HEMOLYSIS AND CELL FUSION BY RHABDOVIRUSES

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1. Introduction

Fusion of certain enveloped viruses with cellular membranes is thought to be the mechanism by which the viral genome penetrates host cells. This has been clearly demonstrated with paramyxoviruses, where F protein activated by proteolytic cleavage is playing a critical role not only for the cell fusion and hemolysis, but also for infectivity [1–3]. Influenza viruses and togaviruses such as Semliki forest virus (SFV), Sindbis virus and rubella virus have been demonstrated capable of causing hemolysis and cell fusion at low pH [4–9]. The virus may be taken into phagocytic vesicles and reach the secondary lysosome, where the viral envelope fuses with lysosomal membrane in its acidic environment, resulting in the release of viral genome into the cytoplasm.

Penetration of host cells by rhabdoviruses has been studied mostly by electron microscopy. In [10] vesicular stomatitis virus (VSV) penetrates the cells by phagocytosis within minutes after adsorption of the virus. In contrast, in [11] VSV penetrates the cells by fusion of viral envelope with the plasma membrane of the cells. In [12] rabies virus penetrates the cells by these two kinds of entry pathway. However, the electronmicroscopic studies have limitations for interpreting the observation such that only a small fraction of the input viruses can be detected despite extremely high multiplicities of infection.

Here, we describe that VSV and rabies virus incubated at low pH with human O erythrocytes cause high hemolysis and cell fusion. This system might be a useful model to analyse the entry mechanism of rhabdoviruses.

2. Materials and methods

VSV, Indiana serotype, was grown in HEL-R66 cells with Eagle minimum essential medium (MEM) containing 0.2% bovine serum albumin (BSA). The CVS strain of rabies virus was grown in murine neuroblastoma cells (N-18 clone) with serum free Eagle MEM. Viruses were pelleted by ultracentrifugation and the resulting pellets were resuspended in small amount of PBS (–) of pH 7.4 to yield 100-fold concentration. Rabies virus was used for experiments after purification in 20–60% linear sucrose gradient centrifugation.

Hemagglutination was carried out in an ice bath at final pH 6.4 as in [13] except that the concentration of erythrocytes increased to 1.0% and the concentration of BSA in borate saline (pH 9.0) decreased to 0.2%.

Hemolysis was determined by following manner. Appropriately diluted virus in borate saline in test tubes was mixed with an equal volume of 10% erythrocytes suspended in virus adjusting diluent of pH 6.4 and incubated in ice bath for 30 min. The erythrocytes were then pelleted by low speed centrifugation and the supernatant buffer was replaced with 1 ml hemolysin buffer. For the buffer ranging from pH 5.0–5.4, 0.02 M acetate-buffered saline and for that of pH 5.8–7.0, 0.02 M phosphate-buffered saline was used, respectively. Test tubes were further incubated for 20 min at 37°C and after low speed centrifugation, the absorbance of the supernatant was measured at 575 nm by spectrophotometer.

3. Results

3.1. *Hemagglutination of human erythrocytes by VSV and rabies virus*

Goose erythrocytes were first used for hemagglutination and hemolysis experiments. Both viruses agglutinated goose erythrocytes well, however, only a slight hemolysis was caused in the system. We found

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in the next experiment that hemagglutination by these viruses was markedly enhanced with human erythrocytes which were pretreated with fungal semialkali protease (Seikagaku-kogyo) at 1 mg/ml final conc. for 60 min at 37°C. Hemagglutinating titers reached almost the same level as with goose erythrocytes.

Preliminary experiments with SDS–polyacrylamide gel electrophoresis showed that glycoproteins of the erythrocytes membrane were almost completely removed by this treatment.

3.2. *pH-dependent hemolysis and fusion of human erythrocytes by viruses*

Hemolysis studies were carried out with 64 hemagglutination units (HAU) of VSV and 256 HAU of rabies virus in hemolysing buffer of various pH-values after virus adsorption on protease-pretreated human erythrocytes at pH 6.4.

As shown in fig.1, both viruses caused marked pH-dependent hemolysis. Within the pH tested, the maximum hemolysis occurred at pH 5.0 and the hemolysis decreased with the increase of pH. However, profile of pH dependency of the hemolysis appeared to be slightly different between these viruses. Hemolysis of rabies virus almost completely diminished at pH > 6.2, while the hemolysis by VSV did not completely disappear at pH > 6.2 and even in neutral pH.

These viruses caused extensive fusion of erythrocytes at low pH. Fig.2 shows the fusion of erythrocytes by rabies virus. Similar fusion was also caused by VSV. Fusion of erythrocytes could not take place if the pH was raised to neutrality.

