Cell Fusion by Semliki Forest, Influenza, and Vesicular Stomatitis Viruses

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ABSTRACT  Representatives of three families of enveloped viruses were shown to fuse tissue culture cells together. These were: Semliki Forest virus (SFV, a togavirus), vesicular stomatitis virus (a rhabdovirus), and two myxoviruses, fowl plaque virus and Japan influenza virus (Japan/A/305/57). Unlike paramyxoviruses, whose fusion activity is known to occur over a broad pH range, fusion by these viruses was restricted to mildly acidic pH. The pH thresholds for the four viruses were 6.0, 6.1, 5.5, and 5.1, respectively. The precursor form of Japan influenza, which is not infectious and which contains the uncleaved hemagglutinin, had no fusion activity. This result suggested a role for the influenza hemagglutinin in the low-pH-dependent membrane fusion activity. Taken together, our results show that low-pH-induced fusion is a widespread property of enveloped animal viruses and that it may play a role in the infective process.

The fusion reactions with all four viruses were fast, efficient, and easy to induce. With UV-inactivated SFV, the fusion was shown to be nonlytic and the polykaryons were viable for at least 12 h. 30 ng of SFV/1 x 10^6 BHK-21 cells were required for 50% fusion, and 250 ng sufficed to fuse the entire culture into a single polykaryon.

MATERIALS AND METHODS
Viruses and Cells

A prototype strain of SFV (unlabeled and [35S]methionine-labeled) was grown in BHK-21 cells, and purified as described (1, 7, 8). Fowl plaque virus (FPV) was grown in the allantoic cavity of 11-d embryonated chicken eggs infected with 3 x 10^6 plaque-forming units (pfu). After 24 h at 37°C, the allantoic fluid was collected and centrifuged (8,000 g, 30 min, 4°C) in a Beckman J6 centrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) to remove cellular debris. The supernate was concentrated in a Dow C/HFV 10 Mini-Plant Ultrafilter with a molecular weight cutoff of 30,000 daltons (Dow Chemical Co., Indianapolis, Ind.). The virus was pelleted onto a cushion of 50% glycerol in TN buffer (50 mM Tris-base, 0.1M NaCl, pH 7.4) by centrifugation at 81,500g for 90 min, 4°C, and resuspended overnight in a small volume of TN. After five strokes in a loose-fitting Dounce homogenizer, the virus was isopycnically banded on 5-50% (wt/vol in TN) potassium tartrate gradients (81,500 g, 90 min, 4°C). The virus bands were collected and the virus pelleted and resuspended as above in TN. Electrophoretic analysis on polyacrylamide gels indicated that the hemagglutinin was present in the completely cleaved form (9). Vesicular stomatitis virus (VSV, Indiana strain) was grown in BHK-21 cells in 600-ml NUNC flasks (NUNC GmbH, Wiesbaden, W. Germany), using Eagle's minimal essential medium (MEM) with Earle's salts supplemented with 2 mM glutamine, 100 U/
ml penicillin, and 0.1 mg/ml streptomycin sulfate. After the virus inoculum (0.01 pfu/cell) was adsorbed to washed cells in 2 ml of medium for 1 h in a 37°C, 5% CO₂ incubator (Forma Scientific Inc., Mallinckrodt, Inc., Marietta, Ohio), 35 ml of fresh medium was added, and the cells were incubated for 20 h in the CO₂ incubator. After centrifugation at 8,000 g for 30 min in a Beckman Jr centrifuge to remove cellular debris, the medium was concentrated through an Amicon DC Hollow Fiber device (cartridge H1 x 50; Amicon Corp., Scientific Sys. Div., Lexington, Mass.) with a 50,000-dalton mol wt cutoff. The remaining purification was identical to that described for FPV. The human influenza virus Japan/A/305/75 and its precursor are the generous gifts of Dr. John Skehel (Medical Research Council, Mill Hill, London, England). These purity was confirmed at the time of assay for fusion activity by SDS polyacrylamide gel electrophoresis (9). All viruses were stored at concentrations of 10⁻²⁻⁰ mg of protein/ml of TN at −80°C.

