ACTIVATION OF INFLUENZA VIRUS BY ACIDIC MEDIA CAUSES HEMOLYSIS AND FUSION OF ERYTHROCYTES

Toyozo MAEDA and Shun-ichi OHNISHI
Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan.

Received 27 October 1980

1. Introduction

Although myxoviruses share many biochemical and morphological features with paramyxoviruses, their infection mechanism is not completely elucidated. It is established that paramyxoviruses penetrate the plasma membrane via envelope fusion [1], eventually inducing lysis and cell fusion. These activities have not been reported for influenza virus. In [2] morphological evidence appeared for envelope fusion of influenza virus with the cell membrane, but in [3] an infection mechanism via viropexis was suggested.

In [4,5] infectivity of influenza virus harvested from tissue culture cells was greatly enhanced when influenza virus HA protein was split into two fragments by trypsin. In [6] the amino-terminal region formed by proteolysis was found to be similar to the hydrophobic sequence of the F protein of hemagglutinating virus of Japan (HVJ) (also known as Sendai virus). The F protein is involved in the envelope fusion, hemolytic, and fusion actions of HVJ [7]. These activities may be caused by direct interaction of the amino-terminal hydrophobic sequence with hydrophobic regions of the target cell membrane. The HA protein of influenza virus may function as the F protein of HVJ does in certain conditions, causing hemolysis and cell fusion. Here, we found sonicated virus incubated with cells in acidic medium of pH 5.2 caused high hemolytic and fusion activities.

2. Materials and methods

Influenza virus (A_0PR8 and WSN), HVJ(z) and their spin-labeled derivatives were prepared as in [8]. Sonication of influenza virus was carried out for 90 s at 0°C under a nitrogen stream by a tip-type sonifier (Kaijo Denki Co., T-A-4201) with a setting of 100 mA. Buffers contained 0.85% (w/v) NaCl in addition to 20 mM sodium acetate for pH 4.5–5.8, 10 mM sodium phosphate for pH 6.0–8.0 and 20 mM glycine–NaOH for pH 9.0–10.0. Protein concentration was determined by Lowry's method [9] and hemagglutination assay was carried out using Salk's pattern method [10]. Hemagglutination activity/mg virus protein was 1.3×10^5 for A_0PR8, 3.0×10^4 for WSN and 6.3×10^3 for HVJ(z). Hemolysis was assayed spectrophotometrically at 540 nm. The spin-label assay method for phospholipid transfer was described in [8].

3. Results

3.1. *pH profile of hemagglutination*

Hemagglutination activity of influenza virus and HVJ was measured in various buffers from pH 4.5–10.0. Both viruses aggregated chicken erythrocytes over a wide pH range (pH 7.0–10.0) as shown in fig.1. The activity of influenza virus decreased below pH 6.0 and increased slightly in alkaline pH. HVJ was active over a wider pH range with slightly smaller aggregation activity in alkaline pH. The molecule cause of hemagglutination should therefore be different for the two viruses even though they both bind to neuraminic acid.

3.2. *Hemolysis and fusion by influenza virus*

Hemolytic activity of sonicated influenza virus (A_0PR8) was examined over a wide pH range. The virus caused extensive hemolysis between pH 5.0–5.5. The pH dependence of hemolytic activity had a very distinct profile as shown in fig.2. On the other hand,

