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Proteolytic Activation of Hemolysis and Fusion by Influenza C Virus

Brief Report

By

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With 1 Figure

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Summary

Influenza C virus has been found to cause pH-dependent hemolysis and fusion of chicken erythrocytes. For these activities, treatment of the virus with proteolytic enzymes, e.g., trypsin and elastase which were known to cause cleavage of gp88 was specifically required.

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It seemed established in paramyxoviruses that virus-induced hemolysis and cell fusion are caused by fusion of the viral envelope with cell membrane (1, 4). Recently, it has also been shown that influenza A and B viruses (3, 6, 7, 10, 15), Semliki Forest virus (6, 12—15), vesicular stomatitis virus (15), Sindbis virus (12, 13), rubella virus (13) and rhabdovirus (9) caused hemolysis and fusion of cells in acidic conditions. Among them, proteolytic activation of the hemagglutinin glycoprotein was shown to be required for influenza A and B viruses to induce these activities (3, 10, 15). No information has been available, however, about hemolysis and cell fusion of influenza C virus.

We have previously shown that proteolytic enzymes, trypsin and elastase, enhanced the infectivity of influenza C virus by cleaving a large glycoprotein, gp88, into subunit glycoproteins gp65 and gp30 whereas chymotrypsin and thermolysin did not (11). In this paper, we describe the proteolytic activation of hemolysis and fusion activities of influenza C virus, the phenomenon being associated with the activation of the infectivity.

The JJ/50 strain of influenza C virus was grown in MDCK line of canine kidney cells and purified as described before (11). This virus has a glycoprotein entirely in uncleaved form (11) and exhibits none of the hemolytic and cell fusion

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activities. The virus was then subjected to a treatment with 20 μg/ml each of the proteolytic enzymes listed in Table 1, in the same manner as described before (11) except that a phosphate-buffered saline (PBS) at pH 7.0 was used as a reaction buffer. The WSN strain of influenza A virus, grown in embryonated eggs and purified by differential centrifugations, was used for reference. Hemolysis studies were carried out in a saline buffered with 10 mM MES [2-(morpholino)ethanesulfonic acid], at the pH range between 5.00 and 7.25. Within this pH range, spontaneous hemolysis did not occur. This buffer system was preferable to acetate buffer which had been used for influenza A and B viruses (3, 7, 10), because in the acetate buffer spontaneous hemolysis occurred in the pH range from 5.00 to 5.75 (7).

Table 1. *pH-Dependent hemolysis induced by influenza C and A viruses*[a]

Viruses	Treatment with	Optical density at 540 nm, at pHs									
		5.00	5.25	5.50	5.75	6.00	6.25	6.50	6.75	7.00	7.25
C/JJ/50[c]	None	0[b]	0	0	0	0	0	0	0	0	0
	Trypsin	0.78	0.65	0.50	0.42	0.37	0.32	0.29	0.25	0	0
	Chymotrypsin	0	0	0	0	0	0	0	0	0	0
	Thermolysin	0	0	0	0	0	0	0	0	0	0
	Elastase	0.62	0.50	0.45	0.27	0.22	0.17	0.14	0.10	0	0
A/WSN[d]	None	0.88	0.48	0.31	0.19	0.11	0	0	0	0	0

[a] A 200 μl of the virus suspension at 128 HAU/ml was added to 1 ml of 2 per cent chicken erythrocytes in PBS at pH 7.0, and incubated at 0° C for 30 minutes. The mixtures were centrifuged and the pellets were suspended in 1 ml of saline buffered with 10 mM MES of various pHs and incubated at 37° C for 60 minutes. The mixtures were then centrifuged and the supernatants were measured for the optical density at 540 nm

[b] The values less than 0.10

[c] MDCK cell-grown virus

[d] Embryonated egg-grown virus

As shown in Table 1, hemolysis was caused by the reference strain of WSN and by influenza C viruses treated with either trypsin or elastase, but neither with chymotrypsin nor with thermolysin. This spectrum of the protease for activation of the hemolysis was the same as that for activation of the infectivity in which the cleavage of gp88 into gp65 and gp30 occurred (11). In contrast with paramyxoviruses, the hemolysis caused by many strains of influenza A and B viruses was shown to be pH-dependent (3, 6, 7, 10). We confirmed the above observation with the reference strain of WSN and furthermore found that the hemolysis caused by the proteolytically activated influenza C virus was also pH-dependent, though the latter pH was somewhat wider than the former one. In the course of hemolysis study, we found that influenza C virus was able to fuse chicken erythrocytes. Then we attempted to determine whether the protease treatment was also prerequisite to this phenomenon. Influenza C virus was treated with the various proteases as described above. A 200 μl of the virus suspension at 256 HAU/ml in PBS at pH 7.0 was added to the pellet of chicken erythrocytes with 4×10^6 cells and mixed. After adsorption for 30 minutes at 0° C, the mixtures

were centrifuged and the pellets were suspended in the saline buffered with 10 mM MES of various pHs, containing 2 mM $CaCl_2$ and 0.2 per cent (w/v) bovine serum albumin, the latter of which was included to prevent colloid osmotic hemolysis (2, 5). The suspensions were then incubated at 37° C for 20 minutes, and examined for the fusion under the microscope at a magnification of ×200. Some of the representative patterns of the interactions between viruses and erythrocytes are shown in Fig. 1. With viruses treated with either trypsin or elastase, fused chicken erythrocytes with multiple nuclei appeared within 10—20 minutes after incubation at 37° C (Fig. 1 a). The amount of virus necessary to detection of the cell fusion was over 30 HAU/4×10^6 cells in our experimental conditions. The pH range for the cell fusion was very limited to between 5.00 and 5.25. Furthermore, we noticed that each of the cells became rounded and swollen prior to the cell fusion (Fig. 1 b). This occurred at rather wide pHs ranging from 5.00 to 6.00. Though the mechanism involved in this phenomenon has not been fully studied, this might be a reflection of the fusion between the viral envelope and cytoplasmic membrane of the chicken erythrocytes. At pHs

