Infectious Entry Pathway of Influenza Virus in a Canine Kidney Cell Line

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ABSTRACT The entry of fowl plague virus, an avian influenza A virus, into Madin-Darby canine kidney (MDCK) cells was examined both biochemically and morphologically. At low multiplicity and 0°C, viruses bound to the cell surface but were not internalized. Binding was not greatly dependent on the pH of the medium and reached an equilibrium level in 60–90 min. Over 90% of the bound viruses were removed by neuraminidase but not by proteases. When cells with prebound virus were warmed to 37°C, part of the virus became resistant to removal by neuraminidase, with a half-time of 10–15 min. After a brief lag period, degraded viral material was released into the medium. The neuraminidase-resistant virus was capable of infecting the cells and probably did so by an intracellular route, since ammonium chloride, a lysosomotropic agent, blocked both the infection and the degradation of viral protein.

When the entry process was observed by electron microscopy, viruses were seen bound primarily to microvilli on the cell surface at 0°C and, after warming at 37°C, were endocytosed in coated pits, coated vesicles, and large smooth-surfaced vacuoles. Viruses were also present in smooth-surfaced invaginations and small smooth-surfaced vesicles at both temperatures.

At physiological pH, no fusion of the virus with the plasma membrane was observed. When prebound virus was incubated at a pH of 5.5 or below for 1 min at 37°C, fusion was, however, detected by ferritin immunolabeling. At low multiplicity, ~90% of the prebound virus became neuraminidase-resistant and was presumably fused after only 30 s at low pH.

These experiments suggest that fowl plague virus enters MDCK cells by endocytosis in coated pits and coated vesicles and is transported to the lysosome where the low pH initiates a fusion reaction ultimately resulting in the transfer of the genome into the cytoplasm. The entry pathway of fowl plague virus thus resembles that earlier described for Semliki Forest virus.

For enveloped animal viruses, two separate pathways of entry into the host cell have been recognized (1). Sendai virus and other paramyxoviruses are known to fuse their membranes directly with the plasma membrane of the host cell at physiological pH (2). Their nucleocapsids are thereby introduced into the cytoplasm. Fusion is mediated by a viral spike glycoprotein (F) which, to be active, must be present in a proteolytically cleaved form consisting of two sulfhydryl-linked polypeptide chains (F1 and F2) (3, 4). The second pathway, so far best exemplified by Semliki Forest virus (an alphavirus), depends on adsorptive endocytosis and membrane penetration at an intracellular site (5, 6). After binding to the cell surface, Semliki Forest virus is rapidly internalized in coated vesicles and routed into secondary lysosomes via endosomes (large prelysosomal vacuoles). The low pH in the lysosomes apparently triggers a fusion reaction between the virus membrane and the lysosomal membrane that releases the nucleocapsid into the cytoplasm. Membrane fusion thus plays a central role in both pathways of virus entry but the site of fusion and the pH required to activate it differ.

In the case of orthomyxoviruses, the available data on entry are contradictory. A number of studies with influenza virus have shown that virus particles enter the cell by endocytosis (7–11). Moreover, amantadine, an inhibitor of influenza virus infection (12), has been shown to act at an intracellular location (13–16). Other studies suggest, however, that influenza virus, like Sendai virus, may fuse directly with the plasma membrane of the cell (17).

Here we examine the entry of fowl plague virus, an avian influenza A virus, by combined biochemical and morphlogical approaches in an attempt to reconcile the contradictory observations. The Madin-Darby canine kidney (MDCK) cell line,

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which resembles in culture a differentiated epithelium, has been used throughout. These cells possess distinct apical and basolateral surfaces separated by junctional complexes (18). Our studies show that fowl plague virus infects MDCK cells primarily by endocytosis, using an entry pathway remarkably similar to the one we have earlier described for Semliki Forest virus.

MATERIALS AND METHODS

Cells

MDCK cells, obtained from Dr. Daniel Louvard of this institute, were grown at 37°C in 5% CO_2 and routinely passaged twice a week in 75 cm^2 plastic bottles (Falcon Plastics, Oxnard, Calif.) in Eagle's minimal essential medium with Earle's salts (Earle's MEM) supplemented with 10 mM HEPES, pH 7.3, 10% (vol/vol) fetal calf serum (FCS), penicillin (110 U/ml), streptomycin (100 μg/ml), and fungizone (0.025 μg/ml). Cells were released from the plastic using trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA/l in Puck's saline).

Viruses

STOCK VIRUSES: Fowl plague virus, obtained from Dr. H.-D. Klenk (Institut fuer Virologie, Universitaet Giessen), was plaque purified three times on MDCK cells. For virus stock, MDCK cells were infected in a 5% CO_2 atmosphere (0.01 plaque-forming units (pfu)/cell) for 20 h at 37°C in Earle's MEM containing 0.2% (wt/vol) bovine serum albumin (BSA) and penicillin/streptomycin. The stock virus was concentrated by centrifugation at 116,000 g_{max} for 90 min in an SW 27 rotor (Beckman Instruments, Palo Alto, Calif.), resuspended at 4°C overnight in PBS (Dulbecco formulation), containing 0.2% BSA, and stored in aliquots at −80°C.

RADIOACTIVE VIRUS: Fowl plague virus labeled with [^{35}S]methionine was prepared by infecting slightly subconfluent bottles of MDCK cells with stock virus (20 pfu/cell) in Earle's MEM containing 0.2% BSA and penicillin/streptomycin at 37°C in 5% CO_2. After 1 h, the inoculum was aspirated and replaced with 5 ml of Earle's MEM containing 5 μM methionine (one-tenth of the amount present in normal MEM) and 15 mM HEPES, pH 7.3. In another 1.5 h, 1 mCi [^{35}S]methionine was added to each flask and incubation continued for 7.5 h. The virus was concentrated by centrifugation at 116,000 g_{max} for 90 min in an SW 27 rotor and resuspended overnight at 0°C in 50 mM Tris-Cl (pH 7.4) containing 100 mM NaCl (TN). Finally, the virus was purified on sucrose gradients prepared by overlaying a 5.0 ml 25–50% (wt/vol) gradient with an 8.0 ml 10–20% (wt/vol) gradient, both in TN. The gradients were centrifuged for 3 h at 284,000 g_{max} in a Beckman SW 40 rotor. The peak of radioactivity in the lower part of the gradient was pooled and stored in aliquots at −80°C. This material was identified as virus and judged free of contaminating labeled material by SDS PAGE and autoradiography. The titer of preparations was ~1×10^7 pfu/ml which corresponded to 1 pfu/cpm.

UNLABELED VIRUS: Large quantities of fowl plague virus were grown in the allantoic cavity of 11-d-old embryonated chicken eggs and purified as described earlier (19). The purity was monitored by SDS PAGE. Preparations contained ~3×10^7 pfu/μg protein and had a particle-to-pfu ratio of ~100, assuming a particle weight of 3×10^{-16} g (20).

Plaque Titrations

Plaque titrations were performed in duplicate on MDCK cell monolayers (21) grown in 60-mm plastic petri dishes. Experimental samples were serially diluted in PBS/0.2% BSA, and 0.25 ml aliquots were adsorbed to cell monolayers for 1 h at 37°C in 5% CO_2. The inoculum was then removed and the cells were overlayed with 4 ml of Earle's MEM containing 0.1% BSA, 0.1% FCS, penicillin, streptomycin, and 0.9% (wt/vol) agarose (Litex, Denmark). After a 2-d incubation at 37°C in 5% CO_2, plaques were detected by staining with 0.02% neutral red in PBS.

Binding and Uptake Assays

To measure virus binding, MDCK cells were grown to confluence (1–2 × 10^6 cells/dish) in 35-mm diameter plastic petri dishes, washed twice with 1 ml of binding medium, and cooled for 10–15 min. Approximately 40,000 cpm of radioactive fowl plague virus in 200 μl of the appropriate ice-cold binding medium were added to each dish, and the dishes were gently shaken for various times at 0°C. After this incubation, the free virus was removed and the cell monolayers were washed twice with 1 ml of cold binding medium. Cells were then scraped from the dish, the dish was washed twice with 1 ml of cold binding medium, and the cells were pelleted in a conical glass centrifuge tube at 2,000 rpm for 5 min at 4°C. Bound virus is defined as that virus which was cell-associated at this point. After removal of the supernate, pellets were solubilized directly in Rotiszint 22 (Carl Roth KG, Karlsruhe, W. Germany) a detergent-based scintillation fluid, and counted in a Mark III liquid scintillation counter (Searle Analytic, Des Plaines, Ill.).

Variable pH binding media were prepared by supplementing Earle's MEM containing 0.2% BSA with the series of buffers suggested by Eagle (22). Media of pH <6.0 were supplemented with 20 mM succinate. Bicarbonate was omitted from all media used at 0°C or at pH's other than 7.4. Adjustment of pH was at room temperature.

