JOURNAL OF VIROLOGY, June 1982, p. 1076–1079
0022-538X/82/061076-04$02.00/0

Vol. 42, No. 3

Demonstration of Hemolytic and Fusion Activities of Influenza C Virus

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Received 16 November 1981/Accepted 1 February 1982

Influenza C virus showed a marked hemolytic activity when incubated with murine erythrocytes at 37°C in acidic medium. The virus-specific hemolysis was most efficient at pH 5.0. Extensive cell fusion also occurred when the erythrocytes were treated with the virus at acidic pH. When propagated in MDCK cells, the virus had an extremely low infectivity and did not display hemolytic activity in any pH range. When the inactive virus was subjected to mild trypsin treatment, hemolytic activity was drastically manifested, accompanying a drastic increase in infectivity. The glycoprotein in the inactive virus was cleaved into smaller components by trypsin treatments. These results indicated that the envelope of influenza C virus can fuse with the cellular membrane under acidic conditions and that the activation of influenza C virus by cleavage was due to the appearance of this envelope fusion activity.

It was reported that liposomes containing the cleaved hemagglutinin (HA) protein of influenza A virus are able to fuse with cellular membranes; in contrast, liposomes containing the uncleaved HA protein are merely adsorbed to the cell surface (4). Recently, several workers demonstrated that influenza A virus displays high hemolytic activity and causes extensive cell fusion in acidic medium (5, 10, 11). These results suggest that influenza A virus can manifest envelope fusion activity under limited circumstances, such as in liposome or at acidic pH. The fusion activity of influenza A virus observed at acidic pH is currently thought to play an important role in the entry of the virus.

Although influenza C virus lacks neuraminidase and has an entirely different receptor from that of influenza A and B viruses (6, 13, 14), the profile of the activation of this virus by proteolytic cleavage of the viral glycoprotein is quite similar to those of influenza A and B viruses (2, 7, 9, 15). The increase in infectivity by the cleavage of viral glycoprotein is much more clearly observed in influenza C virus than in influenza A and B viruses (15). In the present study, we found hemolytic and fusion activities in influenza C virus at acidic pH and demonstrated the intimate relationship between these activities and infectivity.

MATERIALS AND METHODS

Cell and virus. MDCK cells (an established line of canine kidney cells) were grown in Eagle minimal essential medium supplemented with 10% bovine serum. The JJ/50 strain of influenza C virus was propagated in the amniotic cavity of a 9-day-old chicken egg at 33°C for 2 days. The amniotic fluid containing the virus was harvested and centrifuged at 4,000 × *g* for 30 min to remove cell debris.

Preparation of MDCK-grown virus. The JJ/50 strain of influenza C virus was first propagated in MDCK cells in the presence of trypsin as described by Nerome et al. (12). The virus thus obtained was then injected into MDCK cells at a multiplicity of 10 times the mean egg infective dose per cell and was incubated in the absence of trypsin. The propagated virus was concentrated from the culture fluid by differential centrifugations at 4,000 × *g* for 30 min and 100,000 × *g* for 30 min and by suspension of the pellet in phosphate-buffered saline (pH 7.2).

Titration of HA and assay of infectivity. HA titration was performed in a microtiter system with a 1% suspension of chicken erythrocytes. The infectivity was assayed in the MDCK cell cultures by the microscopic counting of hemadsorption-positive cells 3 days after virus infection and was expressed as cell infectious units.

Buffers. For buffers, we used 0.02 M acetate-buffered saline from pH 4.8 to 5.6 and 0.02 M phosphate-buffered saline from pH 5.8 to 7.2.

Antiserum. The mice were injected intraperitoneally with the JJ/50 strain of influenza C virus three times at weekly intervals and were bled 2 weeks after the last injection.

Polyacrylamide gel electrophoresis. The analysis of viral proteins was performed by polyacrylamide gel electrophoresis as described by Laemmli (8). Viral glycoproteins were stained by the periodic acid-Schiff technique as described by Zacharius et al. (16).

