Differentiation of Acid-pH-Dependent and -Nondependent Entry Pathways for Mouse Hepatitis Virus

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Early events of infection of MHV were studied in comparison with those of VSV, which is known to enter cells by an endocytic pathway. Treatment of mouse L-2 fibroblasts with ammonium chloride, chloroquine, or dansylcadaverine inhibited infection of MHV to a much lesser degree than that of VSV, suggesting a relatively minor role for the endocytic pathway and functional endosomes in MHV infection. Endocytosis of MHV and VSV into L-2 cells was assayed by the recovery of infectious (i.e., not uncoated) viruses from homogenates of cells harvested within the first few minutes of infection (and treated with protease to remove surface-bound virus). The results thus suggest that while a small proportion of the MHV inoculum is internalized by endocytosis, productive infection does not depend on functional endocytosis as utilized by VSV. Studies on direct virion-mediated cell fusion showed that MHV can induce fusion at pH 7.4, whereas VSV causes fusion at pH 5.0. Taken together, the above results suggest that MHV enters L-2 cells predominantly by membrane fusion with a non-acidified compartment such as the plasma membrane, endocytic vesicles, or endosomes (prior to their acidification). Results obtained from cell lines which differed in permissiveness to MHV infection suggested that the ability to support MHV infection does not correlate with endocytosis. Rather, nonpermissive cells, such as rat astrocytoma (C-6) and Vero cells, showed higher levels of recoverable internalized MHV than did fully permissive L-2 cells. Cells which are normally nonpermissive to MHV, could be rendered MHV-susceptible by PEG-induced fusion of cell surface-bound virus. Such PEG-mediated susceptibility to MHV infection was insensitive to inhibition by ammonium chloride, supporting the idea that host cell restriction of MHV infection in C-6 and Vero cells may be due to a block in nonendosomal membrane fusion. Thus endocytic internalization of MHV, which clearly occurs in a variety of cells, does not guarantee productive infection. © 1991 Academic Press, Inc.

INTRODUCTION

In general, enveloped viruses enter cells by one of two mechanisms: plasma membrane fusion or endocytosis (reviewed in Hoekstra and Kok, 1989). While most enveloped viruses appear to be internalized by a receptor-mediated endocytic event followed by low pH-dependent endosomal fusion, some viruses, notably Sendai virus, human immunodeficiency virus (HIV), and herpes simplex virus (HSV), appear to penetrate the cell plasma membrane by a neutral pH-dependent fusion mechanism.

The entry mechanism for the coronavirus mouse hepatitis virus (MHV) remains uncertain. Ultrastructural studies of infected cells have indicated the presence of virus inoculum within plasma membrane-proximal vesicles (David-Ferreira and Manaker, 1965; Amheiter *et al.*, 1982), suggesting a receptor-mediated endocytosis pathway. In addition, MHV infection is sensitive to partial inhibition by lysosomotropic agents (Mallucci, 1966; Krzystyniak and Dupuy, 1984; Mizzen *et al.*, 1985) which are known, in other virus systems, to ele-

vate endosomal/lysosomal pH and prevent low pH-dependent membrane fusion.

Despite the above, there is evidence which is not entirely compatible with an endosomal mode of MHV entry. For example, treatment of MHV-infected cells with the lysosomotropic agent, ammonium chloride, delays rather than inhibits virus replication (Mizzen *et al.*, 1985). Also, there has as yet been no demonstrated low pH-dependent fusion activity associated with MHV or the MHV S protein, as has been the case with other viral proteins which are known to facilitate the process of fusion between virion envelope and the endosomal membrane. Low pH may in fact even inhibit the expression of viral fusion at least "from within" in MHV-infected cells (Sawicki and Sawicki, 1986).

In an effort to more closely define the entry pathway of MHV, we undertook comparative studies of virus internalization and ammonium chloride sensitivity using MHV and vesicular stomatitis virus (VSV), the latter of which is known to enter cells by an endosomal, low pH-dependent fusion mechanism (e.g., Helenius *et al.*, 1980; Fan and Sefton, 1978; Schlegel *et al.*, 1982). The results point to important differences in the uptake mechanisms between the two viruses, in particular, that low pH fusion is not a requirement for MHV infec-

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tion. Moreover, using cells of different permissiveness to MHV, we show that virus internalization by a likely endocytic pathway does not necessarily lead to productive infection.

