Biological studies of the fusion function of California serogroup Bunyaviruses

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Like other enveloped viruses, La Crosse virus is capable of inducing membrane fusion after exposure to mild acid. This function is known to have biological significance at the level of the whole organism, since it has been related to infection in a mouse model. In this report the process of fusion-from-within (FFWI) for LAC and other members of the California serogroup of Bunyaviruses is characterized. Like fusion-from-without, FFWI is dependent on pH, temperature, and number of virus particles present in the supernatant of fusing cells. Electron micrographs demonstrate that LAC mediated cell membrane fusion is a rapid, multi-point event, and that other than fusion of their plasma membranes, the cells do not show any morphological change. In agreement with theory, lysosomotropic agents were capable of inhibiting La Crosse virus infection. This inhibition was not due to non-specific toxic effects on infected cells. Finally, fusion studies of other California serogroup members revealed minor differences in the pH of fusion induction in some strains. These differences were consistent with the known subtyping within the serogroup.

Key words: Bunyavirus; fusion; lysosomotropic agents.

Introduction

Many enveloped viruses are capable of mediating the fusion of cell membranes during active infection. This biological feature is most prominent in the herpesviruses, coronaviruses and in the paramyxoviruses,[1,2] though it is also characteristic of some retroviruses like visna and LAV/HTLV III.[3] It is thought that fusion may facilitate the cell-to-cell spread of virus, and it may be particularly relevant in infections where exposure to the extracellular fluid may diminish the production of virus, by exposing it to antibody, for example. The phenomenon of pH dependent fusion, which requires acidification of virions or infected cells, is characteristic of other agents which do not appear to fuse whole cells during routine tissue culture infection. pH dependent membrane fusion plays an essential role at an early stage in the process of infection, where it probably mediates the fusion of the viral envelope with intracellular vesicles, which have been shown to be acidic.[4,5] Fusion of the viral envelope with the membrane surrounding the intracellular vesicle then allows the discharge of the viral nucleocapsid into the cytoplasm, thus beginning the process of replication.[6]

The fusion of viruses with intracellular vesicles has been observed through electron microscopy,[7] but the process is so rapid that the detailed study of viral fusion with

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this methodology is impractical. The fusion function can instead be demonstrated by (1) fusing cells that are undergoing an active infection by exposing them to acidic pH for brief periods of time (fusion from within, FFWI), (2) by adsorbing virus to cells and then acidifying them before infection (fusion from without, FFWO) and (3) by observing the hemolysis of red blood cells that have been coated with virus and then acidified.[8, 9] The physical chemistry of fusion is not understood well, but the proteins responsible for this function have been identified in some systems. In the orthomyxoviridae, where fusion has been studied in great detail, fusion of cells and hemolysis of erythrocytes has been shown to correlate with respect to dependence on pH, temperature, and conformational changes in the hemagglutinin, the protein that mediates this function in these viruses.[10, 11]

The Bunyaviridae comprise a large number of enveloped viruses which contain segmented RNA genomes of negative polarity. The fusion function of La Crosse (LAC) virus, a member of the Bunyavirus genus within the Bunyavirus family has been described in an FFWO system[12] and it has been shown to be an important determinant of the biological behavior of this agent.[13]

In this report we describe the process of FFWI in La Crosse and other California serogroup bunyaviruses and relate fusion to the process of entry of these viruses into tissue culture cells. FFWI allows the study of more strains, since it does not require large scale preparation and purification of viruses and their proteins, and the parameters of fusion can be measured much more efficiently than with FFWO.