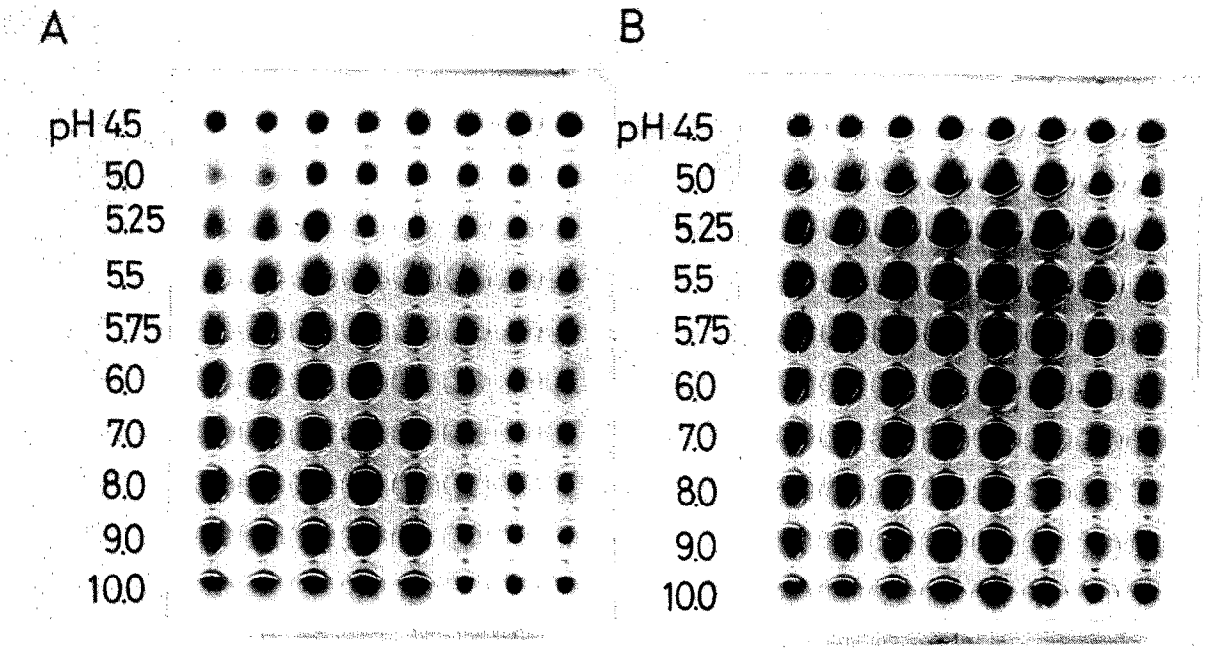

Fig.1. pH dependence of hemagglutination by influenza virus (A) and HVJ (B). Using chicken erythrocyte, hemagglutinating activity of 6×10^{-2} μg A_0PR8 and 5 μg HVJ(z) was determined in various buffer systems from pH 4.5–10.0.

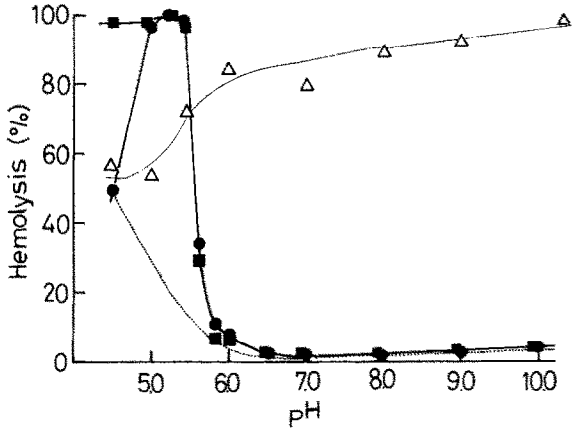

Fig.2. pH profile of hemolysis activity caused by sonicated influenza virus, A_0PR8 (50 μg/ml, •), WSN (28 μg/ml, ▪) and HVJ (20 μg/ml, △). Each virus was mixed with human erythrocytes (2.5%, v/v) and allowed to adsorb for 10 min at 0°C. Then, the mixture was incubated for 30 min at 37°C. Extent of hemolysis was determined spectrophotometrically at 540 nm. A dotted line shows spontaneous lysis in the absence of virus.

