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Fusion of influenza virus in an intracellular acidic compartment measured by fluorescence dequenching

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The fusion of influenza virus with cultured cells has been investigated. The virus was labelled with the fluorescent probe octadecyl rhodamine B and fusion was monitored as fluorescence dequenching due to dilution of the probe from the viral into a cellular target membrane. Fusion with the plasma membrane does not occur, unless the extracellular pH is temporarily lowered. At neutral pH fusion occurs only after a lag phase of 10–15 min, the time required for virus internalization, and the reaction is inhibited by NH_4Cl, indicating that it takes place in an intracellular acidic compartment, most likely the endosome. This suggests that influenza virus infects cells via the endocytic pathway.

Enveloped animal viruses are believed to enter their host cells by either one of two pathways (for a review, see Ref. 1). Paramyxoviruses, e.g. Sendai virus, fuse directly with the plasma membrane of the cell, releasing the viral nucleocapsid into the cytoplasm [2]. Most other enveloped viruses probably enter cells by receptor-mediated endocytosis, after binding to a specific receptor on the cell surface. Induced by the low pH inside the endosomes [3,4], the viral membrane subsequently fuses with the endosomal membrane, extruding the viral nucleocapsid into the cytoplasm. It is thought that this route of infectious entry is used by togaviruses, rhabdoviruses and orthomyxoviruses [1].

For influenza virus, evidence indicating that the virus infects a cell via the endocytic pathway is based upon the following observations. The virus fuses with artificial or biological membranes at low pH [5–11]. Fusion is mediated by the viral spike glycoprotein hemagglutinin [12]. At low pH a conformational change in this protein occurs exposing a hydrophobic segment of the molecule which is crucial for fusion [13]. Furthermore, it has been demonstrated that influenza virus particles appear in the endosomes of the cell on a time scale corresponding to that of infection [4]. Finally, Richman et al. [14], using radioactively labelled influenza virus, have shown that the virus particles enter the cell as intact entities and do not leave their spike proteins behind on the plasma membrane, arguing against fusion of the viral envelope with the plasma membrane. However, direct evidence for the fusion of influenza virus within an intracellular membrane compartment on the endocytic pathway is lacking.

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; R_{18}, octadecyl rhodamine B chloride.

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The above view of the infectious entry of influenza virus has recently been challenged by Haywood and Boyer [15]. It was shown that influenza virus is able to fuse with liposomes at neutral pH. Furthermore, fusion of reconstituted influenza virus envelopes with cell membranes at neutral pH has been reported [16]. Thus, Haywood and Boyer [15] suggest that influenza virus may infect a cell by direct fusion of the viral envelope with the cellular plasma membrane.

Previously, we have monitored fusion of influenza virus with erythrocyte ghosts [11], utilizing an assay based upon dequenching of the fluorescent membrane probe octadecyl rhodamine B [17]. In the present study we have employed the same assay to examine the fusion of influenza virus with nucleated cells in culture. The results indicate that influenza virus fuses intracellulary with the membrane of an acidic compartment. The virus fuses only directly with the plasma membrane, in a non-physiological reaction, when the pH in the external medium is temporarily lowered.

Influenza virus strain X-47 was grown in the allantoic cavity of embryonated eggs, purified, handled and stored essentially as described before [10]. Viral phospholipid was determined, after extraction [18], by phosphate analysis [19] and protein according to Peterson [20]. Virus was labelled with octadecyl rhodamine B chloride (R_{18}, Molecular Probes, Junction City, OR). Before use the virus was centrifuged for 2.5 min at $12\,000 \times g$ to remove any aggregates. BHK-21 (C13) cells were maintained in Glasgows modification of Eagles minimal essential medium containing 10% tryptose phosphate broth and 5% fetal calf serum; LLC-MK_2D cells were maintained in Eagles minimal essential medium containing 10% fetal calf serum. Subconfluent monolayers of logarithmically growing cells in 35-mm plastic petri dishes were used ($(1–1.5) \cdot 10^6$ LLC-MK_2D or $(1.5–2) \cdot 10^6$ BHK-21 cells per dish, respectively). The cells were washed extensively in Hanks/Hepes buffer (137 mM NaCl, 5.4 mM KCl, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.41 mM $MgSO_4$, 0.40 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose and 10 mM Hepes, pH 7.4) at 37° C to remove all traces of serum. Usually, 40 μg of virus (10 nmol of membrane phospholipid) in 0.5 ml of buffer was added per dish. This corresponds to about $7 \cdot 10^{10}$ virus particles. After the experiment, cells were scraped off with a rubber policeman and transferred to a fluorimeter cuvette. For the induction of direct fusion with the plasma membrane a buffer containing 135 mM NaCl, 10 mM Mes, 5 mM Hepes, 15 mM sodium citrate at pH 5.0 was used. Fluorescence was measured at excitation and emission wavelengths of 560 and 590 nm, respectively, in an SLM-8000 fluorimeter (SLM/Aminco, Urbana, IL). For the calculation of the percentage R_{18} fluorescence dequenching the residual fluorescence of the labelled virus was taken as the zero level and the fluorescence at infinite probe dilution as 100%. This latter value was determined 30 min after addition of Triton X-100 (0.5% v/v) to the samples.