RESULTS

Demonstration of hemolytic activity of influenza C virus at acidic pH. Aliquots (0.1 ml) of virus suspensions with HA titers of 1:256 were adsorbed to 1-ml portions of a 2% murine erythro-

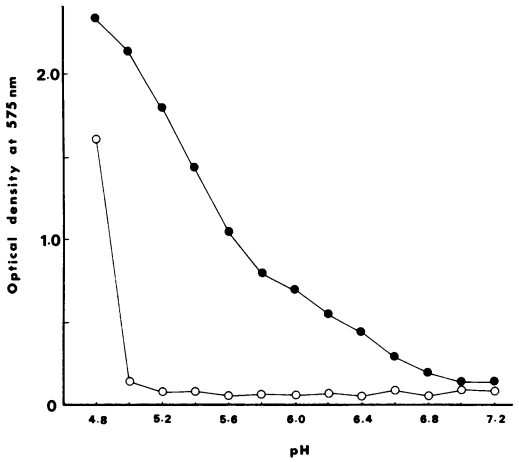

FIG. 1. pH dependency of hemolysis by influenza C virus. Aliquots (0.1 ml) of virus with an HA titer of 1:256 were adsorbed to 1-ml portions of a 2% murine erythrocyte suspension for 10 min at 0°C. After low-speed centrifugation, the supernatants were replaced with 1-ml portions of buffered salines of pH 4.8 to 7.2. Hemolysis was measured after incubation for 10 min at 37°C and expressed as the optical density at 575 nm. Symbols: ●, virus-specific hemolysis; ○, hemolysis of mock-treated erythrocytes.

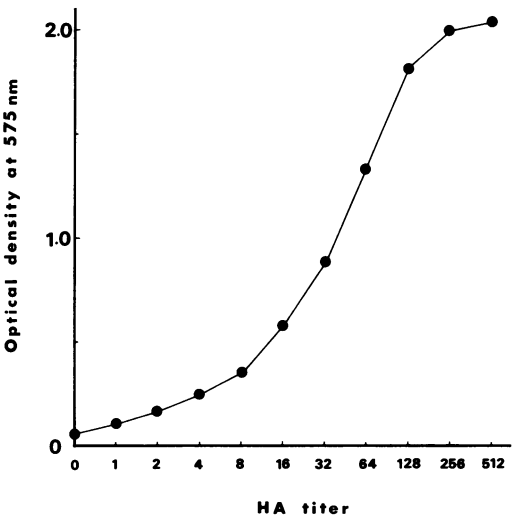

FIG. 2. Hemolysis as a function of virus concentration. Serial dilutions of influenza C virus were adsorbed to murine erythrocytes and incubated for 10 min at 37°C and pH 5.0. Procedures were as described in the legend for Fig. 1.

cyte suspension at 0°C for 10 min. After centrifugation of the mixtures at 2,000 rpm for 15 s, the supernatant fluids were replaced with 1-ml portions of buffered salines of pH 4.8 to 7.2, and the erythrocytes were suspended gently and incubated at 37°C for 10 min. After low-speed centrifugation of the suspension, the optical density of the supernatant fluid was measured at 575 nm. The virus exhibited a high hemolytic activity at acidic pH but none at neutral pH (Fig. 1). The virus-specific hemolysis was most evident at pH 5.0. The hemolytic activity was completely inhibited by pretreatment of the virus with antiviral serum (data not shown). No hemolysis occurred in any pH range at 0°C (data not shown), indicating the temperature dependency of the hemolytic activity. At pH lower than 5.0, the erythrocytes spontaneously caused lysis. Therefore, the hemolysis by the virus was performed at pH 5.0 in the following experiments.

The dose response of hemolytic activity was next examined in relation to HA titers. The extent of hemolysis closely correlated with the HA titer of the virus (Fig. 2). The fact that the hemolysis was observed even with the lowest HA titer indicates that the hemolytic activity of the virus was highly efficient.

Erythrocytes from several species of animals were examined for their sensitivity to hemolysis caused by the virus. The experimental conditions were identical to those described above. The most efficient hemolysis was observed with murine erythrocytes (Table 1). Although the human B and chicken erythrocytes were well agglutinated by the virus, they showed only a slight hemolysis.