Results

pH and temperature of FFWI

FFWI was performed with BHK-21 (clone 13) monolayers infected with LAC virus by briefly exposing the cells to mildly acidic pH and then incubating them at 37°C for 30 min. For all experiments, the fusion index (FI—see materials and methods), a measure of fusion based on the number of nuclei within a polykaryon, was determined at least twice. The pH at which a LAC virus infected monolayer fused was found to be similar to the pH at which fusion was observed in the FFWO assay,[12] where the pH was dropped after adsorbing large quantities of virus to cells at 4°C. Fusion of BHK-21 cells began when the pH was dropped to 6.4 and was maximal at pH 6.2 or
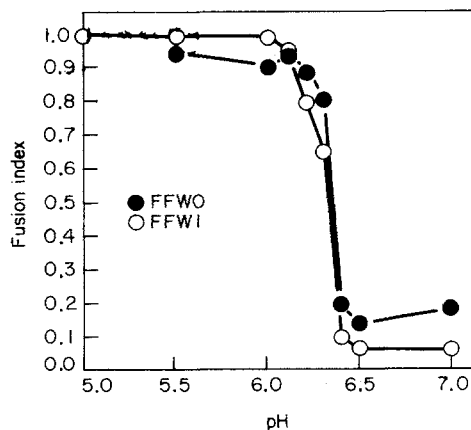
Fig. 1. pH of activation of FFWI and FFWO. FFWO was performed as described previously, and FFWI as described in 'Materials and methods'. The FI was determined as described for approximately 1000 nuclei per data point.
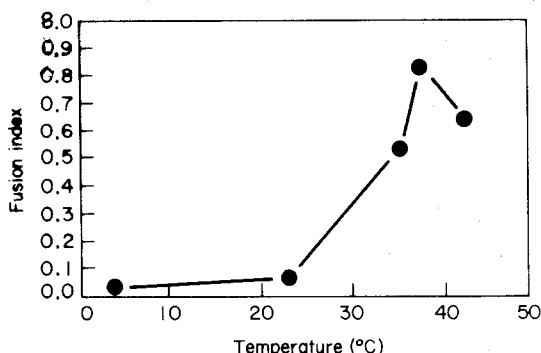
Fig. 2. Temperature dependence of FFWI. Monolayers of BHK-21 cells in 24 well plates were infected with LAC virus at MOI = 5 and incubated at 35°C for 8 hours. The media was then removed and briefly exposed to pH 5.5 media (see 'Materials and methods') pre-equilibrated to the indicated temperature. The cells were then refed with maintenance media and maintained at the indicated temperature for 30 min. The FI was then determined for 800–1000 nuclei.

below. There were slight variations in the pH of maximal fusion with different passage cells. Early passage BHK-21 cells fused more extensively with LAC virus, and reached maximal fusion at a slightly higher pH. The pH curves for FFWO and FFWI are represented in Fig. 1.

As we had noted previously for FFWO[12] there was an absolute requirement for temperatures above 4°C for the fusion of cells (Fig. 2). At room temperature FFWI proceeded slowly, and the assays were routinely done at 37°C. At temperatures above 42°C the monolayers degenerated somewhat.

Parameters of infection

Fusion mediated by LAC could could be demonstrated with monolayers infected with a range of multiplicities of infection (MOI) provided the infection was allowed to proceed until a threshold titer of virus was reached. The pH optima were not affected by differences in the MOI. As illustrated in Fig. 3, the fusion index related to the titer of the virus in the supernatant of infected cultures and fusion was observed once the supernatant reached a threshold titer of around 10^5 plaque forming units (pfu)/ml.
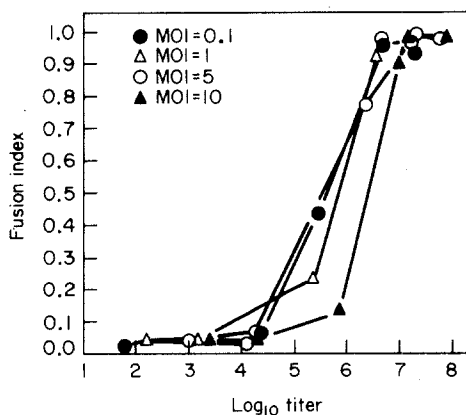

Fig. 3. Relationship between viral titer and FFWI. BHK-21 cells in 24 well plates were infected with LAC virus at different multiplicities, as indicated. At 2-hour intervals the supernatants from duplicate wells were harvested and frozen for titration at a later time and the FI was determined as described in the 'Materials and methods' section, using >800 nuclei per data point.