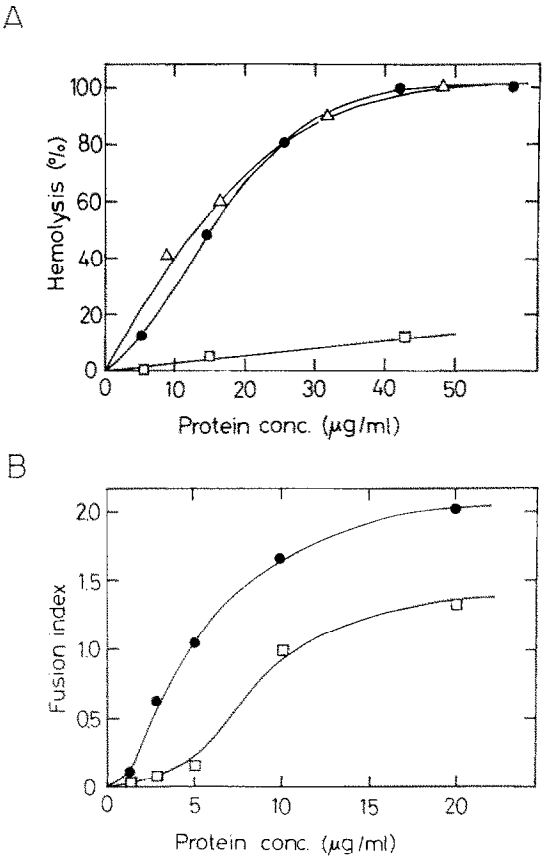

Fig.3. Dose dependence of hemolysis (A) and cell fusion (B) caused by sonicated A_0PR8 (•), unsonicated A_0PR8 (□) and HVJ (△). The buffers used were isotonic acetate buffer (pH 5.2) for influenza virus and isotonic phosphate buffer (pH 7.0) for HVJ, respectively. 10 μg virus protein correspond to 325 HAU for A_0PR8 (pH 5.2) and 63 HAU for HVJ (pH 7.0).

the hemolytic activity of HVJ was almost independent of pH over 5.0–10.0. Another influenza virus strain, WSN, also showed the same sharp pH profile as A_0PR8, except that there was no hemolytic activity inhibition at pH <5.0.

Sonication effectively caused intact virus to become more hemolytic and non-sonicated virus had only low hemolytic activity.

The hemolytic activity of sonicated influenza virus increased with dose as shown in fig. 3A. The specific activity based on protein weight is comparable to that of HVJ. The amount of virus necessary for 50% hemolysis was 126 HAU and 20 HAU/10^8 cells for A_0PR8 and HVJ in our experimental conditions, respectively.

The sonicated influenza virus caused extensive fusion of erythrocytes together with hemolysis at pH 5.2 (fig.4). The pH profile qualitatively paralleled that of hemolysis. Fig.3B shows the fusion activity as a function of viral dose. Unsonicated virus also showed some fusion activity at higher doses, in contrast to hemolytic activity. Sonication enhanced the fusion activity of unsonicated virus.

3.3. *Phospholipid transfer and structural modification of erythrocyte membranes*

Sonicated influenza virus was densely labeled with spin-labeled phosphatidylcholine and mixed with erythrocytes at various pH values. The resulting aggregates were put in a quartz capillary tube and ESR spectra was recorded continuously at 37°C. The exchange-broadened signal rapidly changed into a sharp spectrum over pH 4.5–5.4 in the same way as for a HVJ–erythrocyte system [8]. However, the spectrum remained unchanged at other pH where influenza virus was non-hemolytic. The rate of spec-