Fusion of erythrocytes by influenza C virus. A portion (1 ml) of a 2% murine erythrocyte suspension was incubated with 0.1 ml of virus suspension with an HA titer of 1:512 for 3 min at pH 5.0 and 37°C. After incubation, the erythrocytes were sedimented by low-speed centrifugation and observed under a microscope. Extensive cell fusions were observed at pH 5.0 (Fig. 3A). On the other hand, no cell fusions were observed in erythrocytes treated with the virus at neutral pH (Fig. 3B). This result indicated that the envelope of influenza C virus can fuse with cellular membrane under acidic conditions. It is likely that the hemolysis was caused by this fusion.

TABLE 1. Hemolysis of various erythrocytes by influenza C virus[a]

Erythrocyte	Hemolysis (OD[b] at 575 nm)
Mouse	1.97
Human[c]	0.10
Chicken	0.05

[a] Aliquots (0.1 ml) of influenza virus with an HA titer of 1:256 were incubated with 1-ml aliquots of a 2% erythrocyte suspension for 10 min at 37°C and pH 5.0.
[b] OD, Optical density.
[c] Type B erythrocyte.

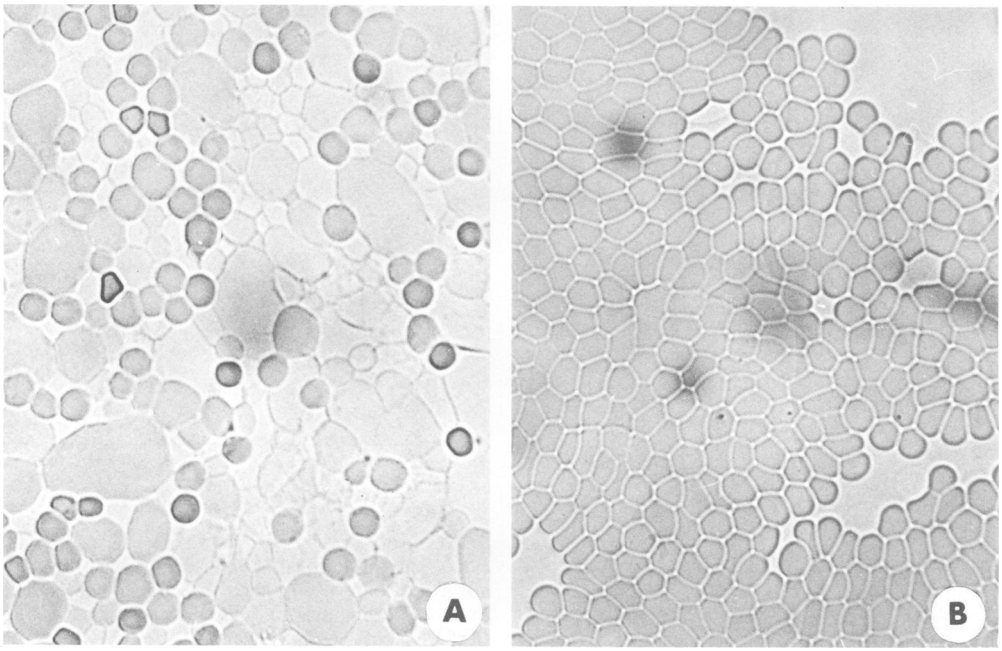

FIG. 3. Cell fusion of erythrocytes treated with influenza C virus. Influenza C virus was adsorbed to murine erythrocytes at 0°C, and then the erythrocytes were incubated at 37°C for 3 min at (A) pH 5.0 and (B) pH 7.2. Magnification, ×400.