Electron microscopy

Electron micrographs of cells undergoing FFWI were obtained in order to visualize the fusion of cell membranes and to determine whether whole virions were present in the vicinity of the fusing membranes.

Infected cell cultures showed numerous virions within smooth intracellular vesicles, or maturing into such vesicles in perinuclear areas, or trapped within extracellular spaces. We did not see any virions budding at the plasma membrane. This morphology is characteristic of cultures infected with LAC or other members of the Bunyaviridae.[14, 15]

**Fig. 4.** Early fusion of BHK cells. A confluent BHK monolayer was infected with LAC virus and 8 hours later it was exposed to acidified media for 60 s, then neutralized and incubated at 37°C for 7.5 min. The cells were then fixed *in situ* as described. The efficient and synchronous fusion of adjacent cells is clearly evident from the many interruptions in the cellular plasma membranes (arrowheads). Bar represents 4 μm.

   We looked at infected cells which had been acidified briefly and then placed at 37 °C
for varying time points. Control cultures consisted of cells that had been infected but
maintained at neutral pH (with buffer as described in the Methods section) or
uninfected cultures exposed to acid buffers. Infected, acid treated cultures demon-
strated fusion of plasma membranes with neighboring cells such that the cytoplasm
of adjacent cells could be clearly seen as continuous. Thin sections of monolayers
fixed at 7.5 min after the induction of fusion by acidification showed multipoint fusion
events in which gaps along adjacent peripheral membranes of varying lengths were
apparent (Figs 4 and 6(a)). At this early time point, the cells maintained their relative