All measurements of virus uptake were done after warming sample dishes for various periods at 37°C in media buffered to pH 7.4 containing sodium bicarbonate. After incubation of labeled virus with cells, the medium was collected and the medium and cells were analyzed separately. The cells were washed, scraped from the dish, pelleted, and counted directly, or washed and incubated with neuraminidase to remove surface-associated virus. In most cases, the neuraminidase was added at a concentration of 5 mg/ml in 1.0 ml PBS, and the dishes were shaken at 0°C for 90 min. After neuraminidase treatment, the neuraminidase solution was removed and the monolayers were scraped and pelleted in PBS/0.2% BSA. Finally, the cell pellet was washed two times with 5 ml of PBS/0.2% BSA and counted. Medium samples were divided into two aliquots. One aliquot was counted directly, the other aliquot was precipitated with an equal volume of 10% (wt/vol) trichloroacetic acid (TCA) on ice for 1 h, centrifuged for 5 min in a microfuge (Eppendorf, Hamburg, W. Germany), and the supernate counted. Insoluble radioactivity was calculated by subtracting radioactivity in the TCA supernates from the total radioactivity in the medium.

Infection by Internalized Virus

To determine whether cell-associated, neuraminidase-resistant virus was capable of infecting the host cell, MDCK cells were grown to confluence in two 24-well plastic trays (5–6 × 10^5 cells/well). The cells were washed with pH 7.4 binding medium and cooled to 0°C. Stock virus was suspended in 0.2 ml of ice-cold binding medium with or without 20 mM ammonium chloride at three different multiplicities and allowed to bind to the cells as described for binding assays above. After 1 h, the inocula were removed from one of the two trays and 1.5 ml of prewarmed, pH 7.4 medium (with bicarbonate and with or without ammonium chloride) was added to each well. The tray was incubated for 20 min at 37°C in a 5% CO_2 atmosphere and then cooled to 0°C by placing it on ice and replacing the warm medium with cold medium. The second tray was not taken through this warming step. Both trays were then processed together through the rest of the experiment. To remove the remaining surface-associated viruses, neuraminidase (10 mg/ml in Earle's MEM with or without 20 mM ammonium chloride) was added to each well, and the trays were shaken at 0°C for 90 min. After digestion, the neuraminidase solution was removed and the monolayers were washed three times in cold pH 7.4 medium with or without ammonium chloride. Next, prewarmed complete MDCK cell growth medium with or without ammonium chloride was added to each well for 1 h to help the cells recover and, finally, pH 7.4 medium with bicarbonate was added and the trays were incubated for 5 h at 37°C in 5% CO_2. The amount of virus produced was determined by plaque titration. A diagrammatic description of this experiment is given in Table I.

Antibodies

Antibodies raised against fowl plague virus spike proteins were prepared by immunizing rabbits with spike protein complexes. These were prepared by the following modification of a procedure used for the preparation of protein micelles (23). Fowl plague virus (1.5 mg) was solubilized at room temperature in 2% Triton X-100 containing 50 mM Tris-Cl (pH 8) and 200 mM NaCl, mixed for 15 min, and centrifuged over a 20–50% sucrose gradient having a zone of 15% (wt/vol) sucrose and 1% (wt/vol) Triton X-100 in the same buffer for 24 h at 284,000 g_{max} in an SW 40 rotor at 20°C. The spike protein peak was located by gel electrophoresis, dialyzed against 500 vol of 50 mM ammonium carbonate at 5°C, and lyophilized. To purify the complexes further, they were redissolved in 50 mM Tris-Cl (pH 8) containing 200 mM NaCl and centrifuged over a second gradient identical to the first except for the absence of the Triton X-100 zone. Spike protein complexes isolated in this manner were judged free of M protein and other viral components by SDS PAGE. We injected 10–20 μg of protein in Freund's complete adjuvant directly into the popliteal lymph nodes of rabbits (24), and boosted this injection subcutaneously 3 wk later with the same amount of protein in Freund's incomplete adjuvant, bleeding the rabbits after 10 d. Anti-fowl virus spike IgG was prepared by standard procedures of ion-exchange chromatography (25).

Immunofluorescence

Immunofluorescence was performed by the indirect technique. MDCK cells were grown to slightly subconfluent density on glass cover slips in 35-mm plastic dishes. The dishes (one cover slip per dish) were washed in pH 7.4 binding medium and cooled on ice. Fowl plague virus (3 μg/dish) in ice-cold binding medium was added and allowed to bind in the cold for 1 h. After removal of the free virus, two cover slips were washed, covered with cold fixative, and brought slowly to room temperature. The other dishes were warmed at 37°C for various times and fixed. To fix the cells, the cover slips were washed three times with PBS (Eisen formulation), incubated for 20 min in formaldehyde (3% (wt/vol) formaldehyde, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ in PBS), and quenched for 10 min in 50 mM NH_4Cl in PBS. Viral antigens on the cell surface were visualized by inverting the cover slips over 25 μl of anti-spike protein IgG (300 μg/ml), washing, and then staining with goat anti-rabbit IgG conjugated to rhodamine. To see both surface and intracellular antigens, fixed monolayers were freeze-thawed once on dry-ice and stained with antibodies (26, 27). Coverslips were mounted in 90% (vol/vol) glycerol in PBS and viewed through a Zeiss photomicroscope III equipped with a Planapo 63 oil immersion objective (Zeiss, Oberkochen, W. Germany). The rhodamine conjugated antibody, a gift of Dr. Daniel Louvard, was affinity-purified and selected by ion-exchange chromatography to have two to three rhodamine molecules per IgG molecule (28, 29).

Electron Microscopy

GENERAL TECHNIQUES: To visualize virus bound to cells and internalized, cells in 35-mm petri dishes were washed, and 60 μg of virus were allowed to bind at 0°C in 200 μl of binding medium, pH 7.4, for 1 h. The samples were then either fixed directly with cold 2.5% glutaraldehyde in 50 mM sodium cacodylate, pH 7.2, 50 mM KCl, 2.5 mM $MgCl_2$ for ~30 min, or warmed at 37°C and then fixed at room temperature. Postfixation with osmium tetroxide, dehydration, and embedding were done as described earlier (30), except that the cell monolayer was scraped with a teflon blade, osmium fixed, dehydrated, and embedded in Epon 812 after centrifugation in BEEM capsules.

IMMUNOLABELING WITH FERRITIN: To detect fusion at the plasma membrane, MDCK cells were grown on microscope slides and the slides washed in binding medium and cooled on a metal plate fitted over an ice bath. A virus suspension containing 60 μg of virus in 200 μl of ice-cold binding medium was added to the cells, a cover slip was placed over the suspension, and the virus binding was conducted for 1 h. After binding, the slides were plunged into 37°C medium at either pH 5.0 or pH 7.4 for 1 min or less and then fixed immediately in formaldehyde (3% formaldehyde, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ in PBS, pH 7.4) at room temperature for 30 min. Binding controls were placed in cold formaldehyde and slowly brought to room temperature. After fixation, unreacted formaldehyde was quenched with 50 mM ammonium chloride in PBS for 10 min, and the cells were conditioned with 0.2% (wt/vol) gelatin in PBS and stained with antibodies by the indirect method. The first antibody was rabbit anti-fowl plague virus spike protein IgG (399 μg/ml in 0.2% gelatin/PBS). This was followed by ferritin conjugated goat anti-rabbit IgG (affinity purified) prepared according to Kishida et al. (31). After washing, the cells were fixed for 1 h at room temperature with 4.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, scraped with a teflon blade, and centrifuged for 5 min in a Beckman microfuge. The pellet was postfixed for 2 h at 4°C in 2% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4, stained in block overnight in 0.5% magnesium uranyl acetate, dehydrated in graded ethanol, and embedded in Epon. Thin sections were cut on a Reichert OmU_3 ultramicrotome (Reichert-Jung, Vienna, Austria), double-stained with 2% aqueous uranyl acetate and lead citrate (32), and observed with a Philips 400 or Zeiss EM 10A electron microscope.

Low pH–dependent Association

To measure neuraminidase-resistant association of fowl plague virus with MDCK cells after low pH treatment, radioactive fowl plague virus was allowed to bind to the cells at 0°C and the monolayer washed free of unattached virus. The dishes were then transferred to the surface of a water bath at 37°C and flooded with warm medium at various pH's for different times. The medium was then aspirated and the monolayers were rapidly cooled on ice, treated with neuraminidase, washed, scraped, and counted as described above.

Miscellaneous Techniques

Solutions of ammonium chloride and chloroquine were freshly prepared before each experiment by dissolving the salts directly in the appropriate medium and, when necessary, correcting the pH to the original value. Protein determinations were performed as described by Lowry et al. (33). Virus preparations were analyzed on SDS polyacrylamide gels, using the Laemmli buffer system (34). The gels were prepared with a 10–15% (wt/vol) acrylamide gradient (acrylamide/bisacrylamide = 75) and contained 8 M urea for improved resolution (35).