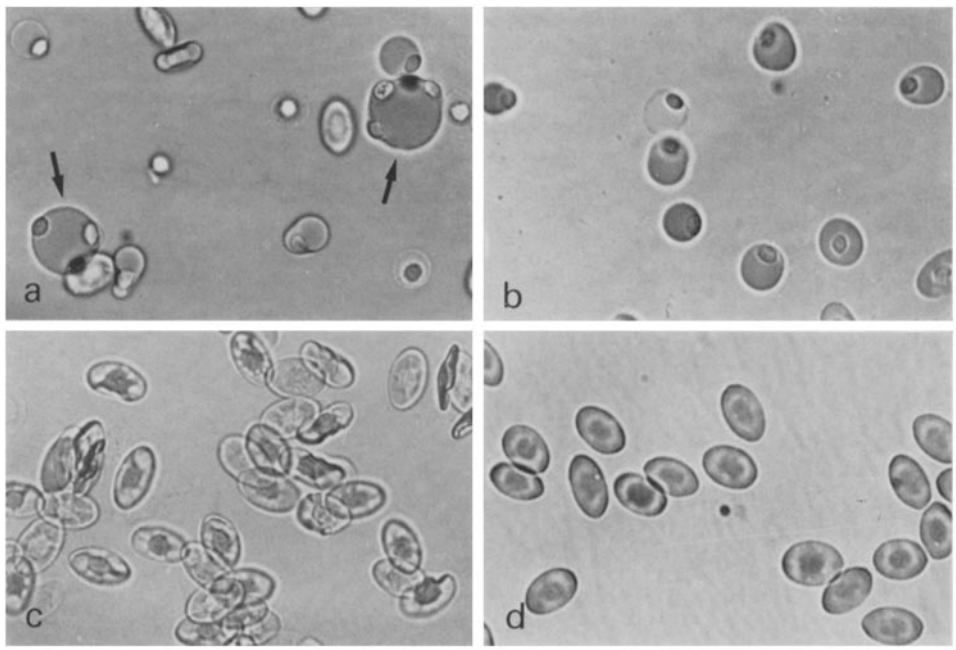

Fig. 1. Interactions between influenza C virus and chicken erythrocytes. The C/JJ/50 strain grown in MDCK cells was used after treatment with 20 μg/ml of trypsin as described in the text. A 200 μl of the virus suspension at 256 HAU/ml was mixed with 4×10^6 cells in PBS at pH 7.00. After adsorption for 30 minutes at 0° C, the mixtures were centrifuged and the pellets were resuspended in 100 μl of the saline buffered with 10 mM MES of various pHs, containing 2 mM $CaCl_2$ and 0.2 per cent (w/v) bovine serum albumin. The suspensions were then incubated at 37° C for 20 minutes and examined for the fusion under the microscope. *a* Cells after incubation at pH 5.00, showing fused cells (arrows) with multiple nuclei. *b* Rounded and swollen cells, observed after incubation at pH 6.00. *c* Aggregated cells, observed after incubation at pH7.00. *d* Control cells without virus. Incubation at pH 5.00. Magnification ×200

over 6.25, only aggregated cells were observed (Fig. 1c). Neither rounding and swelling of the cells, nor fusion of the erythrocytes was seen with viruses treated with chymotrypsin or thermolysin (data not shown).

The overall results showed that treatment with special proteolytic enzymes, e.g., trypsin and elastase, was necessary for influenza C virus grown in MDCK cells to cause hemolysis and fusion of the chicken erythrocytes. This spectrum of protease action was very similar to the one observed for the restoration of the infectivity (11). Since proteolytic cleavage of gp88 into gp65 and gp30 was shown to be required for restoration of the infectivity of influenza C virus, the cleavage of gp88 may also be responsible for hemolysis and fusion activities of this virus as was the case with HA glycoprotein of influenza A and B viruses (3, 10, 15).

Our present results on influenza C virus together with others on influenza A and B viruses (3, 6, 7, 10, 15), now reveal that acidic pH-dependent hemolysis and cell fusion are common feature of the family Orthomyxoviridae. However, whether hemolysis and fusion activities of influenza virus are necessary requisite for the infection to proceed in a physiological condition in the cell is not known at present. MAEDA *et al.* (7) and MATLIN *et al.* (8) suggested that influenza A virus is phagocytized into phagosomes and reaches the secondary lysosomes, and then the acidic environment in these organelles activates fusion of the phagocytized influenza virus with the lysosomal membrane, resulting in the transfer of influenza virus genetic material to the cytoplasm. This view, however, remains to be verified.

Acknowledgments

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