spacing vis-a-vis neighboring cells and otherwise maintained a normal ultrastructural
appearance. At 15 min after acid treatment, the gaps within peripheral membranes
were more numerous and extensive, and residual stretches of unfused plasma mem-
branes could often not be assigned to individual nuclei with certainty. Later time
points demonstrate a progressive disappearance of residual plasma membranes with
a concomitant randomization of cytoplasmic organelles and nuclei (Fig. 5). Many
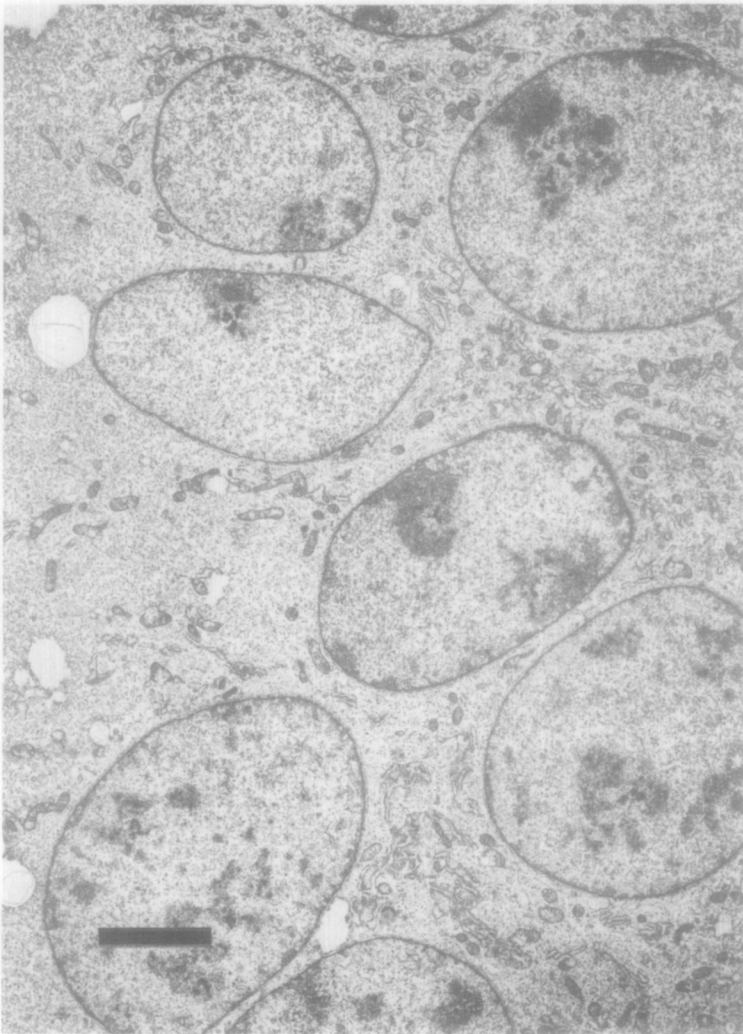
Fig. 5. Late fusion of BHK cells. Thin section of BHK monolayer which was infected and treated as
described in Fig. 4 but incubated for 45 min after the induction of fusion. Note large syncytia with random-
ization of nuclei and without evidence of intervening plasma membranes. Bar represents 4 μm.
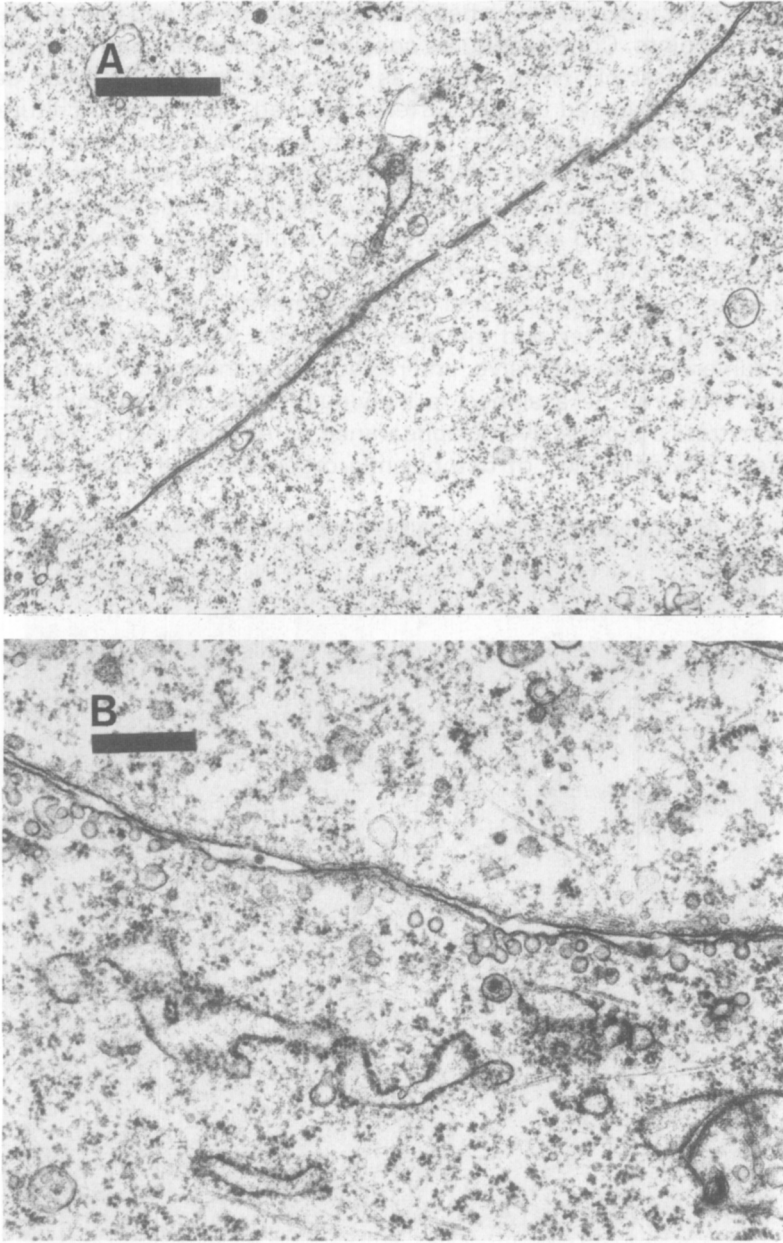
Fig. 6. High magnification view of fusing membranes. Higher magnification of residual plasma membrane fragments within developing syncytia revealed numerous independent fusion events (a) and highly active membranes with many vesicles in association with the discontinuous membranes (b). Fixation was 7.5 min after the induction of fusion. Bars represent 1 μm (a) and 0.5 μm (b).

areas of the monolayers fixed at 30 min, and most areas of the monolayers fixed at 45 min after induction of fusion showed extensive syncytia consisting of 10–20 nuclei with no evidence of residual intervening membrane (Fig. 5). In contrast to the dramatic changes occurring at the plasma membranes of fusing cells, the ultra-structural appearance of individual cytoplasmic organelles, internal membranes, and intravesicular virions remained essentially unchanged.