Sources of Reagents

Cell culture media and reagents were purchased from Gibco Biocult (Glasgow, Scotland). FCS was obtained from either Gibco or Boehringer/Mannheim (Mannheim, W. Germany). Neuraminidase (Type V from *Clostridium perfringens*, purified, 0.86 U/mg using NAN-lactose) was obtained from Sigma Chemical Co. (St. Louis, Mo.). All buffers and chloroquine were also obtained from Sigma Chemical Co. Reagents for electron microscopy were purchased from either Serva Biochemicals (Heidelberg, W. Germany) or Ladd Research (Burlington, Vt.). Other chemicals were reagent grade. [^{35}S]methionine (>600 mCi/mmol) was purchased from Amersham Corp. (Amersham, England).

RESULTS

Association of Fowl Plague Virus with Cells at 0°C

To examine the association of fowl plague virus with MDCK cells at 0°C, trace quantities (0.03 pfu/cell) of [^{35}S]methionine-labeled virus were incubated with monolayers for various periods of time at pH 7.4. Since MDCK cells were used, binding was expected to occur only to the apical surface (18). As shown in Fig. 1, binding increased immediately upon addition of virus and reached a plateau of ~40% of the added virus in 60–90 min. To assess the effect of pH on virus binding, trace quantities of labeled virus were incubated with MDCK cells at 0°C in medium buffered from pH 5.0 to 8.3 (Fig. 2). After 1 h, 35–55% of the applied virus was cell-associated at all pH's tested, with somewhat higher binding at acidic than at neutral pH. As the effect of pH was not very dramatic, binding of fowl plague virus in all further experiments was conducted at pH 7.4.

Fowl plague virus was tightly and specifically associated with cells at 0°C and pH 7.4. Incubation with cold medium or PBS failed to elute the virus (Fig. 3). Proteinase K, which efficiently removes Semliki Forest virus from the surface of baby hamster kidney 21 cells (BHK-21) (5, 6) and vesicular stomatitis virus from MDCK cells (unpublished observations), had no effect (Fig. 3); nor did other proteases, such as trypsin and chymotrypsin. We found, however, that neuraminidase in the cold released up to 96% of the cell-associated virus (Fig. 3) without affecting the integrity or viability of the cells. In addition, MDCK cells pretreated with neuraminidase failed to bind virus. These results are consistent with the known role of sialic acid in the viral receptor (36). The efficient release by

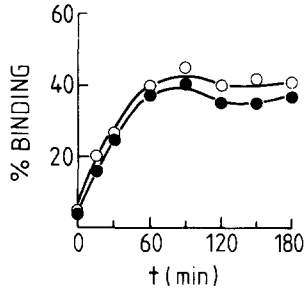

FIGURE 1 Kinetics of fowl plague virus binding at pH 7.4. Trace quantities of [^{35}S]-fowl plague virus (0.03 pfu/cell) were added to MDCK cell monolayers at 0°C in Earle's MEM, pH 7.4, with (●—●) or without (○—○) 20 mM ammonium chloride for various times. At the end of the binding period, cell-associated radioactivity was determined. Data is expressed as percent of added radioactivity bound.

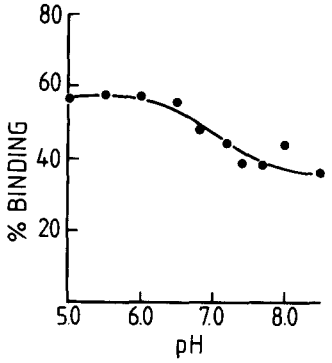

FIGURE 2 Binding of fowl plague virus at different pH's and 0°C. Trace quantities of [^{35}S]-fowl plague virus (0.03 pfu/cell) were bound to MDCK cell monolayers at 0°C for 1 h and cell associated radioactivity determined. Data is expressed as percent of added radioactivity bound.

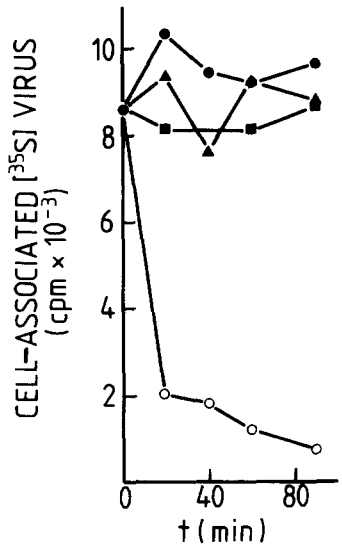

FIGURE 3 Effect of proteinase K and neuraminidase on cell-bound fowl plague virus. Trace quantities of [^{35}S]-fowl plague virus were bound to MDCK cell monolayers at 0°C for 1 h at pH 7.4. After washing the cells with cold binding medium, they were incubated with either binding medium (●—●), neuraminidase at 5 mg/ml in PBS (○—○), proteinase K at 1 mg/ml in PBS (▲—▲), or with PBS (■—■) at 0°C. At the indicated times cells were washed and scraped in PBS containing 0.2% BSA and 1 mM PMSF, and cell-associated radioactivity determined.

neuraminidase indicates that virtually all of the virus remains at the cell surface at 0°C. The radioactive virus which failed to dissociate after neuraminidase treatment was probably located on the surface as well, since prolonged neuraminidase treatment or higher neuraminidase concentrations further reduced the background radioactivity. Neuraminidase treatment using 5 mg of enzyme/ml for 90 min in the cold was routinely used in our further experiments to differentiate between surface-bound virus and either endocytosed or fused virus (see below).

Virus Association with Cells at 37°C

When cells were incubated with virus at 37°C instead of 0°C, we expected to observe internalization of viruses and possibly fusion of virus with the plasma membrane. We first investigated the fate of prebound virus. Cells, to which trace quantities of [^{35}S]methionine-labeled fowl plague virus had been bound for 1 h in the cold, were rapidly warmed to 37°C. At various times after warming, the medium was collected and aliquots were precipitated with TCA and counted. The cells were treated with neuraminidase to remove the surface-bound virus, washed, scraped, and counted.

As shown in Fig. 4*a*, two effects were observed during the first 15 min at 37°C: ~70% of the viruses were converted into a form no longer releasable by neuraminidase treatment; and ~30% of the viruses dissociated into the medium in an acid-precipitable form. From 30 min onward, acid-soluble radioactivity appeared in the medium, increasing steadily throughout the experiment (Fig. 4*a*). The most likely interpretation of these results is that, upon warming, part of the cell-bound virus became irreversibly associated with the cells by either endocytosis or fusion. Some of this virus was degraded after a short lag period. The release of acid-precipitable virus into the medium is probably a consequence of the viral neuraminidase activity. At 37°C, this neuraminidase may digest sialic acid from the viral receptors before internalization can occur. This interpretation is supported by the observation that the amount

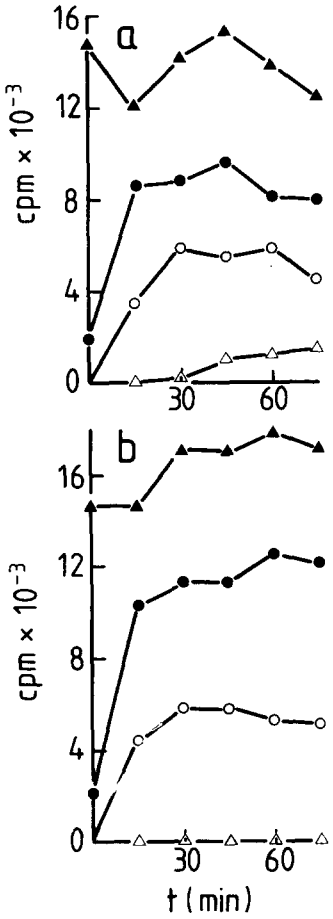

FIGURE 4 Synchronous uptake of fowl plague virus by MDCK cells. Trace amounts of [^{35}S]-fowl plague virus were bound for 1 h to MDCK cell monolayers at 0°C and then warmed rapidly to 37°C in medium without (*a*) or with (*b*) 20 mM ammonium chloride. At the indicated times, the medium was removed and the cells treated with 5 mg/ml neuraminidase for 90 min at 0°C. Cell associated and TCA soluble and precipitable radioactivities in the medium were determined. Binding in this experiment was 30% of the added radioactivity. Cell associated, neuraminidase-resistant radioactivity (●—●); TCA-precipitable radioactivity in the medium (○—○); TCA-soluble radioactivity in the medium (△—△); total recovered radioactivity (▲—▲).

of TCA-precipitable material in the medium decreases during continued incubation at 37°C (Fig. 4), probably by rebinding of released virus to the cells and internalization. Since only trace quantities of virus were originally bound (<1 pfu or 100 particles/cell), many undigested viral receptors should be available to the free virus.