We first determined the binding of virus to cells at 4° C. At this temperature, endocytosis does not occur [21]. R_{18}-labelled influenza virus was added to subconfluent monolayers of BHK-21 or LLC-MK_2D cells. After an incubation for various periods of time the monolayers were washed extensively to remove unbound virus, scraped off from the dishes and transferred to a fluorimeter cuvette at 4° C. The cell-associated fluorescence (after addition of Triton X-100) is shown in Fig. 1. The binding did not saturate over a period of 60 min. At that time about 10.5% of the virus was associated with the BHK-21 cells, and about 4.5% with the LLC-MK_2D cells. These values correspond to approximately 4200 virus particles per BHK-21 cell and 2100 particles per LLC-MK_2D cell.

The binding of the virus was partially reversed by a subsequent treatment of the cells with neuraminidase either at 4° C or at 37° C (Table I), indicating the involvement of sialic-acid-containing receptors in the association. Incubation at 37° C also caused spontaneous release of the virus (Table I), probably due to the action of the viral neuraminidase. In contrast with these results, Richman et al. [14] reported that the virus is not released from LLC-MK_2D cells by neuraminidase treatment in the cold. However, our observations are in agreement with those of Matlin et al. [22].

It is important to note that during the binding of the R_{18}-labelled virus to the cells at 4° C no fluorescence dequenching was observed (results not shown), indicating that neither fusion nor

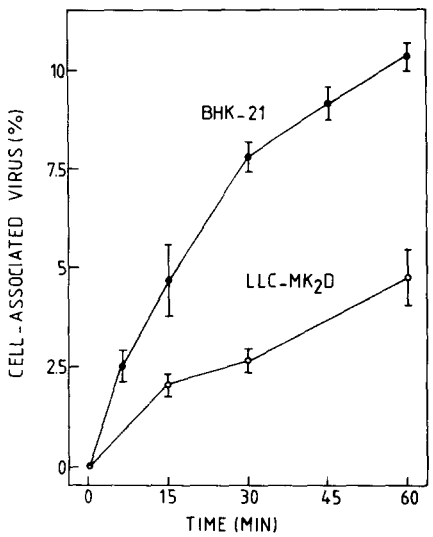

Fig. 1. Binding of influenza virus to BHK-21 (●) or LLC-MK_2D (○) cells. $(1.5–2)\cdot10^6$ BHK-21 or $(1–1.5)\cdot10^6$ LLC-MK_2D cells on 35-mm plastic petri dishes were incubated with 40 μg R_{18}-labelled influenza virus in 0.5 ml of Hanks/Hepes buffer at 4°C for the indicated periods of time. The cell-associated fluorescence was determined in the presence of Triton X-100 (0.5%, v/v) as a measure of the cell-associated virus (shown relative to the total applied virus). Error bars represent standard error of the mean.

transfer of the fluorophore occurs under these conditions.

R_{18}-labelled virus can be induced to fuse with the cellular plasma membrane by a brief exposure to a mildly acidic pH at 37°C, as revealed by an increase of R_{18} fluorescence. Virus was allowed to bind to LLC-MK_2D cells at 4°C for 30 min. Subsequently, the medium was replaced by pre-warmed buffer adjusted to either pH 5.0 or pH 7.4. The cells were incubated at 37°C for 5 min, the cell monolayer was washed with Hanks/Hepes buffer at 4°C, scraped off and transferred to a fluorimeter cuvette. An increase of fluorescence was observed for the cells incubated at pH 5.0. Relative to the fluorescence intensity at infinite probe dilution the increase was 48 ± 9%, indicating that half of the cell-associated virus, corresponding to about 500–700 particles per cell, had actually fused. No fluorescence increase whatsoever was observed for the cells incubated for 5 min at pH 7.4, 37°C. This result clearly shows that at neutral pH the virus does not fuse with the cellular plasma membrane. It also shows that transfer of R_{18} does not occur.