Samples in which discontinuous, fusing membranes were sectioned perpendicu-larly often revealed numerous membrane vesicles adjacent to, or continuous ,with,

residual membrane structures (Fig. 6(b)). Similarly, coated vesicles were also seen continuous with these membranes (not shown). It therefore appears likely that membrane dissolution is accomplished by microvesiculation, consistent with accepted mechanisms of membrane transfer.

FFWI of other CE viruses

We found that other members of the California serogroup of viruses were equally capable of mediating FFWI. The pH of activation of the fusion function was similar to that of the prototype LAC for 4 of 6 strains tested (Snowshoe hare, San Angelo, California Encephalitis BFS-283, and Inkoo). Two viruses, Jamestown canyon and trivittatus had a pH of activation of fusion that was slightly but reproducibly below that of LAC virus, as noted in Fig. 7. We had previously observed that some of the variants of LAC virus selected with neutralizing monoclonal antibodies had alterations in the pH of fusion activation,[16] however the variation noted in Jamestown canyon and trivitattus was much less pronounced.
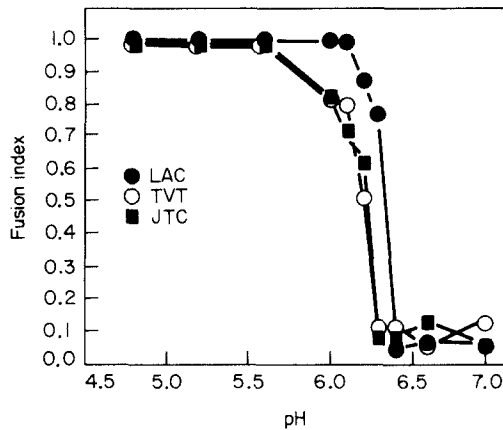

Fig. 7. FFWI by LAC and other California serogroup viruses. California serogroup viruses were obtained from the Yale Arbovirus Research center collection, and have been described previously.[22] The pH of activation of FFWI was determined in at least two separate experiments, performed with uniform multiplicities of infection and time course of fusion, as described in 'Materials and methods'. The FI was determined by counting approximately 1000 nuclei per data point. The figure represents the curves obtained with LAC virus as well as Jamestown canyon virus (JC) and trivitattus virus (TVT). Other California serogroup viruses studied which had curves identical to LAC were: Snowshoe hare, San Angelo, California Encephalitis BFS-283 and Inkoo.