The BHK-21 and HeLa B cells used for the fusion experiments were grown as previously described (4). For the fusion experiments, the cells (1 x 10⁶ in 2 ml of medium) were plated 24 h in advance in six-well Greiner trays (Greiner and Sohne, Nürtingen, W. Germany) containing three glass coverslips (diameter, 11 mm) per well and grown in a 37°C, 5% CO₂ incubator.

**UV Inactivation of SFV**

An ultraviolet light treatment was developed that renders SFV noninfectious but does not inhibit its fusion activity. Samples of the virus diluted to 1 ml of protein/ml of TN buffer were placed in the shallow wells of a glass tray that was kept on ice in a laminar flow hood (Sterilgard Hood, Baker Co., Inc., Sanford, Maine). The virus was then exposed to a 30-W germicidal lamp at a distance of 50 cm. The output radiation at this distance was measured to be 1.2 x 10⁶ ergs/cm²/sec, using a Blak-Ray ultraviolet meter (short wave, type J225; Ultra-Violet Products, Inc., San Gabriel, Calif.). As shown in Table I, 30 min of UV irradiation decreases the infectivity of the SFV by at least 10⁷ orders of magnitude while having virtually no effect on the ability of the virus to induce cell fusion. Higher irradiation doses inhibited the fusogenic capacity of the virus (Table I).

**Fusion Assay**

The viruses used for the fusion were diluted from concentrated stocks into binding medium (RPMI 1640 without bicarbonate) containing 0.2% bovine serum albumin (BSA) and 10 mM MES (2-(morpholinoo)ethanesulfonic acid) and 10 mM HEPES, pH 6.8, and kept on ice. Cover slips of cells were cooled to 0°C, washed twice with 2 ml of ice-cold binding medium, drained of excess medium, and transferred to a 24-well plastic tray (Flow Laboratories, Linbro Div., Hamden, Conn.). The virus suspension (5 µl) was applied to the cells on ice, and after a 1-h incubation, 2 ml of prewarmed 37°C binding medium adjusted to the indicated pH were added and the tray was placed in a 37°C water bath for 30-60 s. The media were then removed and replaced with 2 ml of binding medium adjusted to pH 7.2, and the tray was returned to the 37°C incubator. After 30 min, the cells were washed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 1.0 ml of 100% cold methanol. Filtered Giemsa stain (E. Merck, Darmstadt, W. Germany) diluted 20-fold into PBS was used to stain the cells (2 x 20 min at room temperature). After being washed twice with PBS, the stained cover slips were dehydrated in acetone, acetone-xylol (1:1), and xylol; mounted on glass slides with Entellan (E. Merck); and viewed with a Leitz microscope.

By photography, Kodak Tri-X film was used. Fusion was quantitated by counting the number of cells and nuclei present in a microscope field after fusion. A fusion index (f) was computed according to the equation f = [1 - (C/N)], where c is the number of cells in a field after fusion and n the number of nuclei. An average field at a magnification of x 250 contained 150-250 nuclei. Duplicate cover slips were prepared for each experimental condition and four to six fields were scored.

**Electron Microscopy**

For electron microscopy, confluent monolayers of BHK-21 cells on 11-mm glass cover slips were incubated for 1 h with either 1 µg of SFV or 20 µg of FPV or VSV in 20 µl of binding medium on ice. The cover slips were then dipped rapidly into RPMI medium at the indicated pH for 30 s and then either immediately washed with ice-cold pH 7.3 medium and fixed using 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, or incubated for 30 min at 37°C in pH 7.3 medium before fixation. Dehydration, embedding, sectioning, and staining with uranyl acetate were performed as described previously (8, 10). An Philips 400 or a Zeiss 10 A electron microscope was employed.