3.3. *Inhibition of hemolysis by antiserum*

VSV was incubated for 30 min at room temperature with mouse antiserum before adsorption of the virus to erythrocytes. Mouse antiserum with 1:6400 neutralizing antibody titer was treated with cold acetone and 25% kaolin to remove non-specific inhibitor before use as in [14]. Normal mouse serum was used as a control. Hemolytic activity of VSV was completely inhibited by antiserum (not shown).

3.4. *Fusion of BHK-21 cells by VSV*

Cell fusion activity of VSV was also tested with somatic cells instead of erythrocytes. BHK-21 cell monolayers, not completely confluent, were infected with the virus at a multiplicity of ~80 plaque forming units (p.f.u.)/cell. After 1 h adsorption, the mono-

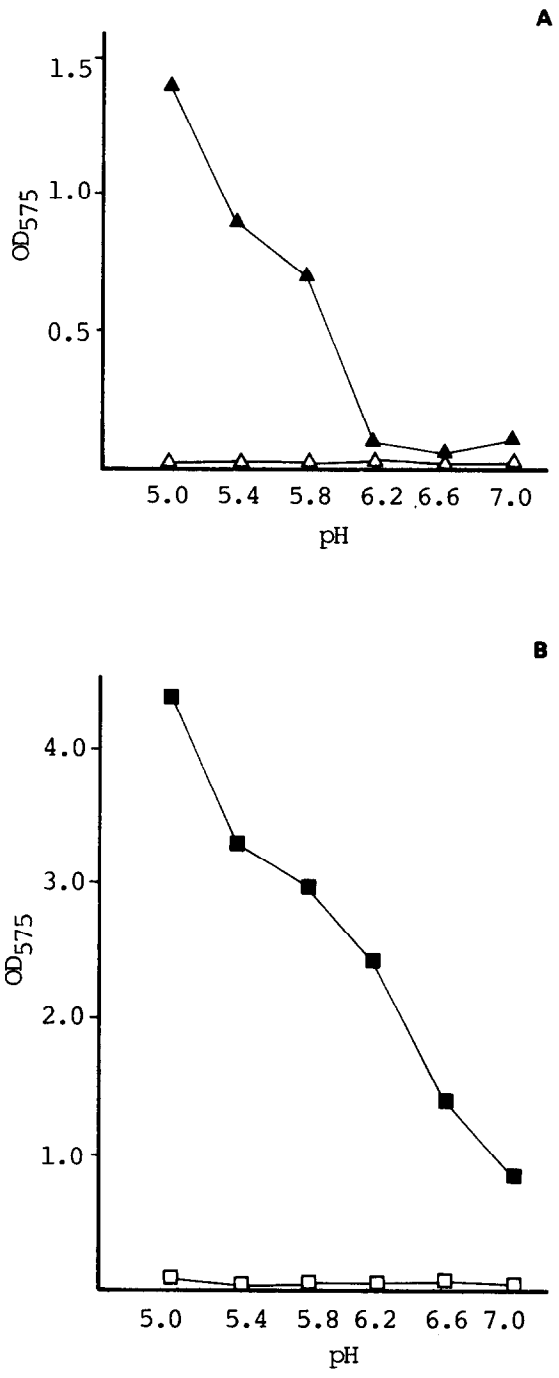

Fig.1. pH-Dependence of hemolysis by rabies virus (A) and VSV (B). Human erythrocytes (10%) pretreated with fungal semialkali protease were incubated for 20 min at 37°C with hemolysing buffer from pH 5.0–7.0 after adsorption with 256 HAU of rabies virus (▲——▲) and with 64 HAU of VSV (■——■) in ice bath for 30 min. (△——△) or (□——□) means A_{575} with control erythrocytes without rabies virus or VSV.