MATERIALS AND METHODS

Cells and virus

Monolayer cultures of mouse fibroblast L-2 (Rothfels *et al.*, 1959), rat astrocytoma (Benda *et al.*, 1968), and African monkey kidney Vero (Yasumura and Kawakita, 1963) cells were propagated in "medium" consisting of minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS) unless otherwise noted. The A59 strain of MHV (Manaker *et al.*, 1961) and the Indiana serotype of VSV were used.

Effect of ammonium chloride and chloroquine on MHV and VSV production

Duplicate cultures of L-2 cells in 35-mm plates were pretreated with medium containing 0 or 20 mM ammonium chloride, or 0, 50, 100 μM chloroquine for 60 min at 4°. During the latter 30 min, the cultures were inoculated with MHV or VSV (multiplicity of infection, m.o.i. of 1), adsorbed, washed three times with medium (with or without NH_4Cl or chloroquine) to remove excess inoculum, and warmed to 37° by the addition of prewarmed medium (with or without NH_4Cl or chloroquine). Supernatants were removed and replaced at 2-hr intervals. Supernatant samples were stored at −70° until assayed for infectious virus by plaque assay (e.g., Lucas *et al.*, 1977).

Effect of dansylcadaverine on MHV and VSV production

Duplicate cultures of L-2 cells in 35-mm plates were cooled to 4° for 1 hr. The cultures were then infected with MHV or VSV (m.o.i. of approx 2), adsorbed for 30 min, washed three times with cold medium, treated with cold medium containing dansylcadaverine (0, 50, 100, or 200 μM) for 5 min, and warmed to 37° by the addition of prewarmed medium with or without dansylcadaverine. Supernatants were removed and replaced with medium containing the appropriate concentration of dansylcadaverine (0, 50, 100, or 200 μM). The supernatants were stored at −70° and titrated for MHV and VSV as described above.

Assay of MHV and VSV internalization

Cell cultures in 35-mm plates were adsorbed for 30 min at 4° with MHV or VSV at an m.o.i. of approx 5, washed extensively to remove inoculum, and then

warmed to 37° by the addition of prewarmed medium. At various intervals, the cultures were treated with proteinase K (0.5 mg/ml) in MEM for 45 min at 4° in order to remove external virus (Helenius *et al.*, 1980). Subsequently, treatment was stopped by the addition of 1 mM PMSF, 3% bovine serum albumin (BSA) in MEM. As a control, the proteinase K step was omitted from one replicate series. Cells were transferred to centrifuge tubes and centrifuged (2000 g for 5 min) and the resultant pellets were washed twice with 0.2% BSA in MEM. The final pellets were resuspended in reticulocyte storage buffer (RSB) and left on ice for 5 min, and an equal volume of 2× MEM, 10% FCS was added. The samples were freeze(−70°)-thawed twice and dounce homogenized to release internalized virions, which were quantitated by plaque assay.

Low-pH treatment of VSV and MHV

Media used for these studies consisted of MEM supplemented with 10% FCS. Confluent monolayers of either L-2 or Vero cells in 35-mm plates were incubated in either control medium or cholesterol-supplemented medium for 24 hr as previously described (Daya *et al.*, 1988). Parallel cultures were set up and analyzed for cholesterol/fatty acid content by gas chromotography.

Low pH treatment of virus-adsorbed cells was carried out according to Metsikko *et al.* (1986). Briefly, either 12 PFU/cell of MHV-A59 or 3 PFU/cell of VSV were added to either the L-2 cells or Vero cells, respectively, and incubated on ice for 1 hr. Unadsorbed inoculum was removed and the cells (1 control and 1 cholesterol plate of Vero and L-2 cells per pH value) were treated with warm (37°) medium, either pH 7.4 or pH 5.0, for 1 min. The medium was removed and replaced with warm (37°) medium (with or without cholesterol), pH 7.4, and the cells were incubated for 1 hr at 37°. The monolayers were then fixed for 30 sec with methanol and Giemsa stained. Photography was done on a Leitz microscope at 10× magnification.

Dot-blot hybridization for MHV-RNA

Cultures (35-mm plates) of L-2, Vero, and C-6 cells were inoculated with MHV at various m.o.i.s and incubated for 0, 4, 8, 12, and 16 hr p.i., at which times total RNA was extracted, applied to nitrocellulose, and probed with a ^{32}P-labeled cDNA prepared against the MHV N-mRNA (Cheley and Anderson, 1984).