**[35S]Methionine Incorporation**

BHK-21 cells on 11-mm-diameter cover slips were washed three times with 1.0 ml of MEM containing 1 µg/ml methionine. 250 µl of MEM containing 1 µg/ml methionine and 5 µCi of [35S]methionine were added per well, and the cells were incubated for 15 min in a 37°C CO₂ incubator. The labeling medium was then replaced with 2.0 ml of MEM containing 0.2% BSA and 150 µg/ml methionine, and the cells were returned to the CO₂ incubator for an additional 15 min. The cells were then placed on ice, and the cover slips were washed once with 2.0 ml of PBS containing 10 mM methionine, and then dipped into a beaker with 200 ml of PBS containing 10 mM methionine before being placed in a new Linbro tray on ice with 2.0 ml of PBS per well for 15 min. The PBS was then removed, and the cells were treated for 1 h on ice with 2.0 ml 10% TCA. The cover slips were finally removed from the wells, drained, and counted with 7.0 ml of Triton-X-100-based scintillation fluid (Rotiszint 22, containing 800 ml of H₂O/10 liter [Roth Chemical Co., Karlsruhe, W. Germany]).

**RESULTS**

**Cell Fusion by SFV**

Light microscopy was used to detect cell fusion. Viruses were bound to cells at 0°C for 1 h, and the cells were then covered with warm (37°C), low-pH medium (usually pH 5.5) for 30–60 s. The low-pH medium was replaced with neutral medium, and the incubation continued at 37°C. During this incubation, massive fusion was observed (Fig. 1). Although the fusion reaction may have already taken place during the low-pH treatment (4), the cell fusion first became readily recognizable after 5–10 min of the second incubation (Fig. 1). Preliminary visualization of the fusion reaction by use of an image intensifier and a time-lapse video recording system revealed that the morphological changes occurred gradually and smoothly, and that the cells did not lyse or come off the support (D. Louvard, J. White, and A. Helenius, unpublished observations).

Cell fusion was equally efficient with UV-inactivated and untreated virus (Table I), and it was totally dependent on both the low-pH treatment and the presence of virus. As shown in Fig. 2, the pH threshold for fusion was ~6, which is the same as that shown previously for SFV fusion with the plasma membranes of tissue culture cells (4) and with liposomes (6). Fusion was confirmed by transmission electron microscopy (not shown).

**Characterization of the SFV-induced Fusion**

For further characterization, it was necessary to quantify the extent of fusion. To do this we used an index of fusion (f), which expresses the extent of fusion as the average number of fusion events per original mononucleated cell. The fusion index was defined (see Materials and Methods) so that f = 0.5 is obtained when the average number of nuclei per cell is 2, and so that f approaches 1 when all of the nuclei in the microscope

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**Table 1**

| Length of irradiation | Dosage | SFV titer | % Fusion activity |
|-----------------------|--------|-----------|------------------|
| min                   | ergs/cm² x 10⁻³ | pfu/ml |                  |
| 0                     | 0.0    | 1.4 x 10¹⁰ | 100              |
| 5                     | 3.6    | 3.8 x 10⁹  | 99               |
| 10                    | 7.2    | 6.2 x 10⁸  | 104              |
| 20                    | 14.4   | 1.5 x 10⁸  | 91               |
| 30                    | 21.6   | <4 x 10⁷   | 93               |
| 5*                    | 40.0   | <4 x 10⁷   | 50               |

SFV (1 mg/ml in TN) was irradiated with a 30-W UV light (1.2 x 10⁶ ergs/cm² per cm²) as described in Materials and Methods. At various times, aliquots were withdrawn, titrated, and assayed for cell-cell fusion activity.

* Irradiation was for 5 min with a Mineralight Lamp, model R-52 (Ultra-Violet Products, Inc., 1.3 x 10⁶ ergs/cm² per cm²).
FIGURE 1 Time dependence of SFV-induced cell fusion. 1 µg of SFV in 20 µl of binding medium, pH 6.8, was adsorbed to 3.3 x 10^5 BHK-21 cells for 1 h in the cold. Prewarmed medium, pH 5.5, was added for 60 s and the cells were postincubated with pH 7.2 medium for the indicated time and then fixed and stained (a, 0 min; b, 5 min; c, 10 min; d, 15 min; e, 30 min) Bar, 50 µm. X 300.