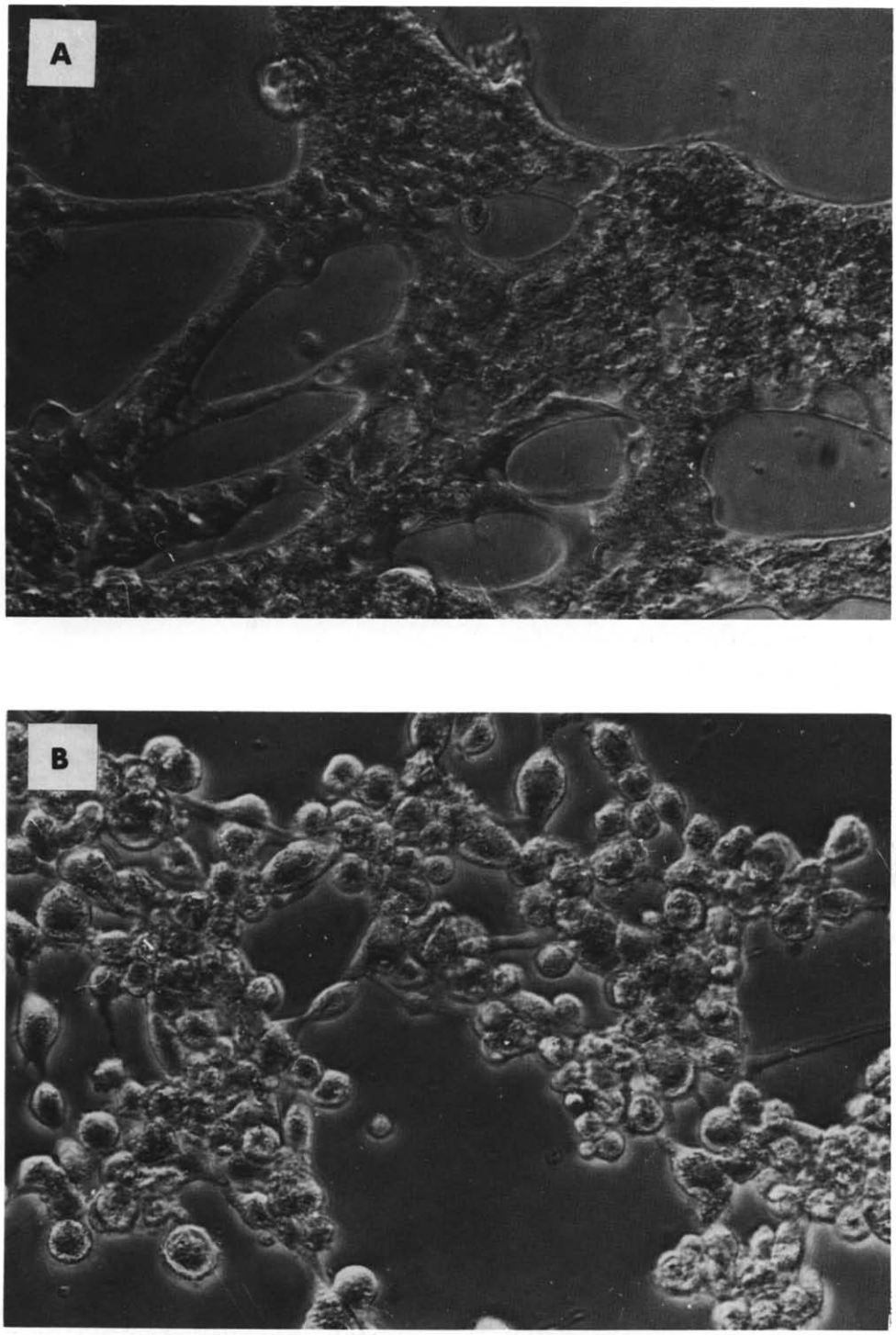

Fig.2. Rabies virus-induced fusion of human erythrocytes. Erythrocytes (10%) were incubated for 20 min at 37°C in hemolysing buffer of pH 5.4 after adsorption at pH 6.4 with (A) or without (B) rabies virus (256 HAU).