Lysosomotropic agents alter infection

Lysosomotropic agents are compounds that can change the pH of endosomes and lysosomes from their usual acidic range towards neutrality.[17] In a number of systems they have been shown to inhibit infection by retarding the initial steps of cellular penetration.[18] Amantadine, one of the most commonly used lysosomotropic agents, has antiviral activity in vivo, and it is used in the prevention of human influenza. However, amantadine (0.5 mM) is reported to raise the endosomal pH to 5.5,[4] a level that is still acid enough to allow fusion to occur with LAC and other bunyaviruses. Methylamine (10 mM) is estimated to raise the pH of endosomes to 6.3,[19] a pH that is just at the borderline for the activation of LAC virus fusion. We used methylamine to test the hypothesis that lysosomotropic agents would inhibit LAC virus internalization, as measured by the subsequent production of virus. The results of these experiments are shown in Figs 8(a) and 8(b).
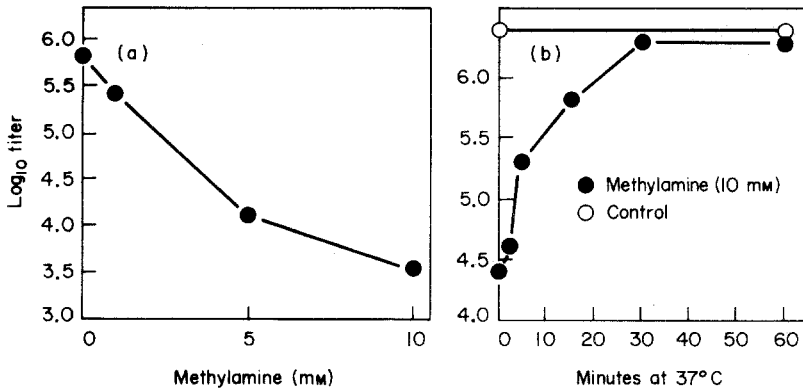
Fig. 8. Methylamine can inhibit infection with LAC virus. (a) BHK-21 cells in 24 well plates were incubated with LAC virus at 4°C at an MOI = 5, washed and refed with maintenance media containing the indicated concentrations of methylamine and placed at 35°C. Eight hours later (approximately one replicative cycle) the supernatants were harvested and frozen for titration. Duplicate wells were inoculated with each concentration of methylamine and titrated separately. (b) Methylamine at 10 mM was added to cultures treated as above at the indicated time points following the shift to 35°C. Duplicate wells were titrated separately.

For these experiments LAC virus was adsorbed on BHK-21 monolayers at 4°C. The virus was then allowed to internalize and begin infection by raising the temperature to 37°C. If methylamine (10 mM) was added at the time of internalization of virus the titer measured at 8 hours after infection dropped by >99% ($10^{7.2}$ pfu/ml vs $10^{4.8}$ pfu/ml). If the drug was instead added 15 min after the beginning of viral internalization, there was minimal effect, with the titer at 8 hours reaching 10^7 pfu/ml. The inhibitory effect is concentration dependent as shown in Fig. 8(a). At a concentration of 1 mM, there is modest inhibition of infection by methylamine added at the time of internalization.

Similar results were obtained with chloroquine (0.5 mM), which like methylamine raises the pH of endosomes to a level incompatible with LAC fusion[16]. When at a concentration (5 mM) that was 10-fold higher than that used in determinations of its effect on lysosomal pH,[4] amantadine also inhibited LAC infection, with decreases in titer of 4 orders of magnitude (results now shown). At lower concentrations it did not have a marked effect on virus titer.

We were able to overcome the effects of methylamine by exposing the adsorbed virus to mildly acidic pH, thus bypassing the endosomal pathway for the entry of LAC virus. Under these conditions virus envelopes presumably fuse with the plasma membrane, discharging the internal components directly into the cytoplasm. If the methylamine effects had been due to direct inactivation of virus or other toxic effects on cellular metabolism, acidification would not have bypassed its inhibition.

Discussion

We have shown that FFWO and FFWI are independent measures of the same phenomenon, the fusion function of the LAC virus glycoproteins. Elsewhere[13] we have demonstrated that this fusion function is an important determinant of the biology of these agents, and that altered fusion activation pH can affect the behavior of LAC in vertebrate and invertebrate hosts (ref. 13 and Sundin & Beatty, personal communication). We can also demonstrate fusion with other California serogroup viruses. In contrast to our experience with some antigenic variants of LAC virus,[16] in our sample of other members of the serogroup there was only minimal variation in the

pH of activation of this function, and this variation was consistent with the accepted serological relationships between these viruses.