The previous experiment, using virus prebound to cells at 0°C, examined virus uptake into cells under synchronous conditions. We also examined the fate of virus simply added to cells at 37°C (Fig. 5). Trace quantities of labeled virus were incubated with cells at 37°C and, at various times, the medium was collected from two dishes and TCA-precipitated. The cells from one dish were washed, scraped, and counted to measure total cell-associated radioactivity. The second dish was incubated with neuraminidase, and the cells were counted to determine the amount of internalized virus. The difference between the two dishes represented the amount of virus associated with the cell surface at each time-point. Neuraminidase-resistant radioactivity increased linearly for ~1 h. Concomitantly, an equivalent reduction in medium radioactivity was seen. No significant amount of TCA-soluble radioactivity could be detected in the medium during the experiment.

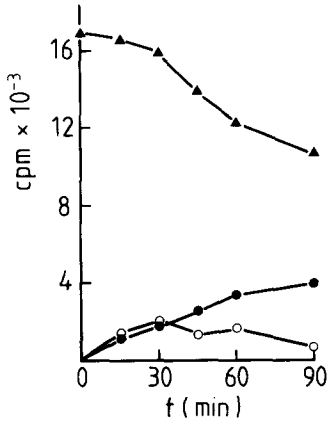

FIGURE 5 Uptake of fowl plague virus at 37°C. Trace quantities of [^{35}S]-fowl plague virus in MEM (0.5 ml/dish), pH 7.4, were added to MDCK cells at 37°C. At various times, the medium was removed and the cells cooled on ice. One set of dishes was washed with cold medium and total cell-associated radioactivity determined. A second set of dishes was treated with 5 mg/ml neuraminidase at 0°C for 90 min, washed, and neuraminidase-resistant counts determined. Aliquots of medium were TCA-precipitated to determine acid-soluble and -precipitable radioactivities. (○—○) Neuraminidase sensitive; (●—●) neuraminidase resistant; (▲—▲) TCA precipitable; TCA soluble counts were negligible.

The results of experiments described so far indicate that fowl plague virus associated with MDCK cells rapidly becomes neuraminidase-resistant at 37°C and is degraded into acid-soluble material after a lag period. These observations are consistent with virus entry by endocytosis, but they do not exclude fusion of the virus with the plasma membrane. To gain more information about the nature of the virus-cell association, virus uptake was examined by immunofluorescence and electron microscopy.

Morphology of Virus-Cell Association at 37°C

Cells with prebound fowl plague virus were warmed to 37°C and examined at different times by indirect immunofluorescence using anti-spike protein antibodies (Fig. 6). Observations were made of both surface staining and surface plus internal staining by freeze-thawing duplicate samples to permit access to intracellular antigens (27). As illustrated in Fig. 6, cells which were fixed immediately after binding virus at 0°C, and stained, were covered with bright spots. Freeze-thawing of equivalent samples did not change this pattern. After 1 h at 37°C, almost no stained material was evident on the cell surface. Internally, fluorescence was associated mainly with perinuclear vesicles. This experiment demonstrated that virtually all the virus antigens originally present on the cell surface had been removed after warming for 1 h, and that a large fraction of them had been internalized. Since no spike protein antigens were detected on the cell surface, extensive fusion of the viral membrane with the plasma membrane seemed unlikely.

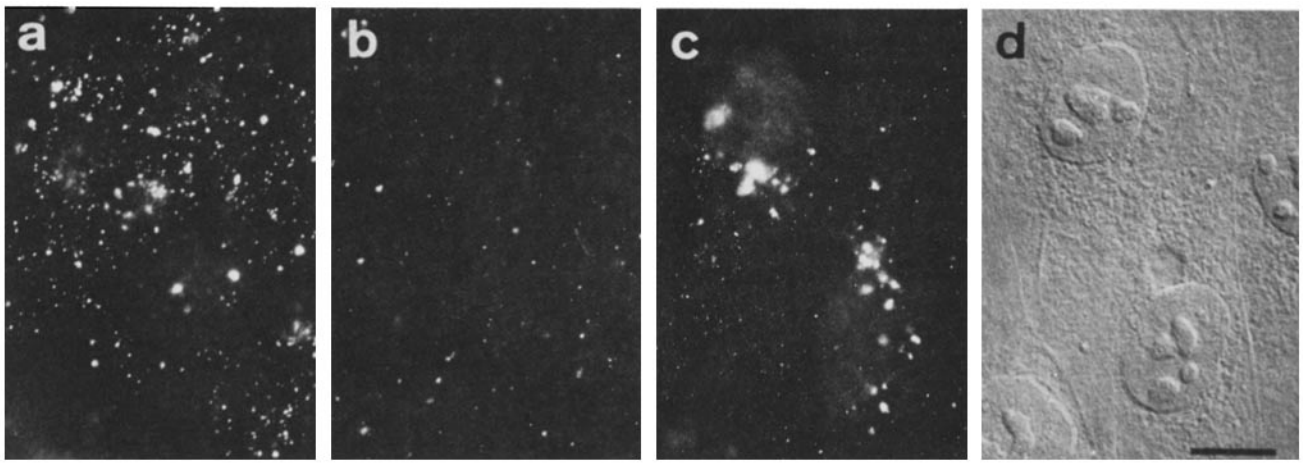

FIGURE 6 Binding and uptake of fowl plague virus into MDCK cells. Fowl plague virus (3 μg) was allowed to bind to MDCK cells grown on glass cover slips for 1 h at 0°C and pH 7.4. Two cover slips were fixed immediately with cold formaldehyde solution. The remaining samples were warmed at 37°C for different times and then fixed. One sample from each time-point was stained with antibodies against fowl plague virus spike glycoproteins and with a rhodamine-conjugated second antibody to visualize viral antigens on the cell surface. A second sample from the same time-point was freeze-thawed to make viral antigens inside the cell also accessible to antibodies and then stained. (*a*) Surface stain after binding but before warming. (*b*) Surface stain after 1 h at 37°C. (*c*) Surface plus internal stain after 1 h at 37°C. (*d*) Nomarski optics of the field shown in c. Bar, 5 μm. × 2,200.

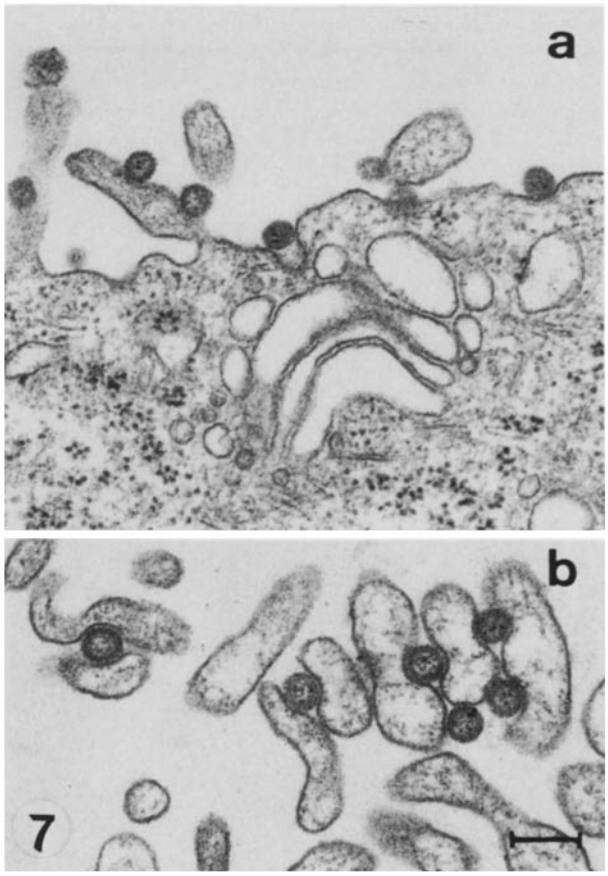

FIGURE 7 Binding of fowl plague virus to the MDCK plasma membrane. Fowl plague virus (60 μg) was allowed to bind for 1 h in pH 7.4 binding medium at 0°C, and the cells were fixed with cold glutaraldehyde. Virus particles were bound to microvilli and other parts of the apical plasma membrane (*a* and *b*). In many instances, microvilli were attached to each other by virus particles. Bar, 0.2 μm. *a*, × 45,000; *b*, × 50,000.

The localization of virus after binding to the cells and warming to 37°C was also investigated by transmission electron microscopy (Figs. 7 and 8). After binding at 0°C, viruses were observed to associate primarily with microvilli (Fig. 7). Often several microvilli were connected to each other by their interaction with common virus particles (Fig. 7*b*), and the microvillar membrane sometimes partially wrapped itself around the virus particles (Fig. 7*b*). Binding to other areas of the plasma membrane was also seen. Consistently, throughout the experiment, viruses were also observed bound to smooth-surfaced invaginations frequently localized at the bases of microvilli (Fig. 8*a*, *b*, and *c*). A characteristic of these was the close (16- to 20-nm) apposition of the viral and plasma membranes all along the perimeter of the invagination.