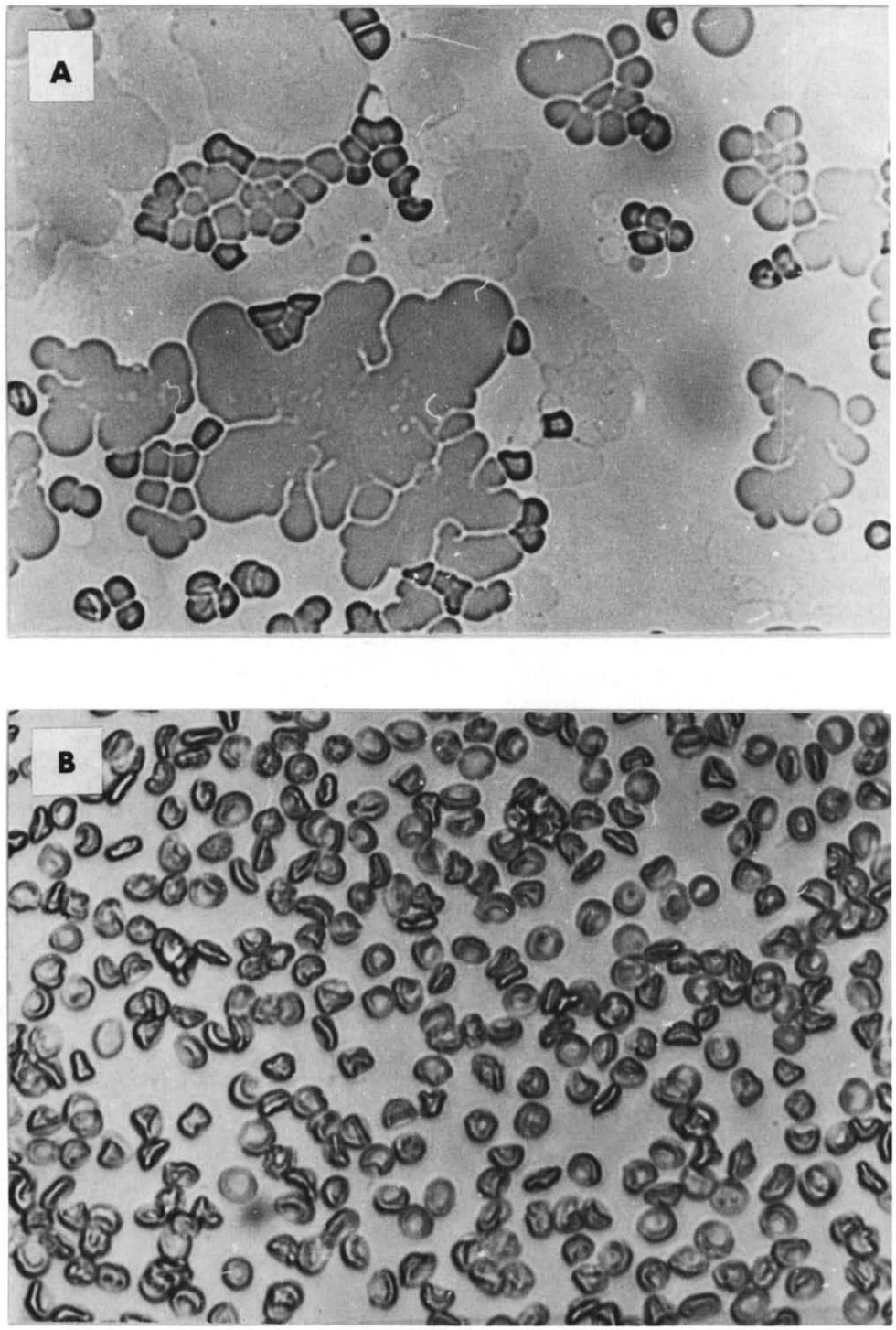

Fig.3. Fusion of BHK-21 cells by VSV. BHK-21 cell monolayers were infected with VSV at a multiplicity of ~80 p.f.u./cell. After 5 h incubation, the monolayers were treated with citrate-buffered saline (pH 5.75) (A) or phosphate-buffered saline (pH 7.4) (B) for 10 min at 37°C. The cultures were again fed with normal culture medium for 1 h at 37°C and observed by phase contrast microscope.

layers were washed twice with PBS (−) to remove unadsorbed virus, refed and incubated for 4 h at 37°C. The culture medium was replaced with citrate-buffered saline (pH 5.75) and the cells were incubated for 10 min at 37°C. The monolayers were then refed with culture medium (pH 7.4) and incubated for 1 h at 37°C and observed by phase contrast microscope.

As shown in fig.3, extensive fusion of the infected cells was observed in the monolayer culture treated with acidic buffer but not with buffer of neutral pH.

4. Discussion

This study shows that VSV and rabies virus have high hemolytic and cell fusion activity at low pH against human erythrocytes pretreated with fungal semialkali protease. This treatment of erythrocytes appeared to expose masked viral receptors on their membrane surface. This hemolysis and fusion would take place not only in such erythrocytes but also in common somatic cells, since VSV-infected BHK-21 cells, from which large amounts of progeny virions are budding, could fuse with each other and produce polykaryocytes with 10 min treatment at low pH. In [15] fusion from without by VSV in BHK-21 cells at low pH was shown.

The hemolytic and fusion activities of rhabdoviruses are quite similar to those of influenza viruses, SFV and Sindbis virus [4–9]. Virus-induced hemolysis and cell fusion are caused by fusion of the viral envelope with cellular membranes. Since acidic environment in cells is limited in lysosomes, the entry mechanism of rhabdoviruses into cells might be mostly via lysosomes as suggested with influenza viruses, SFV and Sindbis virus; e.g., phagosomes containing virus fuse with lysosomes, then viral membrane fuses with lysosomal membrane by its acidic environment, resulting in the release of viral genome to the cytoplasm.

However, our immunolysis studies of cells inoculated with VSV at extremely high multiplicities of infection at neutral pH suggested that a small fraction of the inoculated virus directly fuse with plasma membrane of the cells as in [11,16]. Further detailed studies on the entry mechanisms of rhabdoviruses are in progress.

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