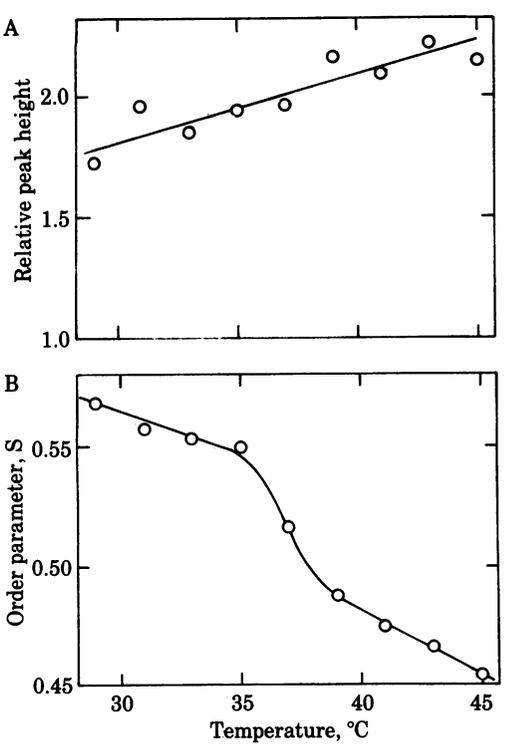

FIG. 6. (*A*) Phospholipid transfer from egg-grown influenza virus to dimyristoylphosphatidylserine liposomes at pH 5.2. Spin-labeled virus (30 μg/ml) was mixed with 1 mM liposomes in isotonic acetate buffer, incubated at various temperatures for 5 min, and electron spin resonance spectra were measured at room temperature (23°C). (*B*) Order parameter of 5-deoxylstearate in dimyristoylphosphatidylserine liposomes at pH 5.2 as a function of temperature. The phase transition temperature, indicated by the sharp change in the parameter, is 37°C.

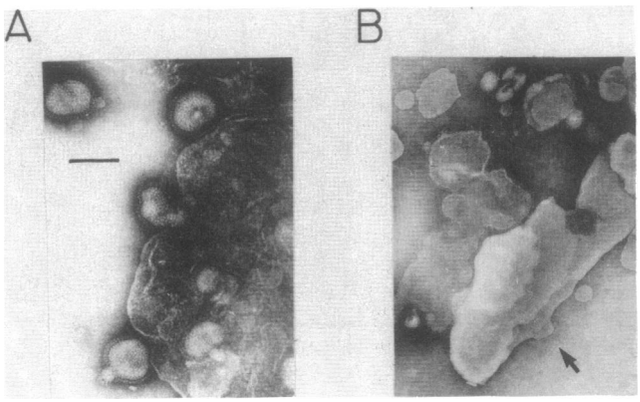

FIG. 7. Electron micrograph of liposomes fused with influenza virus. Influenza virus (50 μg/ml) was incubated for 5 min at 37°C with 1 mM liposomes [PtdCho/PtdEtn/PtdSer/cholesterol, 2:1:1:1 (wt/wt) at pH 7.0 (*A*) or pH 5.2 (*B*)]. Aliquots were negatively stained with 2% phosphotungstate and examined in an electronmicroscope. At pH 5.2, both liposomes uniformly covered with virus spikes and liposomes with spikes on a localized area (see arrow) were observed. Bar, 200 nm.

The interaction of influenza virus with liposomes mimics the essential features of the interaction with erythrocytes. Phospholipid transfer, envelope fusion, and lysis occurred in a similar manner to each other and had the same pH dependence. This similarity suggests that interaction between the HA_2 terminal segment and the lipids in the target cell membrane is an essential key step in the lysis and fusion of cells.