Upon warming to 37°C, the virus particles entered coated pits and coated vesicles (Fig. 8*d–i*). The vesicles seen in Fig. 8*h* and *i* are half-coated and half-smooth. Part of the smooth membrane of the vesicle illustrated in Fig. 8*i* is in the form of a bleb. These vesicles may be in the process of losing their coat and perhaps fusing with other vacuoles. Occasionally, viruses were also observed in small uncoated vesicles with the same membrane proximity seen in the smooth pits (Fig. 8*c*). After 5- or 10-min warming, viruses were also visible in larger, smooth-surfaced vacuoles and multivesicular bodies (Fig. 8*j–l*). The former may be endosomes, prelysosomal endocytotic vacuoles devoid of lysosomal hydrolases (5, 37, 38). At no time was virus fusion with the plasma membrane observed. Several earlier morphological studies of influenza virus entry have also failed to detect fusion (7–11).

From observations of synchronous entry of Semliki Forest virus into BHK cells, it was previously concluded that the virus particles passed sequentially through coated pits, coated vesicles, endosomes, and lysosomes (5). Although fowl plague virus probably follows a similar pathway, it is difficult to reach a definite conclusion from the morphological experiment described here because of the asynchrony imposed by the detachment and rebinding of the virus during the warming period. It is possible, however, to conclude that, at high multiplicities, entry occurs primarily by endocytosis and not by fusion at the plasma membrane. The neuraminidase-resistant viruses observed in the previous experiments with radioactive fowl plague virus were therefore most likely endocytosed.

Inhibition by Lysosomotropic Agents

A number of studies have shown that lipophilic weak bases such as ammonium chloride and amantadine inhibit the entry of influenza virus into their host cells and block infection (12–16, 39–45). Similar inhibitory effects of lipophilic amines have been reported in other virus systems (5, 6, 46, 47). On the basis of our studies with Semliki Forest virus, we have suggested that the inhibition depends on the lysosomotropic character of the agents (5, 6, 47); they are known to accumulate in the lysosomes, to elevate the lysosomal pH, and to inhibit many of the lysosomal hydrolases (48, 49). We have here studied the effects of ammonium chloride and chloroquine on fowl plague virus infection in MDCK cells, thereby confirming and extending the previous findings obtained with other influenza viruses (12–16, 39–45).

As shown in Fig. 9 ammonium chloride inhibited infection as measured by virus production. The optimal inhibitory concentration of ammonium chloride was 10 mM. To be effective, ammonium chloride had to be present during the first hour after virus-cell contact, indicating that its effect was mainly on an early stage of infection. Chloroquine also blocked infection at an optimal concentration of 0.5 mM, but was inhibitory when added one hour after the virus. The binding of the virus to the cell surface and its subsequent internalization (conversion into neuraminidase-resistant form) was not significantly affected by the presence of inhibitory concentrations of ammonium chloride (Figs. 1 and 4*b*). The degradation of virus proteins into acid-soluble material was, however, totally inhibited (Fig. 4*b*), suggesting that the lysosome was important in this degradation. Lysosomal degradation of other endocytosed proteins such as low-density lipoprotein and polypeptide hormones is also blocked by ammonium chloride and other lysosomotropic weak bases (50–52).

The inhibition of viral production by lysosomotropic agents suggests that the lysosomes are involved in the early stages of influenza virus infection. The inhibitors provide, moreover, a convenient tool to study some of the features of the infective pathway.

Infection by Internalized Virus

In a previous section, it was shown that about half of the fowl plague virus bound to MDCK cells in the cold became neuraminidase-resistant after 10 min at 37°C. It was also established that the infection initiated by fowl plague virus was

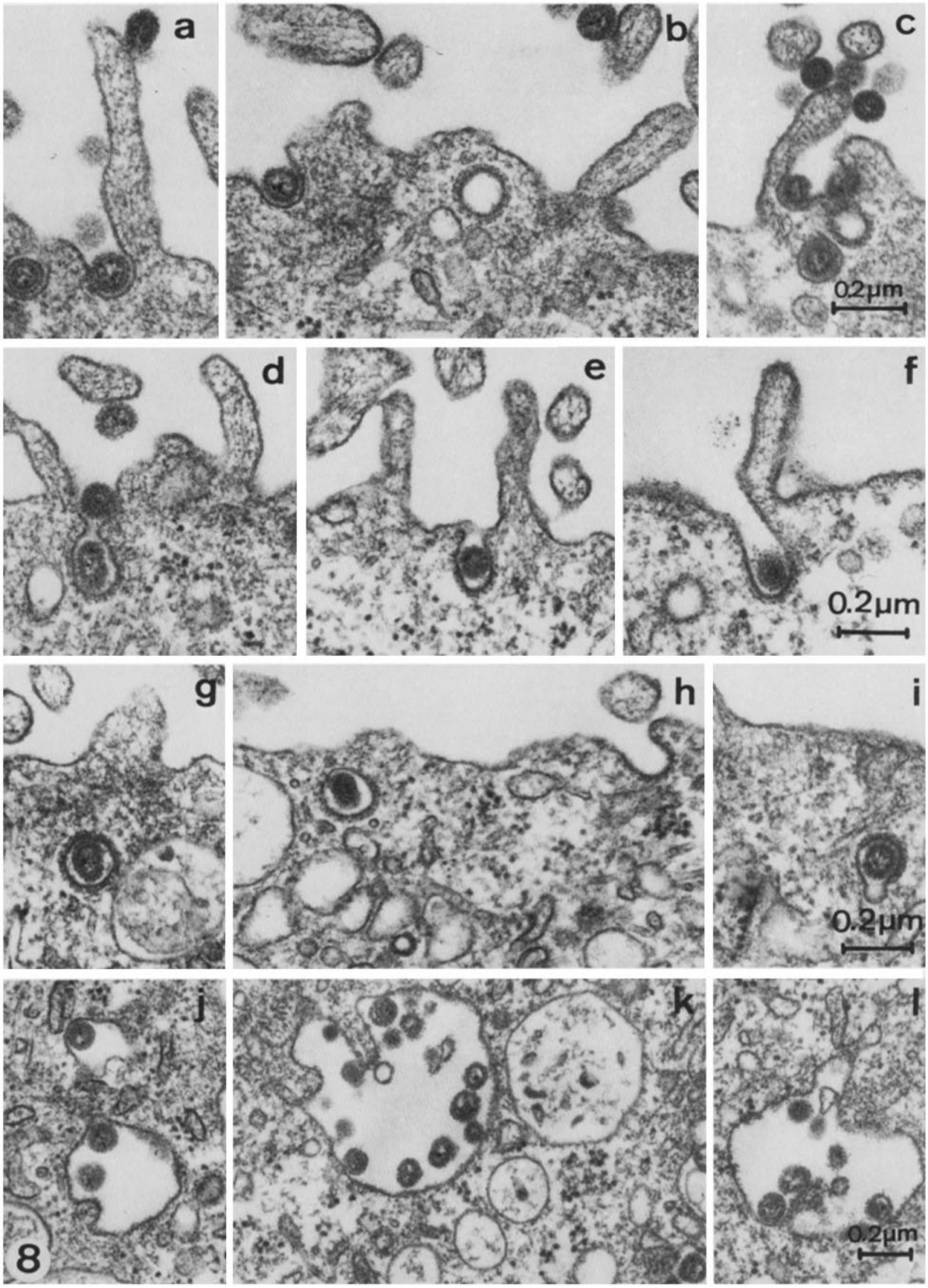

FIGURE 8 Stages of fowl plague virus entry into MDCK cells. Cells with prebound virus were warmed at 37°C for different times and then fixed with glutaraldehyde at room temperature. Within 5 min, virus particles were seen in smooth surfaced pits and vesicles (*a*, *b*, and *c*), coated pits (*d*, *e*, and *f*) and coated vesicles (*g*, *h*, and *i*). In *f*, the sample was stained with anti-fowl plague virus spike protein IgG and then with ferritin-goat anti-rabbit IgG after formaldehyde fixation (see Materials and Methods). This image demonstrates that part of the virus particle was tightly associated with the membrane since only the exposed part is labeled with ferritin. After 10 min, viruses were observed in endosomes (*j*) and multivesicular bodies (*k* and *l*). The images shown in *a*, *b*, and *c* were after 2 min warming; in *d*, *e*, *g*, *k*, and *i* after 5 min warming, in *f* after 1 min warming, and in *j*, *k*, and *l* after 10 min warming. *a–i*, × 62,500; *k–l*, × 50,000.

sensitive to lysosomotropic drugs. To demonstrate directly that virus defined biochemically as neuraminidase-resistant was also capable of infecting MDCK cells through the route sensitive to the lysosomotropic agents, the following experiment was conducted. Confluent MDCK cells grown in two 24-well plastic trays were cooled to 0°C, and fowl plague virus was allowed to bind at three different multiplicities (8, 5, and 2 pfu/cell) in the presence of 20 mM ammonium chloride (see Table I). One tray was warmed to 37°C for 20 min to allow some of the virus to enter the cells while the other was left in the cold. Both trays were then treated with neuraminidase in the cold to remove all virus which still remained on the cell surface. After several washes, warm medium without ammonium chloride was added and the cells were incubated at 37°C for 6 h. Two controls were included in each tray: a positive control in which ammonium chloride was never present, and a negative control with ammonium chloride present throughout the experiment.