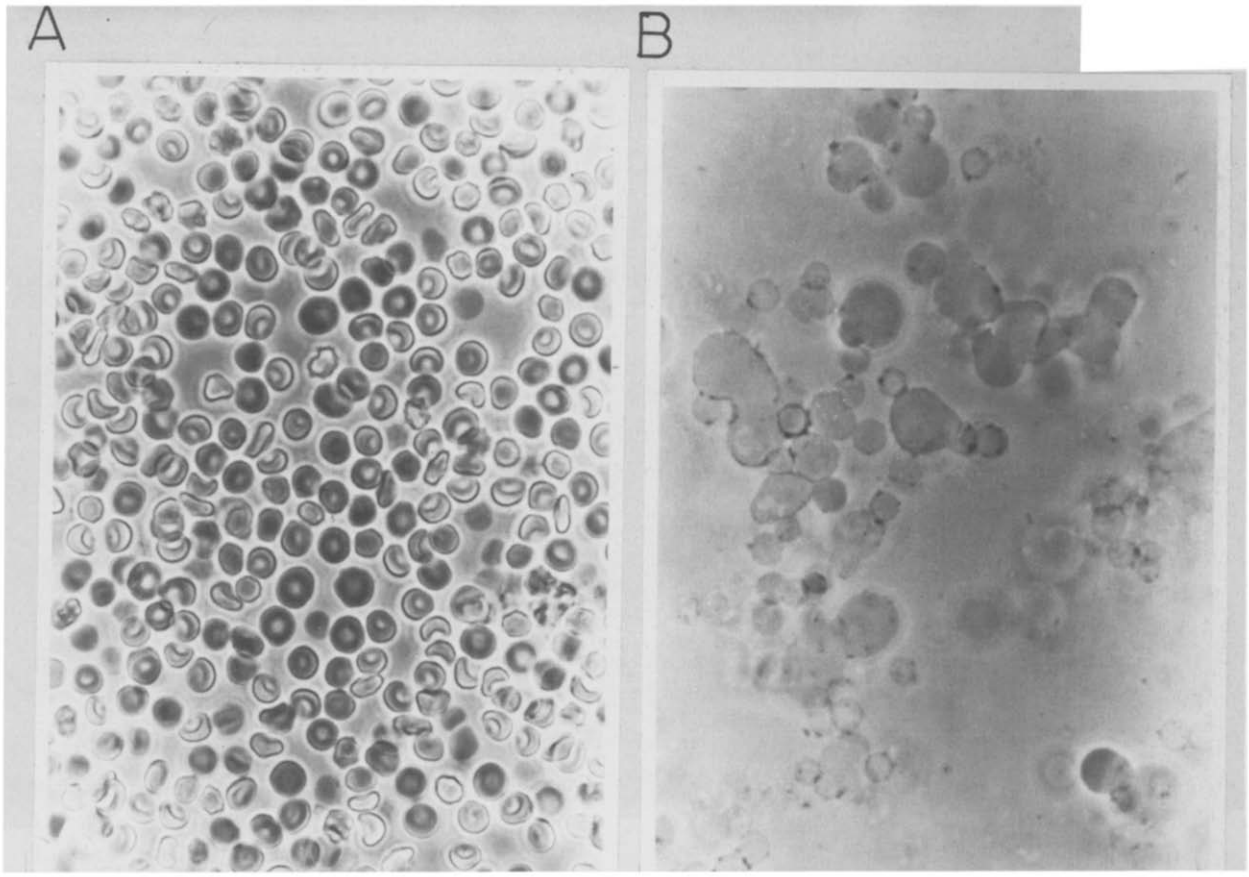

Fig.4. Influenza virus-induced fusion of human erythrocytes. Erythrocytes (2.5%) were incubated for 30 min at 37°C in an isotonic acetate buffer (pH 5.2) in the absence (A) and presence (B) of sonicated A_0PR8 (50 μg/ml).

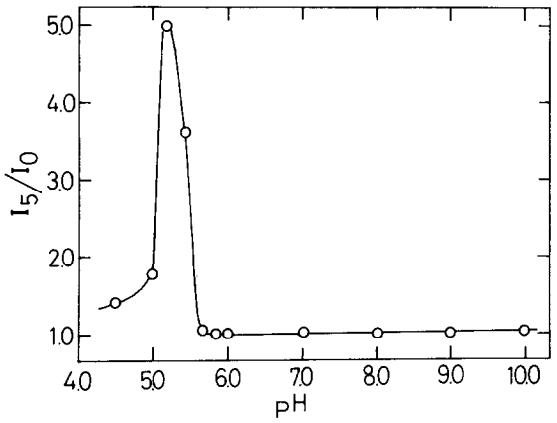

Fig.5. pH dependence of lipid transfer. Spin-labeled influenza virus (33 μg/ml) was added to erythrocyte (2.5%) in acetate buffer (pH 5.2) and ESR spectrum of the resulting aggregates was recorded continuously to assay lipid transfer. Ordinate plots the ratio of the central peak height at t = 5 min to that at t = 0 min.