Contact transfer assay for cell-associated MHV

Cultures of L-2 and Vero cells were adsorbed with MHV for 30 min at 4°, washed extensively with cold medium, and incubated at 37° for various intervals to

permit virus internalization. Immediately following incubation, the cultures were proteinase K treated as described above except that the final pellet was resuspended in 2 ml of medium (cells were not lysed). Aliquots of 0.5 ml from serial 10-fold dilutions were plated onto L-2 cell monolayers in 24-well plates (Falcon) and incubated for 2 hr at 37° to allow cells to settle and attach to the monolayers. Medium was replaced with medium containing 0.5% methylcellulose (4000 cps) and the monolayers were incubated for 24 hr at 37°. Infectious centers were visualized following formaldehyde fixation and crystal violet staining.

Effect of ammonium chloride on PEG-mediated MHV infectivity of C-6 and Vero cells

Cultures of C-6 and Vero cells in 35-mm plates were pretreated with MEM supplemented with 5% FCS and containing 0 or 20 mM ammonium chloride for 60 min at 4°. MHV at an m.o.i. of approx 5 was adsorbed during this time for 30 min. Cultures were washed and treated with PEG (1 g/ml) for 1 min at 37° and subsequently the PEG was slowly diluted out as described by Van Dinter and Flintoff (1987) except the dilutions were made with MEM containing 0 or 20 mM NH$_4$Cl. The diluted PEG was removed and replaced with warm medium containing 0 or 20 mM NH$_4$Cl. The supernatant was removed at 2-hr intervals and replaced with prewarmed medium containing 0 or 20 mM NH$_4$Cl, and the samples were stored at −70° until infectious virus was assayed by plaque assay.

RESULTS

Effect of ammonium chloride on MHV and VSV replication

Lysosomotropic agents such as ammonium chloride are believed to block the replication of viruses that enter cells by an endocytic mechanism by elevating the endosomal pH and thereby inhibiting the fusion of the endosomal membrane with the viral envelope. We thus compared the effect of ammonium chloride on the replication of MHV versus VSV, a virus that enters cells predominantly by an endocytic mechanism. Virus production was monitored in extracellular media which were replaced at 2-hr intervals. Ammonium chloride (20 mM) delayed the production of progeny MHV virions by approx 4 hr in MHV-inoculated L-2 cells (Fig. 1a) as has been previously described (Mizzen et al., 1985). Furthermore, from the times of initial appearance of progeny virions, the kinetics of replication, either in the presence or in the absence of ammonium chloride, are very similar. In marked contrast, ammonium chloride

dramatically suppressed VSV replication in L-2 cells (Fig. 1b) with virus production occurring only to a small degree even at 36 hr. Also, the rate of VSV replication in the presence or absence of ammonium chloride differs significantly, confirming the findings of others that VSV enters cells predominantly by endocytosis and ammonium chloride inhibits this pathway. In contrast, MHV may enter cells by an alternate pathway in addition to an ammonium chloride-sensitive endocytic pathway as suggested by a comparatively rapid recovery of MHV replication in the presence of ammonium chloride.

Further evidence for the ability of MHV to replicate in the absence of low pH-competent endosomes or lysosomes was obtained using another lysosomotropic drug, chloroquine. As shown in Fig. 2, chloroquine at concentrations known to inhibit replication of endocytic viruses (e.g., Helenius et al., 1982; Dille and Johnson, 1982) only delayed the replication of MHV by 2–4 hr, in a manner very similar to that observed with ammonium chloride (Fig. 1). Thus both lysosomotropic drugs indicate a lack of requirement for functional low pH endosomes for MHV replication.

It should be mentioned that the pH of the media described above were carefully monitored and adjusted if necessary to pH 7.4. Inclusion of 10 mM HEPES in all media to optimize their buffering capacity was generally found not to be necessary and did not alter the results described above. It should also be noted that media were changed at 2-hr intervals to ensure constant drug concentrations throughout the experiment.