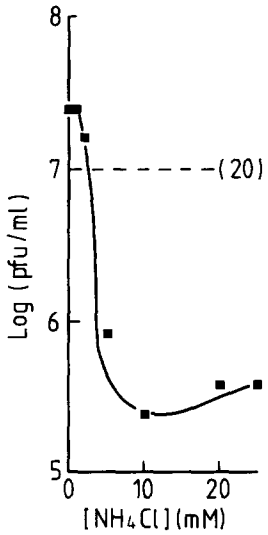

FIGURE 9 Inhibition of fowl plague virus infection by ammonium chloride. Fowl plague virus (20 pfu/cell) was added to MDCK cell monolayers together with ammonium chloride at the indicated concentrations. After 6 h infection, the medium was removed and plaque titrations performed. The dotted line indicates the amount of virus produced when the agent was added at the concentrations given in parentheses 1 h after initial virus-cell contact.

One possible result of this experiment was that only cells which had been both warmed for 20 min and not incubated with ammonium chloride during the final 6 h would produce significant amounts of virus. Such a result would indicate that virus entering the cell during the 20-min warming period was capable of infecting the cells, and that the entry pathway traversed the intracellular compartment sensitive to ammonium chloride. A second possibility was that the neuraminidase-resistant (internalized) virus was not infectious. In this instance only low, background levels of virus would be produced.

As shown in Table I, the first possibility was observed. Viruses which entered cells during the 20-min warming period produced a high level of infection when ammonium chloride was absent throughout the experiment or absent during the last 6 h at 37°C. The infection was completely blocked when ammonium chloride was present throughout the experiment. Samples not warmed for 20 min produced a reduced level of virus infection which could also be abolished by ammonium chloride. The residual infection in the unwarmed tray probably resulted from viruses left on the cell surface after neuraminidase treatment which then infected the cells by the intracellular route. This interpretation is supported by the observed reduction in the background infection at lower multiplicities.

This experiment shows, therefore, that viruses which entered cells during a brief warming period are capable of infecting the cells, and that the infectious pathway probably traverses the lysosome. It strongly suggests, therefore, that the fowl plague virus infection of MDCK cells is initiated by endocytosis of the virus.

Virus Fusion at Low pH

Semliki Forest virus, which infects cells through the endocytotic pathway, has been shown to possess a low pH-induced fusion activity (5, 19, 53, 54). This activity transfers the viral genome from the lysosome to the cytoplasm (5, 6, 54). Semliki Forest virus can also be artificially fused at the plasma mem-

TABLE I

Infection by Internalized Fowl Plague Virus

	A warmed			B not warmed		
Tray	1	2	3	1	2	3
Bind 0°C, 60 min	−*	+	+	−	+	+
Warm 37°C, 20 min	−	+	+		(No warming step)	
Neuramidase 0°C, 90 min	−	+	+	−	+	+
Wash 0°C 1	−	+	+	−	+	+
2	−	+	+	−	+	+
3	−	+	−	−	+	−
MDCK-medium 37°C 60 min	−	+	−	−	+	−
pH 7.4 medium 37°C 5 h	−	+	−	−	+	−
	Virus production as percent of control‡ (Log pfu)					
Multiplicity (pfu/cell)						
8	100 (6.62)	1 (4.68)	120 (6.70)	41 (6.23)	1 (4.79)	46 (6.28)
5	100 (7.06)	0.5 (4.79)	76 (6.94)	12 (6.15)	0.5 (4.76)	10 (6.07)
2	100 (6.58)	0.5 (4.30)	69 (6.42)	7 (5.43)	0.5 (4.26)	6 (5.34)

* + and − are used to denote presence or absence of 20 mM ammonium chloride in the medium.

‡ Virus production is given as percent of the positive control A1 and as log pfu in the medium at the end of the experiment (in parentheses).

brane of cells by lowering the medium pH (5, 19, 54). If fowl plague virus infects MDCK cells by an endocytotic pathway passing through the lysosomes, it might also be expected to fuse at the plasma membrane if exposed to low pH. This seemed especially likely since low pH-dependent hemolysis and cell-cell fusion had been recently demonstrated for influenza viruses (19, 55, 56). To test this, cells with prebound virus were suspended in media of pH 5.0 and pH 7.4 for 1 min at 37°C and examined by transmission electron microscopy after indirect ferritin immunolabeling. In the cells kept at pH 7.4, ferritin was associated only with virus particles and not with the cell surface. No fusion of the virus with the cell surface was observed. In contrast, ferritin was attached to the plasma membrane only in samples exposed to low pH (Fig. 10). In several cases, clear continuity between the cell and virus membranes was observed, with ferritin only bound to the protruding virus profile (Fig. 10 *a* and *b*). Fusion of viruses to membrane vesicles apparently shed from the cells was also observed (not shown). Low pH treatment of MDCK cells in the absence of virus produced some disturbance of the plasma membrane but did not induce artifactual ferritin binding.

To quantitate low pH-induced fusion of fowl plague virus to the MDCK cell plasma membrane, cells with prebound radioactive virus were incubated for 30 s at 37°C with media