Using UV-inactivated SFV and the index f to express the extent of fusion, we determined the dose-, pH-, and the cell density-dependence of cell fusion. The pH dependence was very sharp (Fig. 3); virtually no fusion occurred above pH 6.2, whereas at pH values <5.8 all of the cells were fused together into giant polykaryons (Figs. 2 and 3). The extent of fusion was also critically dependent on the concentration of virus (Fig. 4). With 5 µl as the standard sample volume, 10 ng of SFV bound per cover slip was needed to obtain f = 0.5. This represents ~250 viruses bound per cell (3). To fuse all the cells into very large polykaryons, ~80 ng or 2 x 10^5 bound viruses were required. Fusion was also dependent on cell density with an optimum at 3.3 x 10^5 BHK-21 cells per 11-mm-diameter cover slip (see Fig. 1 a). With more confluent cells, the generation of giant polykaryons required larger amounts of virus and, with more sparse cultures, the polykaryons formed were smaller in size. By variation of the cell density, the amount of inactivated virus, and the pH of fusion, the average size of the field are present in a single cell. The background value for f in the control samples ranged from 0.03 to 0.07. The controls were: (a) non treated cells, (b) cells treated with virus and maintained at neutral pH, and (c) cells without virus treated at low pH (pH 5.5 or 5.0).

FIGURE 2 pH dependence of SFV-induced cell fusion. SFV was bound to BHK-21 cells as described in the legend to Fig. 1. The cells were then treated for 60 s with 2 ml of different pH media and postincubated in neutral pH medium for 30 min before fixing and staining (a, pH 6.5; b, pH 6.2; c, pH 6.0; d, pH 5.8). Bar, 50 µm. X 300.

FIGURE 3 pH titration of SFV-induced cell fusion. BHK-21 cells were fused as described in the legend to Fig. 2 and the fusion index was determined as described in the Materials and Methods section.
polykaryons could be stringently controlled (Table II).

Although extensive studies with different cell types were not performed, HeLa B cells were shown to be similar to BHK-21 cells in their susceptibility to SFV-mediated cell fusion (Fig. 4).

**Cell Viability after Fusion**

We tested the viability of the polykaryons by measuring their ability to incorporate $[^{35}S]$methionine into acid-precipitable material at different times after fusion. Table III shows that for up to 12 h, BHK-21 cells fused to an index of 0.6 incorporated as much methionine as unfused control cells. During the next 20 h, this function gradually declined in the fused cells. Similar results were previously obtained with paramyxovirus-induced polykaryons (11).

**Cell Fusion with FPV, Human Influenza Viruses, and VSV**

To determine whether other enveloped viruses could induce cell fusion at low pH, we tested VSV (a rhabdovirus), FPV (an avian influenza A virus), and two forms of Japan/A/305/57 (human influenza viruses). The two preparations of Japan influenza differed in having either the activated, proteolytically cleaved hemagglutinin (HA consisting of the two disulfide-bonded glycopolyptide chains HA1 and HA2) or the precursor form of the hemagglutinin (HA0) (12, 13). The viruses were allowed to bind to BHK-21 cells in the cold and the pH was lowered as described above for SFV. After a further 30 min at neutral pH, the cells were inspected for fusion. We found that FPV, VSV, and the activated form of the Japan influenza induced massive cell fusion; only the precursor Japan influenza was inactive (Fig. 5). The pH-dependence of the fusion by these viruses was titrated over the pH range 4.6–7.0. It was found that the pH thresholds were quite sharp, but that they varied from one virus to the next. VSV resembled SFV in having a pH threshold close to 6 whereas FPV and Japan influenza had values of 5.5 and 5.1, respectively.