Intracellular fusion of the virus was examined by incubating cells with the virus at 37°C in a medium of neutral pH for a continued period of time, allowing the virus to be internalized. To synchronize the entry, the virus was first allowed to bind to the cells at 4°C. The monolayers were extensively washed to remove unbound virus, and rapidly warmed to 37°C, employing a volume of 2 ml Hanks/Hepes buffer, and incubated for various periods of time at 37°C. Subsequently, the cell monolayers were washed with cold Hanks/Hepes buffer and the cell-associated fluorescence was determined at 4°C. After a lag phase of about 10–15 min, fluorescence dequenching started, resulting in a 30% fluorescence increase after 1 h at 37°C (Fig. 2). The observation of a lag phase, during which the fluorescence did not increase, further supports the above conclusion that the virus does not fuse with the cellular plasma membrane at neutral pH and suggests that the subsequent dequenching of R_{18} fluorescence represents low-pH-induced fusion of the virus within an intracellular acidic compartment.

Further evidence for intracellular fusion was obtained by examining the effects of inhibitors of vacuolar acidification. As shown in Table II, the fluorescence increase was completely blocked by NH_4Cl and strongly inhibited by monensin. NH_4Cl and monensin are known to raise the intra-endosomal and intralysosomal pH [23,24]. Thus, the increase in fluorescence takes place at

TABLE I

EFFECT OF NEURAMINIDASE TREATMENT ON THE RELEASE OF CELL-BOUND VIRUS

LLC-MK_2D cells were incubated for 30 min at 4°C with R_{18}-labelled virus (40 μg), after which the cells were washed and treated with 1.25 mg/ml of *Clostridium perfringens* neuraminidase in Hanks/Hepes buffer at 4°C or 37°C. After the incubation the cells were washed and the amount of cell-associated virus determined at 4°C.

Treatment	Cell-associated virus (%)
None	2.6 ± 0.5
Neuraminidase, 90 min, 4°C	1.2 ± 0.1
Neuraminidase, 30 min, 37°C	1.2 ± 0.1
Control, 90 min, 4°C	2.1 ± 0.1
Control, 30 min, 37°C	1.3 ± 0.1

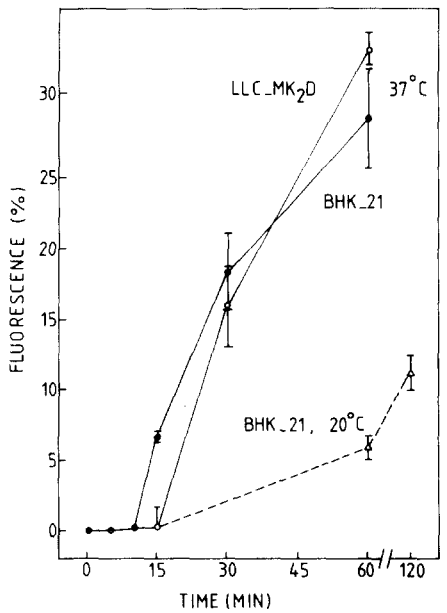

Fig. 2. Intracellular fusion of R_{18}-labelled influenza virus after uptake by BHK-21 (●, △) or LLC-MK_2D (○) cells. Virus was allowed to bind to the cells for 30 min at 4°C (see the legend to Fig. 1). Subsequently, the cells were washed at 4°C, 2 ml of prewarmed Hanks/Hepes buffer was added and the cells were further incubated at either 20°C or 37°C for the indicated periods of time. Then the cells were washed with cold Hanks/Hepes buffer and the cell-associated fluorescence was determined at 4°C. Error bars represent standard error of the mean.

low pH in a compartment of the endocytic pathway. It is well established that inhibitors of vacuolar acidification, such as NH_4Cl, block the infection of cells by influenza virus [22]. Our present observations indicate that this inhibition of infection is due to a lack of fusion of the viral envelope with the endosomal or lysosomal membrane.

To investigate whether influenza virus fuses at the level of the endosomal compartment or within the lysosomes, the interaction of the virus with cells was studied at 20°C. At this temperature there is no transport from endosomes to lysosomes [21,24,25]. Yet, we did observe a significant degree of fluorescence dequenching at 20°C (Fig. 2), suggestive of a reaction between the viral envelope and the endosomal membrane [4]. However, as both endocytosis and fusion are temperature dependent, fusion at 20°C was considerably slower than at 37°C.

TABLE II

EFFECT OF INHIBITORS OF VACUOLAR ACIDIFICATION ON THE INTRACELLULAR FUSION OF INFLUENZA VIRUS

$(1.5–2)\cdot10^6$ BHK-21 cells on 35-mm plastic petri dishes were incubated for 30 min at 4°C with 40 μg of influenza virus in 0.5 ml of Hanks/Hepes buffer with or without inhibitors. After washing, the cells were incubated at 37°C for a further 60 min in 2 ml of Hanks/Hepes buffer with or without inhibitors. The cells were washed with cold Hanks/Hepes buffer and the cell-associated fluorescence determined at 4°C. Monensin was obtained from Calbiochem (La Jolla, CA) and added from an ethanolic solution.