The process of pH dependent fusion has been related to the entry of viruses via endosomes. As such, it resembles other receptor–ligand interactions, where the endosomal pH plays a crucial part in either the recycling of receptors[21] or, in the case of enveloped viruses, the activation of a fusion function that mediates the discharge of viral contents into the cytoplasm. Our experiments with lysosomotropic agents, which raise the pH of endosomes and other acidic vesicles, confirm the role of endosomes in the entry pathway of LAC and other California serogroup viruses. The time course of inhibition by methylamine (Fig. 8(b)) showing that the effect of this antibiotic is prominent only early in the time course of infection, suggests that internalization is a rapid event. Similarly, the inhibition was dependent on the concentration of the compound, consistent with the fact that the effect on intralysosomal pH of these chemicals is related to its concentration.[4] This is in agreement with findings in other systems.[19]

We were able to overcome the effects of lysosomotropic agents by exposing viruses to acid prior to internalization, presumably because the viral envelopes then fused with the plasma membrane, and endocytosis was not required. Since the viruses had been exposed to methylamine prior to and during acidification, this rules out non-specific toxic events by the compound. Experiments now in progress will attempt to visualize these events morphologically.

The fusion events observed here could have resulted from an acid induced activation of viral glycoproteins present on mature particles trapped between adjacent cells, or from glycoproteins present as integral proteins in infected plasma membranes, or both. The extent to which infected BHK cells express LAC viral glycoproteins at surface membranes is unclear. Although numerous extracellular virions were observed between cells, fusion events could not be correlated with the presence or amount of extracellular virions.

Materials and methods

Viruses
The origin and passage history of LAC virus has been described previously.[22] Other California serogroup viruses were obtained from the Yale arbovirus research center.[23]

FFWI
FFWI was performed on LAC and other California serogroup viruses as described previously.[13] A subconfluent monolayer of BHK-21 (clone 13) cells on 96-well plates (3.5×10^4 cells/well) was inoculated at a multiplicity of infection of 0.1 pfu/cell, kept at 4°C for one hour, washed with PBS and incubated with MEM plus 2% fcs (maintenance media) for 18 h at 35°C. At that point the cells were washed again with PBS and then exposed to acidic pH in the form of serum-free MEM with 10 mM morpholinoethanesulfonic acid adjusted to the appropriate pH with NaOH. The acidic media was replaced with maintenance media after 30–60 s and the cells were further incubated at 37°C (or at other temperatures for experiments described in Fig. 2) for 30 min. The cells were then fixed and stained with Geimsa (Difco Laboratories, Detroit MI), and counted. The fusion index (FI) was determined as FI = 1 − (C/N) where C and N are the numbers of cells and nuclei, respectively.

Electron microscopy

Confluent monolayers of BHK-21 cells in 24 well plates were infected with LAC virus at an MOI = 5 and maintained at 35°C. Eight hours after infection the monolayers were washed and exposed for 60 s to pre-warmed buffer (see FFWI, above) adjusted to either pH 7.0 or 5.5, then replaced with maintenance media. The monolayers were maintained at 37°C and fixed at 0, 7.5, 15, 30 and 45 min after the induction of fusion. For fixation the monolayers were washed with MEM and fixed *in situ* at 4°C in 50 mM sodium cacodylate buffer, pH 7.0/2% glutaraldehyde/50 mM $CaCl_2$. Equivalent cultures were stained with Giemsa as described above, to enable monitoring by light microscopy.

After fixation the monolayers were washed in 100 mM phosphate buffer and processed as described,[15] except that dehydration was accomplished in an ascending ethanol series without propylene oxide. The epon embedded monolayers were separated from the plastic plates, selected areas were affixed to epon blanks, and sectioned parallel to the plane of the monolayer. Sections were stained with uranyl acetate and lead citrate and observed and photographed in a Jeol B electron microscope which was calibrated with a grating replica with 2160 lines per millimeter.

Lysosomotropic agents

Methylamine, chloroquine and amantadine were obtained from Sigma. BHK-21, clone 13 cells in 24 well plates were chilled to 4°C for 15 min and inoculated with LAC virus at a multiplicity of infection of 5 pfu/cell and the inoculum and cells then maintained at 4°C for one hour. The monolayers were washed twice with ice cold MEM (Gibco) without fetal calf serum, covered with maintenance medium (MEM with 2% fcs) and placed at 35°C. Methylamine and other lysosomotropic agents were added to the monolayers at the appropriate concentration either just prior to placing the cells at 35°C (time 0) or at the specified time period after warming the cells. The supernatants were harvested after 8 hours and titrated as described.[22]

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