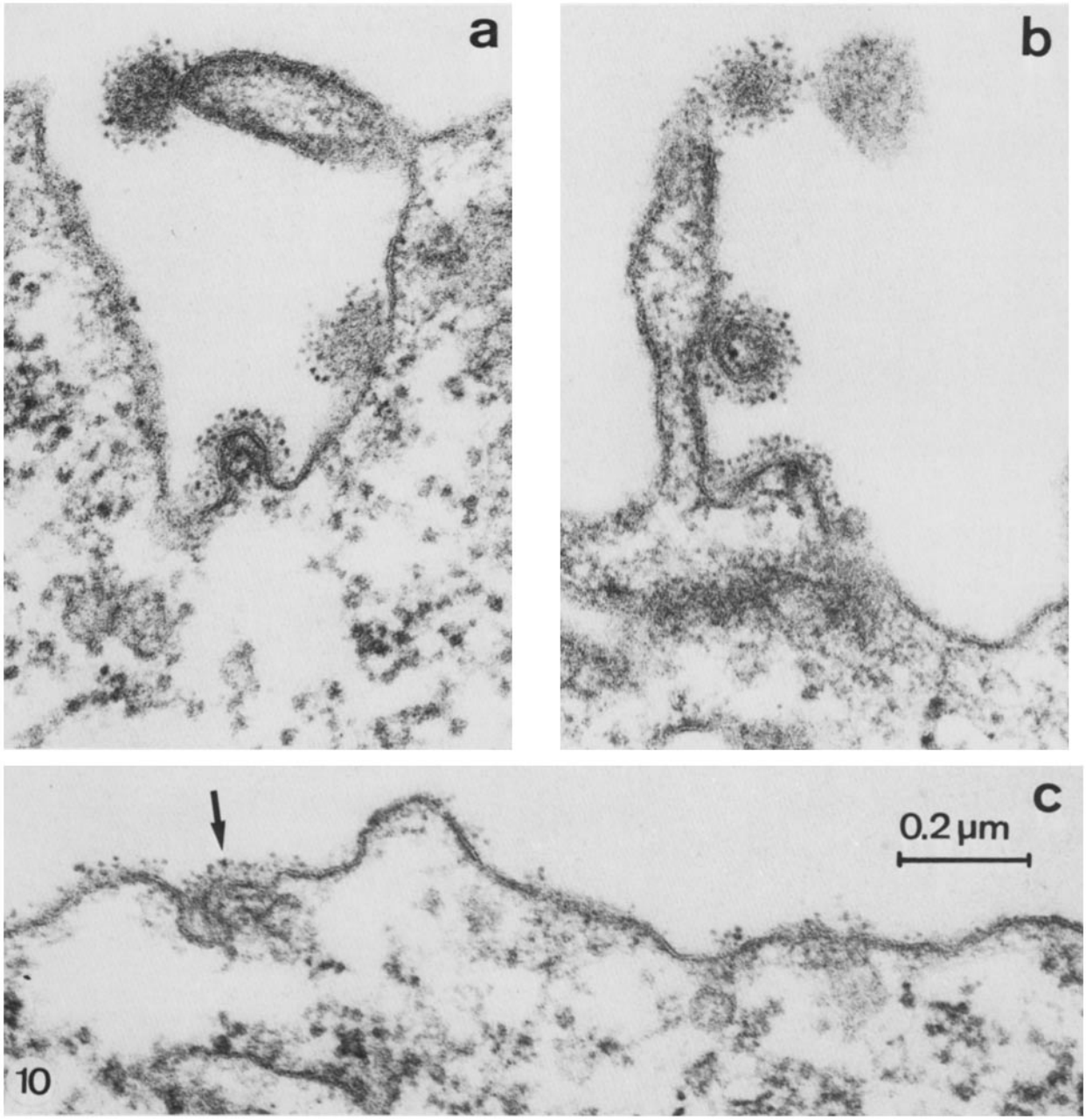

FIGURE 10 Fusion of fowl plague virus at the MDCK plasma membrane. Fowl plague virus (60 μg) was bound to MDCK cells for 1 h at 0°C and fusion was induced by incubating the cells for 1 min at pH 5.0 and 37°C. The cells were then fixed with formaldehyde and immunolabeled with anti-fowl plague virus spike IgG and ferritin-conjugated goat anti-rabbit IgG. Viruses are clearly recognizable by the ferritin attached to their membrane. In *a* the nucleocapsid is still clearly visible. In *b* the capsid is barely recognizable, but the virus shape and ferritin-labeled spikes are still obvious. In *c* the spike proteins have presumably diffused in the plane of the membrane away from the site of fusion. A virus profile is still detectable (arrow). Bar 0.2 μm. × 106,000.

buffered between pH 5.0 and 6.5, and then treated with neuraminidase. We expected both fused and endocytosed viruses to be resistant to removal by neuraminidase. As the results in Fig. 11 illustrate, considerably more radioactivity became resistant to neuraminidase at pH's <5.5 than at pH 7.4. The amount of radioactivity cell-associated at pH 7.4 was presumably due only to endocytosis, since fusion was not seen at this pH by electron microscopy. In a second experiment, the rate of fusion was compared to the rate of endocytosis (Fig. 12). Within 30 s of warming at low pH, 90% of the bound virus radioactivity was neuraminidase-resistant. At pH 7.4 the rate was much slower (Fig. 12, see also Fig. 4).

Semliki Forest virus can be made to infect cells by fusing it to the host cell plasma membrane at low pH (5, 54). Unlike the normal infection through the endocytotic pathway, infection by cell-surface fusion is resistant to lysosomotropic agents (5, 54). Several attempts to infect MDCK cells with fowl plague virus by low pH–induced fusion at the plasma membrane in the presence of ammonium chloride were, however, unsuccessful.

These experiments clearly demonstrate that fowl plague virus is capable of fusing with the MDCK cell plasma membrane at mildly acidic pH. Furthermore, the fusion process is distinct in rate and extent from the uptake by endocytosis observed at physiological pH.

DISCUSSION

Our results suggest that fowl plague virus infects MDCK cells by the following entry pathway: (*a*) binding to the cell surface; (*b*) endocytosis in coated and possibly smooth-surfaced vesicles; (*c*) transport to endosomes and secondary lysosomes; and (*d*) fusion between the virus membrane and the lysosomal membrane which results in release of the nucleocapsid into the cytoplasm.

Binding

Like other orthomyxoviruses (36), fowl plague virus binds to sialic acid-containing molecules on the MDCK cell surface. At 0°C, the proteases tested do not remove attached viruses, but high concentrations of neuraminidase are effective. No virus enters the cells in the cold in contrast to earlier reports (57). After warming to 37°C, large amounts of virus prebound at 0°C spontaneously fall off. This phenomenon is greater when more virus is bound and is probably due to digestion of the sialic acid on the virus receptor by the viral neuraminidase. Similar virus shedding has been seen when hemagglutination reactions with influenza virus are conducted at 37°C (58). The binding of fowl plague virus to MDCK cells is not greatly pH dependent and reaches an equilibrium value of ~40% at pH 7.4 in 60–90 min. In electron micrographs, viruses are bound primarily to microvilli.

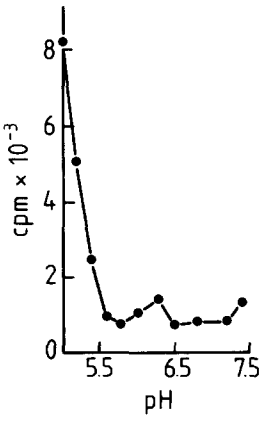

FIGURE 11 Association of fowl plague virus with MDCK cells at acidic pH and 37°C. Trace quantities of [^{35}S]-fowl plague virus were bound to MDCK cells for 1 h at 0°C and pH 7.4. After washing with cold binding medium, cells were treated with media buffered to the indicated pH's for 30 s at 37°C, incubated with 5 mg/ml neuraminidase for 90 min at 0°C, and cell-associated radioactivity determined. In this experiment ~25% of the added radioactivity was bound and 62% of the bound virus was neuraminidase resistant after pH 5.0 treatment.

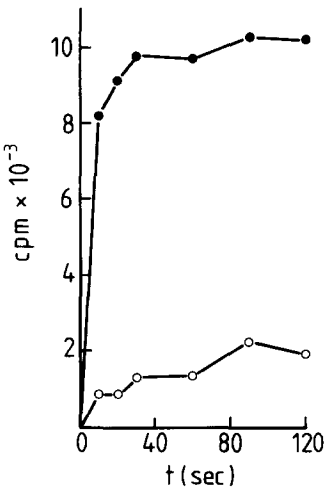

FIGURE 12 Kinetics of low pH induced cell association of fowl plague virus. Trace quantities of [^{35}S]-fowl plague virus were bound to MDCK cells for 1 h at 0°C and pH 7.4. Binding in this experiment was 30%. After washing with cold binding medium, cells were incubated for the indicated times with pH 5.0 medium or pH 7.4 medium at 37°C, rapidly cooled, treated with 5 mg/ml neuraminidase for 90 min, and cell-associated radioactivity determined. About 90% of the bound virus was neuraminidase-resistant after 30 s at pH 5.0. ●—●, pH 5.0; ○—○, pH 7.4.

Endocytosis

Our morphological results indicate that the surface-bound virus is quickly endocytosed. At early times (<10 min), virus particles are seen in coated pits and coated vesicles and, later, in large, smooth-surfaced vacuoles and multivesicular bodies (Fig. 8). These structures are probably endosomes or secondary lysosomes (5, 37, 38). Our electron microscopy findings are in agreement with the previous results of Dales and Choppin (7), Baechi (8), Dourmashkin and Tyrrell (9), and Patterson et al. (11). These authors concluded that influenza virus enters cells of the chorioallantoic membrane and Ehrlich ascites tumor cells by engulfment into vesicles or viropexis (59). Patterson et al., in particular, observed influenza virus entering chick cells in coated pits and coated vesicles and found that the entry process was insensitive to a number of metabolic and cytoskeletal inhibitors (11). The latter study also demonstrated, by ruthenium red staining, that the virus-containing vacuoles were sealed off from the extracellular medium and were, therefore, true vesicles and not fortuitous sections through membrane pits.

In addition to coated pits and vesicles, fowl plague virus is frequently seen in uncoated depressions in the membrane and smooth vesicles completely filled by the virus particle. Similar structures were also noted by Baechi (8) after influenza virus

binding to cells at 0°C. The importance of these smooth-surfaced pits and vesicles to influenza entry is unclear. They are not observed during Semliki Forest virus endocytosis in BHK-21 cells (5, 6) or during infection of MDCK cells by vesicular stomatitis virus (unpublished observations). They may be caused by the plasma membrane progressively wrapping itself around the virus particle as more spikes are bound. Their formation may therefore be induced by the virus particle. The possible involvement of smooth-surfaced vesicles in the antibody-induced endocytosis of histocompatibility antigens has been previously noted (60).

At pH 7.4, we found no evidence for fusion between the virus and the plasma membrane such as clear bilayer continuity. Indirect ferritin immunolabeling also failed to detect spike proteins in the plasma membrane when cell-bound virus was warmed for 1 min at physiological pH. The efficacy of the ferritin immunolabeling technique to detect membrane fusion was confirmed by our observations of fusion when it was artificially induced by low pH (Fig. 11). Our results are therefore in disagreement with those of Morgan and Rose (17) who concluded that influenza virus enters cells by fusion at the plasma membrane at physiological pH. Others have also concluded that influenza viruses do not fuse with the host cell plasma membrane (7–11). Dourmashkin and Tyrrell (9), in particular, ruled out cell surface fusion as an entry mechanism after tilt analysis of the electron microscope specimens.