Characteristics of infectious MHV and VSV internalization

Experiments were pursued in order to compare the endocytic internalization characteristics of MHV and VSV. In order to examine virus uptake from the cell surface, proteinase K was used to strip cell-bound (but not internalized) virions from the cell surface. Cultures of L-2 cells were exposed to MHV or VSV for adsorption (30 min at 4°), warmed to 37° to allow virion internalization, and then proteinase K treated. Cells were then disrupted by homogenization and aliquots assayed for the presence of infectious virus. Thus, this assay would be expected to reflect the relative numbers of infectious virions which are adsorbed to and/or taken up by the cells in plasma membrane invaginations and subsequently endocytic vesicles. [The success of this method of internalized virus assay depends at least in part on the efficient liberation of virus from endocytic and/or endosomal elements during the homogenization procedure. Liberation of endosomal
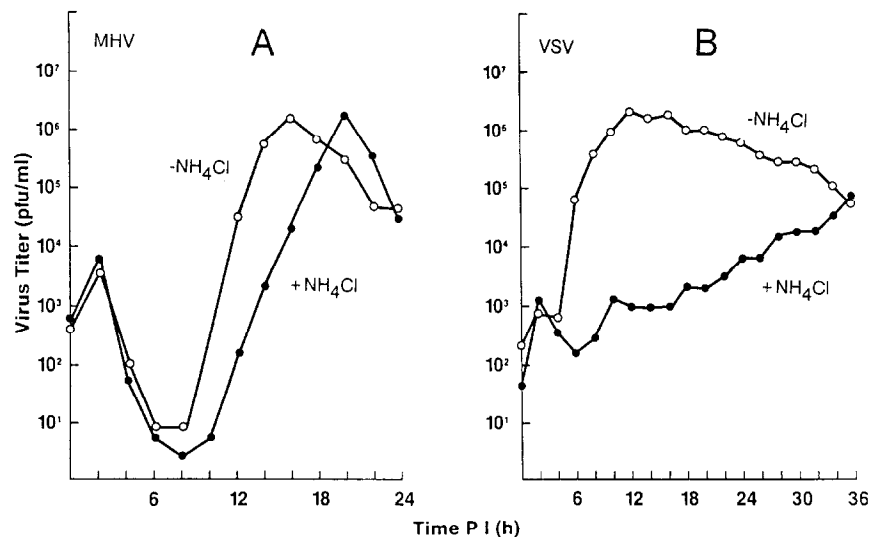
FIG. 1. Production of extracellular virus from MHV-infected (A) and VSV-infected (B) L-2 cells maintained at 37° in the absence (O) or presence (●) of 20 mM ammonium chloride.

marker contents does in fact appear to occur readily during cell homogenization (Gruenberg and Howell, 1986)]. As a control, the proteinase K step was omitted
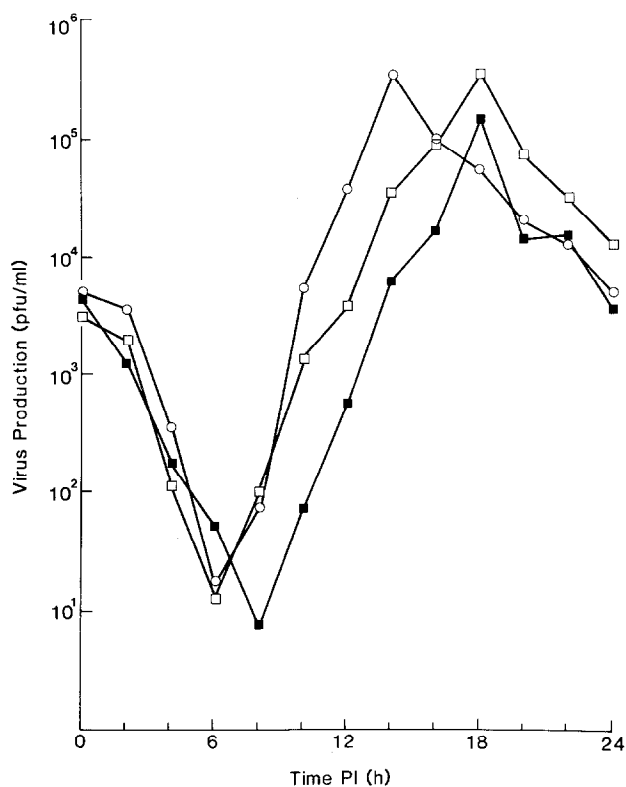

FIG. 2. Production of extracellular virus from MHV-infected L-2 cells maintained at 37° in the absence (O) or presence of 50 μM (□) or 100 μM (■) chloroquine.

from one replicate series in order to differentiate surface-bound from internalized virions. In the case of both MHV and VSV, proteinase K was effective in removing surface-bound virus, as shown by the time zero samples in Fig. 3. From these data, we estimate that proteinase K removed approx 90 and 99% of surface-bound VSV and MHV, respectively.