Inhibitor	Fluorescence increase (%)
None	30.0 ± 1.1
NH_4Cl (20 mM)	0.6 ± 3.1
Monensin (9 μM)	8.5 ± 1.1

The kinetics of fusion shown in Fig. 2 are a composite of the rates of virus internalization, acidification of intracellular compartments and fusion. To investigate the kinetics of fusion of intra-endosomal virus directly, cells were incubated with virus for 60 min at 37°C in the presence of 20 mM NH_4Cl. During the incubation virus is taken up by the cells [22], but can not fuse due to the elevated intra-endosomal pH. Subsequently, the cells were washed and the medium was replaced by Hanks/Hepes buffer at 37°C, resulting in rapid reversal of the accumulation of NH_4Cl [26]. As shown in Fig. 3, fusion occurred immediately, leading to a 12% increase in fluorescence after only 10 min. This result indicates that the actual fusion reaction is a relatively fast event and that the lag phase (Fig. 2) is likely to be due to the time required for virus internalization. Once again, it is important to note that, during the 60-min period of virus internalization at 37°C in the presence of NH_4Cl, dequenching of R_{18} fluorescence does not occur, indicating that the fluorophore is not transferred to cellular membranes.

At 20°C endocytosis and fusion are slowed down considerably (Fig. 2), causing a significant fraction of the associated virus to remain at the cell surface within an incubation period of 60 min. Upon washing and warming of the monolayers to 37°C, fusion started off with much the same lag phase as after binding of the virus to the cells at

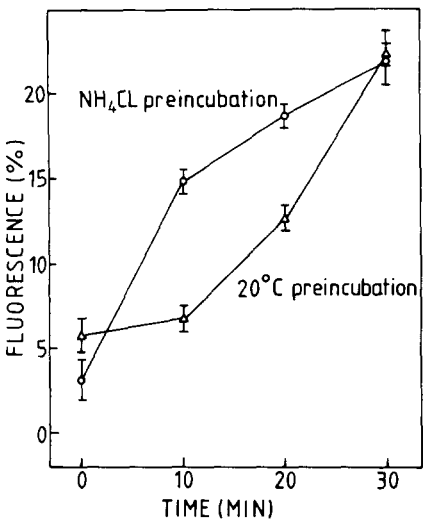

Fig. 3. Intracellular fusion of influenza virus with BHK-21 cells after virus internalization at 37 °C in the presence of NH_4Cl (○) or at 20 °C (△). Virus was allowed to interact with the cells for 60 min at 37 °C in the presence of 20 mM NH_4Cl or at 20 °C. Then the cells were rapidly washed with Hanks/Hepes buffer and the incubation continued at 37 °C for the indicated periods of time. Finally, the cell monolayers were washed with cold Hanks/Hepes buffer and the cell-associated fluorescence was determined at 4 °C. Error bars represent standard error of the mean.

4 °C (Fig. 2). This supports the above view that the lag phase most likely represents the time required for endocytosis, even though the possibility that during an incubation at 20 °C part of the unfused virus accumulates in the endosomes can not be excluded.

In conclusion, in this paper a direct demonstration is given of the intracellular fusion activity of influenza virus after internalization of the virus particles by endocytosis. The fusion reaction is inhibited by compounds that affect the acidification of intracellular vacuoles, indicating that fusion occurs within an acidic compartment. Since the extent of the reaction is still significant at 20 °C, a temperature at which transfer from endosomes to lysosomes is blocked [21], fusion presumably occurs at the level of the endosomes [4]. This conclusion contrasts with the view of Haywood and Boyer [15] who have suggested that influenza virus may infect cells via direct fusion with the plasma membrane at neutral pH.

The increase in fluorescence, resulting from fusion of the virus with endosomal membranes amounts to about 30% after 1 h. Since the fluorescence intensity is proportional to the degree of dilution of the probe [17], this implies that at least 30% of the virus has fused. This represents approximately 500 fused virus particles per BHK-21 cell and about 250 per LLC-MK_2D cell. It is not possible to accurately determine the rate of fusion within the endosomal compartment. However, a lower bound is presented by rate of fluorescence increase after relief of the NH_4Cl block (Fig. 3). Under these conditions a 12% increase of fluorescence occurred during 10 min, corresponding to about 300 virus particles per BHK-21 cell.

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