Because virally induced cell fusion is likely to be a consequence of virus-cell fusion (4, 14, 15), we looked for fusion of VSV and influenza virus with the plasma membrane at low pH. Cells with bound VSV or FPV were treated at 37°C with pH 5.5 or pH 5.0 medium, respectively, for 30 s, and then fixed. Thin sections viewed in the electron microscope showed that, although the majority of the FPV seen on the cell surface remained unfiused (see arrowhead in Fig. 6a), some particles were apparently fusing with the plasma membrane (Fig. 6a). A similar result was observed with VSV where the cytoplasmic location of the electron-dense nucleocapsids made the detection of virus-cell fusion easier. The halo on the outer aspect of the
possess cell fusion activity (22, 23). Indirect evidence suggests cleavage of the inactive precursor F (22-24, 28). Viruses that membrane, F, is known to be involved (22-28). Like the HA of influenza virus (12, 13), the F protein consists of two disulfide-bonded glycopolypeptide chains, F1 and F2, which arise by cleavage of the inactive precursor F0 (22-24, 28). Viruses that have only the precursor F0 are not infective and they do not possess cell fusion activity (22, 23). Indirect evidence suggests a role for the hydrophobic F1 N-terminal moiety in the fusion reaction (24, 29, 30). With paramyxoviruses, fusion occurs over a broad pH range (pH 5-9; see references 31 and 32) either after addition of virus to cells ("fusion-from-without") or by fusion of infected cells ("fusion-from-within") (15).

Our present results show that three other families of enveloped viruses—togaviruses, myxoviruses, and rhabdoviruses—have membrane fusion activities (fusion-from-without and fusion of the viruses with the plasma membrane). Tissue culture cells exposed to these viruses and to mildly acidic pH (<6) fuse into giant polykaryons. Fusion of erythrocytes (fusion-from-without) at low pH has previously been reported for three togaviruses (SFV, Sindbis, and rubella) and for influenza virus (32-34). Fusion-from-within has been observed in myxovirus- and rhabdovirus-infected cell cultures (35, 36), and recent findings with influenza virus suggest that this may also be dependent on low pH (34).

The pH dependence (Fig. 3) of cell fusion with SFV was identical to that previously found for the fusion of SFV with liposomes (6) and with the plasma membranes of intact cells (4). Several lines of evidence suggest that the membrane fusion activity of SFV is required for infection, and that the low-pH environment in the lysosomes triggers the fusion (references 1, 2, 4, 6, and 37; footnote 1). We have shown that agents that increase the lysosomal pH to values >6 (lysosomotropic agents such as chloroquine and ammonium chloride) inhibit the entry of the SFV genome into the cytoplasm (references 1 and 2; footnote 1). It is well known that lysosomotropic agents also inhibit the entry of influenza virus (38, 39) and VSV (40). If we consider, in addition, the morphological observations, which show that influenza (41-43) and rhabdoviruses (44-46) enter cells by endocytosis, an entry pathway similar to that of SFV emerges. In this respect it is significant that the low-pH-induced fusion activity of the Japan influenza virus correlates with infectivity; viruses that contained HA were fusogenic at low pH, whereas those containing HA0 were not. We have recently obtained similar results with the X:31 (Hong Kong/1968) strain (J. White, unpublished observations). These findings show that the HA is essential for fusion; it is also required for low-pH-induced hemolysis (34). This possibility was previously raised on the basis of the sequence homology between the hydrophobic N-terminals of the Sendai virus F1 and the HA2 of influenza virus (24, 29). Indirect support came from two lines of evidence. (a) Low molecular weight peptide analogues of the HA2 N-terminal inhibited influenza virus infectivity (30). (b) Morphological observations with influenza virus (47, 48) and with reconstituted influenza virus membranes (49) suggested fusion with the cell surface at neutral pH, but, in a number of other studies, such fusion was not observed (41-43).

SFV-induced cell fusion has several features that may be advantageous for practical applications. It is simple to induce, it can be synchronized, it is at least as fast and efficient as fusion with Sendai virus (15), it can be performed with inactivated viruses, and it is not toxic or lytic to cells. Relatively few viruses are needed, and the extent of fusion can be easily controlled by adjusting the amount of virus and the pH. We have shown that cell fusion occurs equally well with BHK-21 and HeLa B cells. Because we know that SFV can fuse its envelope with the plasma membranes of a variety of monolayer and suspension cells (4; J. White, unpublished observations), the SFV-induced cell fusion technique should be widely applicable.

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