As shown in Fig. 3 maximal endocytic internalization of MHV and VSV occurred within 20 min. Although the infectious virus recovery was lower than anticipated, the time course data were as would be expected from virion endocytic internalization, i.e., an initial increase in cell-associated infectious virions followed by a decline as virions were processed by uncoating and/or degradative events. These results were surprising in view of the previously observed resistance of MHV infection to lysosomotropic agents (Figs. 1 and 2). We thus conclude that endocytosis of some MHV does occur in L-2 cells but that this pathway does not necessarily correlate with productive infection.

To confirm that the internalization events depicted in Fig. 3 represented acid-pH-dependent processes of the type expected to occur during endosomal/lysosomal processing, we examined these events in the presence or absence of ammonium chloride. With both MHV and VSV, ammonium chloride (20 mM) blocked the decline in infectivity observed after 20 min of internalization (Fig. 4). Since ammonium chloride raises the pH of both lysosomes and endosomes, it is likely that the decline in infectivity normally observed in MHV and VSV infection represents acid-pH-dependent processing events. However, while such acid-pH-dependent
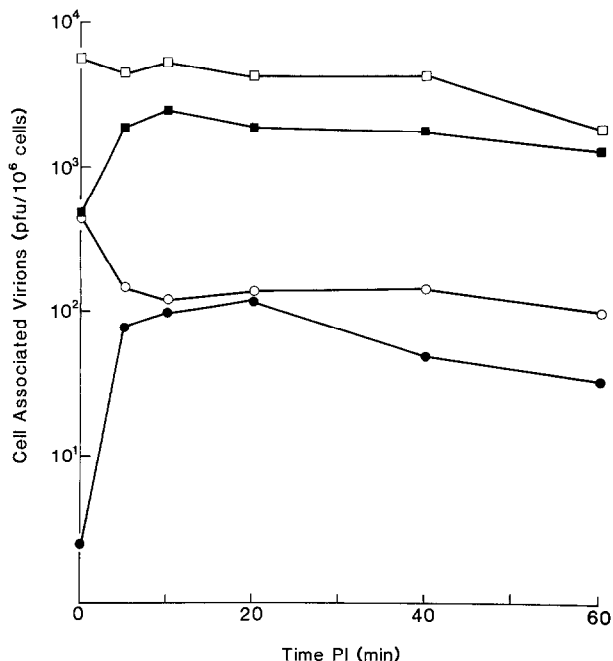
FIG. 3. Binding and internalization of MHV and VSV into L-2 cells. Cultures of MHV-adsorbed (circles) or VSV-adsorbed (squares) L-2 cells were incubated for the times indicated at 37° and washed. One set of samples was then treated with proteinase K to remove surface-bound virions (solid symbols), while the other set was mock-treated (open symbols). Cultures were then lysed and assayed for cell-associated virus by plaque assay. PI (postinoculation) refers to the time following shift to 37°.

processing is apparently indispensable for productive VSV infection, it is apparently not necessary for efficient MHV replication (cf. Fig. 1).

One explanation for the above results (Figs. 3 and 4) is that only a small proportion of the MHV inoculum enters by the endosomal pathway, while the remainder is internalized by an acid-pH-independent mechanism such as direct plasma membrane fusion. It may then be this latter, acid-pH-independent pathway which ultimately leads to productive MHV infection. At present it is difficult to assess absolute numbers of MHV virions entering by either pathway. The low yields of virus recovered by the methods used in Figs. 3 and 4 preclude a quantitative assessment.

Effect of dansylcadaverine on MHV and VSV replication

Dansylcadaverine has been shown to inhibit the cellular internalization of a variety of ligands by apparently blocking receptor-mediated endocytosis (Cheng et al., 1980; Haigler et al., 1980; Maxfield et al., 1979; Schlegel et al., 1982; Svensson, 1985; Svensson and Persson, 1984). Therefore, we used dansylcadaverine to

compare the entry pathways of MHV and VSV. Cultures of L-2 cells were inoculated with either virus and incubated at 37° in the absence or presence (50, 100, or 200 μM) of dansylcadaverine. Media were changed at 2-hr intervals and assayed for infectious virus by plaque assay. Dansylcadaverine inhibited VSV replication in a dose-dependent manner (Fig. 5B), consistent with its endocytic mode of entry. In contrast, dansylcadaverine only delayed by approx 4 hr and slightly reduced MHV virion production (Fig. 5A).