Relationship between the hemolytic activity and infectivity. Influenza C virus grown in MDCK cells shows an extremely low infectivity, but by a mild trypsin treatment, the inactive virus is converted to a highly infectious form (15). The next experiment was performed to examine the relationship between the infectivity and the envelope fusion activity of the virus, using the hemolytic activity as a parameter. A suspension of MDCK-grown virus, prepared as described above, was divided into two groups, and one of them was treated with 20 μg of trypsin per ml at 37°C for 10 min. The reaction was stopped with soybean trypsin inhibitor. The other group was mock treated for a control. A portion was taken from each group to assay the HA titer, hemolytic activity, and infectivity. The remaining portion of each group was ultracentrifuged at 100,000 × *g* for 30 min to sediment the virus, and the pellet was used to analyze the constituent polypeptides by polyacrylamide gel electrophoresis (Table 2 and Fig. 4). The noninfectious virus did not display hemolytic activity, but after the treatment with trypsin, high hemolytic activity and a drastic increase in infectivity appeared. Polyacrylamide gel electrophoresis of the viral proteins revealed that gp88 was cleaved into smaller subunits, gp65 and gp30, by the trypsin treatment. These results indicated that the manifestation of hemolytic activity requires the cleavage of viral gp88 and that the activity is closely correlated with infectivity.

DISCUSSION

The cleavage of viral gp88 may be necessary for influenza C virus to manifest infectivity (2, 15). The present study demonstrated the intimate correlation between infectivity and hemolytic activity. It is likely that the activation of influenza C virus by the cleavage was due to the appearance of envelope fusion activity. Since the fusion activity of influenza C virus is exhibited only under acidic conditions, we speculate that the pathway of virus infection is as follows. The virus is taken into the cell via phagocytosis, and then the phagosome fuses with primary lysosomes. Under the acidic conditions of the lysosome, the virus displays envelope fusion activity, resulting in the release of the nucleocapsid into the cytoplasm by fusion of the viral envelope with the lysosomal membrane. This hypothesis was originally proposed by Helenius et al. (1) for Semliki Forest virus infection, and Maeda and Ohnishi (11) have proposed the same pathway for influenza A virus infection. Further studies may be necessary to determine whether the viral envelope actually fuses with lysosomal membranes and whether the viral antigens can be detected in lysosomal membranes.

Our preliminary results indicated that viral

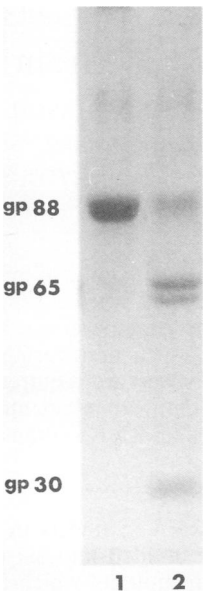

FIG. 4. Glycoprotein composition of MDCK-grown virus before and after activation with trypsin treatment. The suspension of purified influenza C virus was divided into two parts. One of them was treated with 20 μg of trypsin per ml at 37°C for 10 min, and the other was mock treated. Both portions were pelleted by ultracentrifugation and electrophoresed on a 10% polyacrylamide slab gel. Viral glycoproteins were stained by the periodic acid-Schiff technique. Lane 1, mock treated; lane 2, treated with trypsin.

gp88 (and also its cleavage products gp65 and gp30) carries HA activity (unpublished data). Recently, Herrler et al. (3) demonstrated the same conclusion from the analysis of glycoprotein solubilized with octylglucoside. Nevertheless, it is not evident whether the receptor-destroying activity is carried by the same protein as HA activity or by another protein. We have not yet succeeded in separating the activities.

TABLE 2. Enhancement of the biological activities of MDCK-grown influenza C virus by trypsin treatment[a]

Treatment	HA titer	Hemolysis (OD[b] at 575 nm)	Infectivity (CIU[c]/ml)
None	1:128	0.08	2.0×10^4
Trypsin	1:128	2.09	1.2×10^7

[a] The treatment was done with 20 μg of trypsin per ml for 10 min at 37°C.
[b] OD, Optical density.
[c] CIU, Cell infectious units assayed on MDCK cell cultures.

Further detailed analysis of viral glycoprotein function is in progress in our laboratory.

It remains to be resolved why human and chicken erythrocytes scarcely cause hemolysis. Although these erythrocytes are well agglutinated by influenza C virus, the number of receptor sites on these cells appears to be very much fewer than that on murine erythrocytes (unpublished data). The density of receptor sites on the cell surface may considerably affect the efficiency of envelope fusion. Further analysis of this problem may provide a clue for the elucidation of the fusion mechanism of the virus.

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