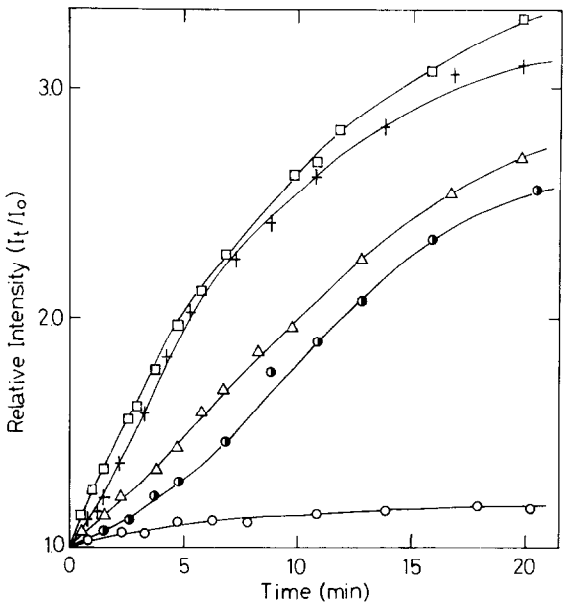

Fig.6. Lipid transfer from inactivated HVJ to erythrocytes mediated by sonicated influenza virus. Varying amounts of sonicated A_0PR8 and spin-labeled and trypsinized HVJ (60 μg/ml) were added to erythrocytes (2.5%) and signal changes in ESR spectrum of the aggregates were recorded at 37°C. Ordinate plots the relative central peak height. A_0PR8: 0 μg/ml (○); 5 μg/ml (◑); 15 μg/ml (△); 30 μg/ml (+); and 60 μg/ml (□).

tral change is plotted as a function of pH in fig.5. The results show that the rate of lipid transfer from the viral envelope to erythrocyte membrane becomes very large at pH 5.2–5.4. The pH range is essentially the same as that of hemolytic activity shown in fig.2.

The ability of the sonicated influenza virus to accelerate lipid transfer from HVJ to erythrocyte membrane was next examined. Non-labeled influenza virus and spin-labeled trypsinized HVJ were added to erythrocytes at pH 5.2 and ESR spectra of the aggregate were measured at 37°C. The results in fig.6 show that the influenza virus greatly accelerated lipid transfer from trypsinized HVJ to erythrocyte membrane. This enhancement indicates that influenza virus may cause membrane structural modifications similar to that caused by HVJ [11].

4. Discussion

This study shows that influenza virus has high hemolytic and cell fusion activity in acidic medium. Sonication of the virus effectively enhanced the hemolytic activity. This may be analogous to the enhancement of hemolytic activity of HVJ [12] and Semliki Forest virus [13] by sonication or freeze–thawing. Such treatment may cause partial disruption or increase of fragility of viral membranes, and integration of the membranes into erythrocyte membrane would introduce permeability defect in the latter, causing hemolysis [14]. Sonication of influenza virus also enhanced the fusion activity in the acidic medium. Unsonicated virus had a considerable level of fusion activity and the extent of enhancement by sonication was apparently smaller than that for hemolysis. The enhancement of fusion activity may be related to the increased permeability by sonication which results in swelling of cells. The role of cell swelling in cell fusion has been discussed [15].

The hemolytic activity of influenza virus is limited to a very narrow pH range around 5.2; in contrast HVJ shows activity over a broad pH range. The transfer of phospholipid from influenza virus to erythocyte membrane and from trypsinized HVJ to erythrocyte membrane in the presence of influenza virus were both activated in the same narrow pH range. Based on our studies of phospholipid transfer between HVJ and erythrocytes [11], the results shown here strongly suggest that envelope fusion between influenza virus and the erythrocyte membrane occurs over a limited pH range. The virus may modify the target cell membrane, enhancing phospholipid transfer and cell

fusion at the acidic pH. Dissociation–association equilibrium of a certain charged amino acid residue(s) with pK ~5.3 should be involved in this characteristic behavior of influenza virus.