In order to examine the effect of dansylcadaverine on the early events of MHV infection, endocytosis of MHV was assayed using the proteinase K-resistant internalization assay described above. As shown in Fig. 6, dansylcadaverine appeared to exert two effects on the endocytosis of MHV. First, endocytosis occurred more slowly than in the absence of drug, and second, the normal decline in virion infectivity (at times greater than 20 min) was inhibited. The latter effect is very similar to that observed with ammonium chloride (Fig. 4), suggesting that dansylcadaverine acts partly in a lysosomotropic manner.
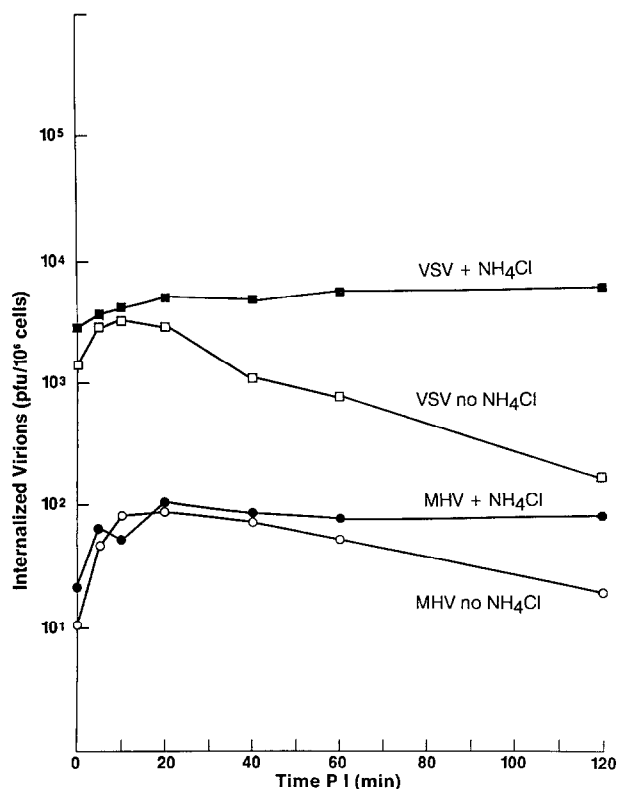