An obvious difference between liposomes and erythrocytes is in the receptor site for virus. Virus binds to sialoglycoprotein on the cell surface, while it directly attaches to the bilayer surface of liposomes. Some hydrophobic interaction may be involved in the virus–liposome binding as the NH_2 terminus of HA_2 contains 10 hydrophobic residues without interrupting acidic residues. This may explain the broad pH dependence of the binding. For further interaction of the terminal segment with lipids to have a significant effect, protonation of the acidic residues is necessary; phospholipid transfer, envelope fusion, and lysis occurred only in acidic media. This effect is strong; the virus can affect lipids irrespective of whether they are in the crystalline or the liquid crystalline states. Virus binding to erythrocytes also showed a broad pH dependence but, quantitatively, was different from the binding to liposomes. The hemagglutination titer at pH 5 was one-eighth that at pH 9 (1). This difference can be ascribed to pH-dependent changes of the cell receptor proteins.

Differences in the rate of phospholipid transfer can also be explained by the different binding sites. Much faster transfer to liposomes may be due to more direct contact of hemagglutinin to the lipids. The transfer was very fast but terminated after 30 s. The termination can be ascribed to the rapid inactivation of influenza virus in acid; incubation of virus at pH 5.2 for 20 s at 37°C abolished hemolysis and fusion activity in acid (unpublished results). Phospholipid transfer to erythrocytes was terminated at a higher extent. This may be due to the greater stability of the HA_2 terminus in acid when bound to the erythrocyte receptors. Virus-induced liposome lysis in acid was also very rapid but terminated at a lower level. The limited lysis may be partly due to the multilayered structure of the liposomes used.

The interaction of HVJ with erythrocytes at neutral pH was similar to that of influenza virus at pH 5.2. However, the interaction of these viruses with liposomes was markedly different. There was almost no interaction between HVJ and liposomes. This difference is due to the weak binding of the virus to liposomes. HANA proteins in the HVJ envelope may prevent F_1 from interacting with lipids as F_1 and HANA are present in equal amounts. On the other hand, HA comprises 80–90% of total envelope protein in influenza virus (19).

Semliki forest virus and influenza virus have similar activities toward erythrocytes and liposomes. Both viruses affect target membranes markedly at low pH. There was one difference, however, between the two kinds of viruses in the interaction with liposomes. Semliki forest virus requires cholesterol (5), but influenza virus does not. Semliki forest virus has three kinds of glycoproteins (E1, E2, and E3) in the envelope, whose amino acid sequence has recently been determined (20). E1 has two hydrophobic stretches near its NH_2 terminus (21–51 and 80–107), and each stretch contains four glutamate residues. The acidic residues must be responsible for the activity. However, close examination of the sequence indicates less similarity between the hydrophobic stretches of E1 and those of HA_2 and F_1. This can explain the individuality of the two classes of viruses which, however, have a common action in acid.

To firmly establish our proposed model for the infection mechanism of influenza virus, we need evidence for phagosome-lysosome fusion and transfer of genetic material by envelope fusion with the secondary lysosome membrane. Dourmashkin and Tyrrell (21) showed entry of influenza virus into cells by viropexis. Recently, we observed inhibition of influenza virus replication in MDCK cells by chloroquine, a lysosomotropic agent that is known to increase the intralysosomal pH to >6 (22) (unpublished). Inhibition of the virus uncoating and replication by adamantane amine derivatives (23–26) also supports the proposed infection mechanism. On the other hand, Huang *et al.* (27) observed fusion of reconstituted influenza virus envelope with cell membranes at *neutral* pH and proposed that envelope fusion with the cell membrane is essential for infection. We do not know the reason for the apparent discrepancy, except for the possibility of modification of the hemagglutinin proteins in the reconstitution.

Enveloped viruses must take off their coat and transfer genetic materials into cytoplasm for infection. Influenza virus (myxovirus) and Semliki forest virus (togavirus) may achieve these processes via envelope fusion with lysosome membranes (envelope fusion from within). HVJ (paramyxovirus) accomplishes these processes via envelope fusion with cell membranes (envelope fusion from without). An advantage for the former may be that it does not cause cytolysis or polykaryon formation. It is interesting to note uniformity and diversity in virus infection mechanisms that are controlled by fine differences in the NH_2-terminal hydrophobic sequences of the viral glycoproteins.

We thank S. B. Sato for electron microscopy.

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