The morphological observations that the majority of viruses are endocytosed are corroborated by our biochemical studies which show that fowl plague virus is rapidly internalized into MDCK cells. The half-life of surface-bound virus is 10–15 min. Uptake is not influenced by the presence of ammonium chloride. Dourmashkin and Tyrrell (9) and Skehel et al. (15) previously demonstrated that the drug amantadine, which blocks influenza infections, does not inhibit endocytosis. Dourmashkin and Tyrrell (9) and Patterson et al. (11) have also shown by electron microscopy that influenza virus endocytosis is unaffected by the cytoskeletal inhibitors colchicine and cytochalasin B.

The endocytosis of influenza virus thus closely resembles the endocytosis of both Semliki Forest virus (5, 6) and many physiological ligands such as low-density lipoprotein (61), asialoglycoproteins (38), polypeptide hormones, (62) α_2-macroglobulin (63), and lysosomal enzymes (64). Two types of endocytosis are recognized: phagocytosis and pinocytosis (65). Phagocytosis is particle dependent and is blocked by cytochalasin B. Pinocytosis, on the other hand, is particle independent, occurs continuously in many cell types, and is not inhibited by cytoskeletal drugs. Pinocytosis is responsible for fluid-phase uptake, and the uptake of ligands bound to the cell surface. The latter process is termed receptor-mediated when a physiological ligand is endocytosed and adsorptive when a nonphysiological ligand is taken up (6). In the case of Semliki Forest virus, which enters (BHK) baby hamster kidney cells by adsorptive endocytosis, uptake is inhibited at low temperature, is not sensitive to colcemid and only marginally sensitive to cytochalasin B, and is partially dependent on oxidative phosphorylation (6). Influenza virus, therefore, like Semliki Forest virus, probably uses a constitutive cellular process to enter cells.

Role of Lysosomes in Infection

Several lines of evidence suggest that a large fraction of the virus particles enters the lysosome or a lysosome-related compartment after endocytosis. Viral antigens are seen by immunofluorescence in perinuclear vacuoles, a distribution characteristic of lysosomes (5). Viral particles are also detectable in various intracellular vacuoles and in multivesicular bodies by electron microscopy. Dourmashkin and Tyrrell (9) also observed influenza virus in large vacuoles. The structures they saw may, however, be endosomes because they failed to stain for acid phosphatase (5, 37, 38).

The participation of lysosomes in the infective pathway of fowl plague virus entry is suggested by the results with lysosomotropic agents. We find that infection of MDCK cells by fowl plague virus is inhibited by ammonium chloride and chloroquine. Similar results have been previously reported for ammonium chloride, alkylamines, and amantadine in other cell systems (12–16, 39–45). All these drugs are less effective if added 1 h after infection and do not significantly change virus binding or internalization. Because the major effect of these agents on the cells is to raise the intralysosomal pH (48, 49), it is reasonable to conclude that they affect a lysosome-dependent stage in the infectious process. This interpretation is supported by detailed studies on amantadine. Kato and Eggers (13), using an assay for uncoating based upon dye sensitivity of the genome, determined that amantadine had slight effect on virus uptake but blocked release of the viral genome. In agreement with this, Skehel et al. (15) found that amantadine inhibits viral gene expression. Recently, rimantadine (an analog of amantadine) was shown to prevent viral RNA from becoming RNAse-sensitive after entry into MDCK cells (16). In control experiments, the viral RNA became degraded by RNAse added to freeze-thawed lysates of cells to which influenza virus had been adsorbed for 90 min at 37°C.

In addition to preventing infection, ammonium chloride also prevents degradation of [^{35}S]methionine-labeled fowl plague virus into acid-soluble radioactivity in the medium after internalization (Fig. 4). This material appears after a lag of 30 min in a synchronous infection in the absence of ammonium chloride and probably results from the action of lysosomal or cytoplasmic hydrolases on viral proteins. During Semliki Forest virus infection of BHK cells, acid-soluble radioactivity is also released into the medium after a similar lag period (5, 6). The study of Marsh and Helenius (6) demonstrated that the released material was, in this case, almost exclusively free methionine with [^{35}S]methionine-labeled virus. Degradation of Semliki Forest virus was, however, only partially blocked by chloroquine. Lysosomotropic weak bases have been shown to prevent degradation of low-density lipoprotein, insulin, and epidermal growth factor (50, 52, 66) which enter lysosomes after receptor-mediated endocytosis.

Further evidence that fowl plague virus infects the cell by way of an intracellular vacuole was obtained from an experiment which showed that infection could be traced primarily to endocytosed viruses (Table I). After inhibiting virus entry by antibodies, Patterson et al. (11) also concluded that endocytosed influenza virus was infectious.

Although the effects of ammonium chloride in blocking the infection and preventing degradation of the virus suggest critical involvement of the lysosome in the infective process, our data do not allow us to exclude the participation of a prelysosomal vacuole. This compartment must, however, have lysosomal characteristics, such as low pH and a complement of degradative enzymes, to account for our observations.

Membrane Fusion

We have demonstrated here that fowl plague virus possesses

a low pH–induced fusion activity which is capable of introducing the nucleocapsid into the cytoplasm of MDCK cells. When virus bound to the cell surface is exposed to a pH of 5.5 or below, a fusion reaction is triggered between the virus membrane and the plasma membrane. This was demonstrated both biochemically (Figs. 11 and 12) and morphologically (Fig. 10). This fusion capacity is probably related to the low pH–dependent hemolytic and cell-cell fusion activities observed in several laboratories, including our own (19, 55, 56). These reports also showed that cleavage of the hemagglutinin (HA) spike to HA1 and HA2, which is necessary for infectivity (67, 68), is also required for fusion. Fusion at the plasma membrane under our conditions did not lead to productive infection of the cells. The reasons for this are unclear. It is possible that low pH is enough to induce membrane fusion but insufficient to permit the genome to uncoat. Lysosomal enzymes, for example, may modify the spike glycoproteins so that their interactions with internal proteins are altered.

The existence of a membrane fusion activity at low pH may explain both how the influenza genome is transferred to the cytoplasm and why lysosomotropic weak bases block uncoating. When the virus particle enters the lysosome or related vacuole, the low pH triggers a fusion between the vacuole membrane and the viral membrane. The genome is thereby introduced into the cytoplasm and initiates replication. When the pH is raised by lysosomotropic drugs, no fusion and thus no uncoating is possible.

Recent amino acid sequence determinations of the HA2 subunit of the influenza hemagglutinin from a number of strains have demonstrated that the aminoterminal segment is both hydrophobic and highly conserved (69). The sequence is homologous to the hydrophobic aminoterminal end of the F1 protein responsible for fusion in Sendai virus (6). Richardson et al. (70) have shown that a series of oligopeptides with amino acid sequences similar to those of the aminotermini of the HA2 and the F1 proteins block virus infection. Moreover, with the paramyxoviruses, cell-cell fusion and hemolysis induced by the viruses was also inhibited. This strongly implicates the hydrophobic aminoterminal segments of HA2 and F1 proteins in the fusion reaction. Maeda and Ohnishi (55) have pointed out that the only charged amino acids in the first 18 residues of the HA2 protein in fowl plague virus are two glutamic acid residues (71), whereas the corresponding positions in the F1 sequence of Sendai virus have uncharged amino acids. If the acidic residues of the HA2 protein were protonated, as might occur in the lysosome, the amino terminal segment would become uncharged and possibly hydrophobic enough to induce membrane fusion. At neutral pH, the acidic residues would be charged and might not allow the protein segment to interact with the lipid bilayer to initiate the fusion reaction. In the three-dimensional structure of the influenza hemagglutinin recently reported by Wilson et al. (72), the aminoterminal region of the HA2 subunit is located near the base of the spike protein. The low pH may induce a conformational change in the hemagglutinin molecule that would move the aminoterminal segment of the HA2 protein into a favorable position for fusion.

The pathway used by fowl plague virus to enter and infect cells may indeed be the 'Achilles heel" by which a number of agents gain access to the cell interior. In addition to Semliki Forest virus, vesicular stomatitis virus also follows this route in MDCK cells (manuscript in preparation). Recently, the effects of diphtheria toxin have been shown to be blocked by lysosomotropic agents and the inhibition overcome by low pH treatment (73, 74). Presumably, the toxin uses the endocytotic-lysosomal route to reach the cytoplasm.

More detailed knowledge of endocytosis and the mechanisms responsible for virus uncoating should lead to a greater understanding of current antiviral therapies and permit the design of more specific drugs. From the work described here, for example, it seems possible that the clinically important compound amantadine (12) inhibits influenza virus infections by raising the lysosomal pH, thus preventing intracellular virus fusion and uncoating. Because this effect of amantadine is on a general cellular rather than specific viral function, use of the drug may cause clinical side effects. This possibility should be kept in mind when future therapeutic applications of amantadine are considered.

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