Many strains of influenza virus have 2 or 3 acidic residues (Glu and Asp) in the amino-terminal segment of HA_2 protein, while the corresponding F protein segment of HVJ consists totally of hydrophobic residues except for the amino-end [6,16]. If the acidic residues were protonated, the terminal segment would become hydrophobic and possibly be the cause of hemolytic and fusion activity at acidic pH. At physiological pH, however, the acidic residues are charged, and might not allow the protein segment to interact with hydrophobic regions of target cells to cause hemolysis. This hypothesis explains the remarkable difference in pH dependence of hemolytic and fusion activity between influenza virus and HVJ. In [13] Semliki Forest virus had hemolytic activity at pH 5.5–6.5, about 1 pH unit higher than that of influenza virus. It would be interesting to determine whether an envelope protein of Semliki Forest virus has a hydrophobic amino-terminal segment similar to those of HVJ and influenza virus and what kind of charged amino acid(s) is (are) in it.

The finding of hemolytic and fusion activity of influenza virus in acidic medium may simplify the controversy about its penetration and infection mechanism. We suggest the following:

> Influenza virus is phagocytized into phagosomes and reaches the secondary lysosomes. 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. Influenza virus adsorbed on the cell surface could not fuse with the plasma membrane because of the neutral pH of the medium.

This mechanism is quite similar to that in [17] for infection by Semliki Forest virus. The mechanism does, however, contradict the report of fusion of reconstituted influenza envelope with plasma membrane of cultured cells at physiological pH [18]. We are attempting to clarify these problems.

Acknowledgements

We thank Dr M. Hamaguchi for giving influenza strains, Dr S. Toyama for his help in virus preparation and Dr R. Lew for a critical reading of the manuscript.

References

[1] How, C. and Morgan, C. (1969) J. Virol. 3, 70–81.
[2] Morgan, C. and Rose, H. M. (1968) J. Virol. 2, 925–936.
[3] Dourmashkin, R. R. and Tyrrell, D. A. J. (1974) J. Gen. Virol. 24, 129–141.
[4] Klenk, H.-D., Rott, R., Orlich, M. and Blödorn, J. (1975) Virology 68, 426–439.
[5] Lazarowitz, S. G. and Choppin, P. W. (1975) Virology 68, 440–454.
[6] Gething, M. J., White, J. M. and Waterfield, M. D. (1978) Proc. Natl. Acad. Sci. USA 75, 2737–2740.
[7] Ishida, N. and Homma, M. (1978) Adv. Virus Res. 23, 349–383.
[8] Maeda, T., Asano, A., Ohki, K., Okada, Y. and Ohnishi, S. (1975) Biochemistry 14, 3736–3741.
[9] Lowry, O. H., Rosebrough, N. Y., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[10] Salk, J. E. (1944) J. Immunol. 49, 87–98.
[11] Maeda, T., Asano, A., Okada, Y. and Ohnishi, S. (1977) J. Virol. 21, 232–241.
[12] Hosaka, Y. (1970) J. Gen. Virol. 8, 43–54.
[13] Väänänen, P. and Kääriäinen, L. (1979) J. Gen. Virol. 43, 593–601.
[14] Homma, M., Shimizu, K., Shimizu, Y.-K. and Ishida, N. (1976) Virology 71, 41–47.
[15] Knutton, S. (1979) J. Cell Sci. 36, 85–96.
[16] Gething, M.-J., Bye, J., Skehel, J., Waterfield, M. (1980) Nature 287, 301–306.
[17] Helenius, A., Kartenbek, J., Simon, K. and Fries, E. (1980) J. Cell Biol. 84, 404–420.
[18] Huang, R. T. C., Wahn, K., Klenk, H.-D. and Rott, R. (1980) Virology 104, 294–302.