FIG. 4. Effect of ammonium chloride on MHV (circles) or VSV (squares) internalization into L-2 cells. Cultures of MHV-adsorbed or VSV-adsorbed L-2 cells were warmed to 37° for various times in the absence (open symbols) or presence (closed symbols) of ammonium chloride. After treatment with proteinase K to remove external virus, cells were lysed and assayed for internalized virus by plaque assay.
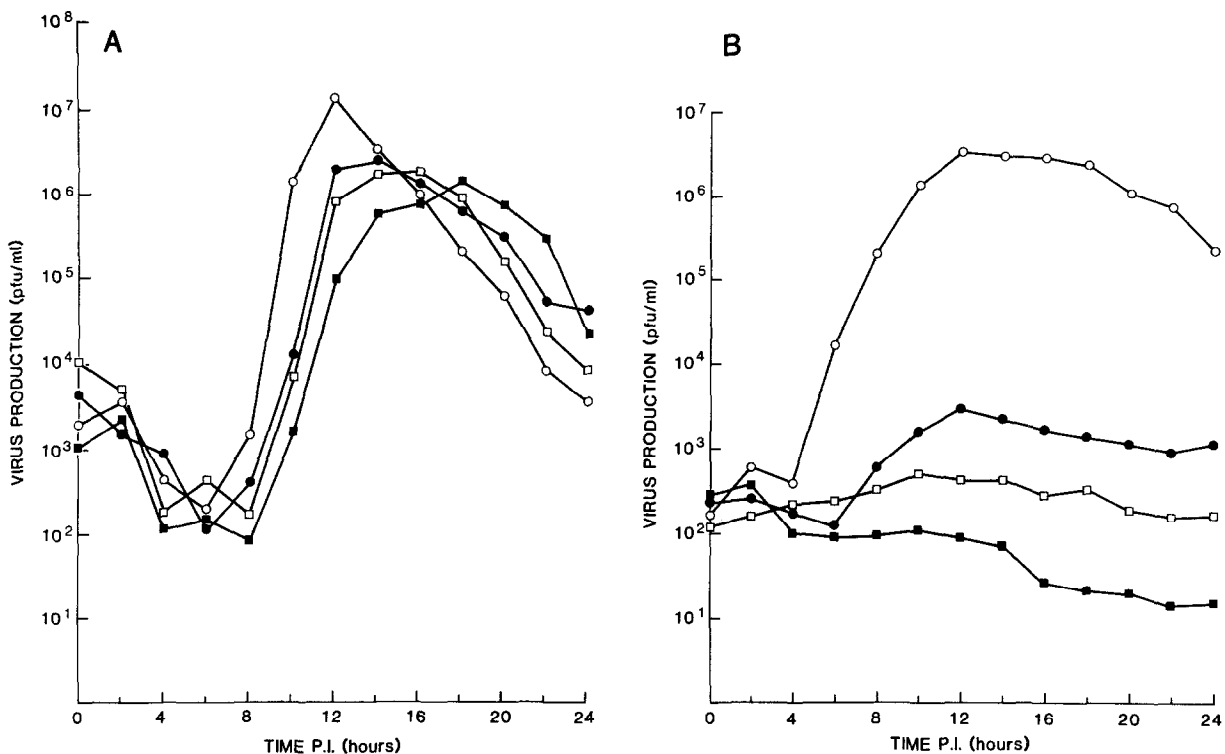
FIG. 5. Production of extracellular virus from MHV-infected (A) and VSV-infected (B) L-2 cells maintained at 37° in the presence of dansylcadaverine at concentrations of 0 μM (O), 50 μM (●), 100 μM (□), and 200 μM (■).

pH-dependence of virion-induced fusion

In an effort to demonstrate a potential for acid-pH-independent virus entry we undertook a study of the ability of MHV virions to induce membrane fusion at both acid (pH 5.0) and neutral pH (pH 7.4). We have previously noted that MHV-infected cells containing a high membrane cholesterol content showed increased cell fusion from within (Daya et al., 1988). We have
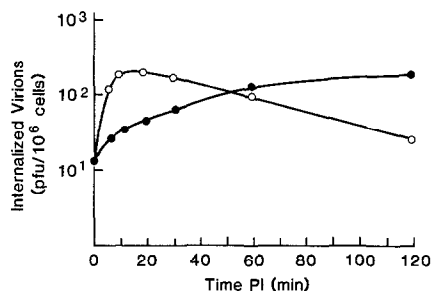

FIG. 6. Effect of dansylcadaverine on MHV internalization into L-2 cells. Cultures of MHV-adsorbed L-2 cells were warmed to 37° for various times in the absence (open symbols) or presence (closed symbols) of 100 μM dansylcadaverine. After treatment with proteinase K to remove external virus, cells were lysed and assayed for internalized virus by plaque assay.

since observed that not only "fusion from within" but also "fusion from without" is enhanced by increased cholesterol content in the target membrane and have exploited this observation in the following study of virion-mediated cell fusion. Cultures of L-2 and Vero cells were supplemented with cholesterol, using our previous procedure (Daya et al., 1988), thereby raising the membrane cholesterol/fatty acid ratios from 0.18 to 0.45 (L-2 cells) and from 0.19 to 0.35 (Vero cells). Little or no fusion was observed in unsupplemented cells (Fig. 7), confirming the amplifying effect of membrane cholesterol on fusion. Cholesterol-supplemented L-2 cells showed rapid cell fusion within 1 hr following exposure to MHV (Fig. 7). Such fusion occurred when cell-bound MHV was briefly incubated (1 min) at both pH 7.4 and 5.0, and then incubated at pH 7.4 for 1 hr. The results clearly show not only that MHV is able to cause fusion from without at physiological pH, but also that brief pH 5.0 treatment does not irreversibly destroy the virion's fusogenicity. VSV was also able to induce early fusion, which was cholesterol-enhanced, but which occurred only at pH 5.0 and not at pH 7.4, in agreement with other studies (White et al., 1981), confirming the low pH-dependent fusion activity of the VSV G protein. By contrast, MHV appears to mediate fusion at neutral pH, which may allow MHV to

pH 7.4

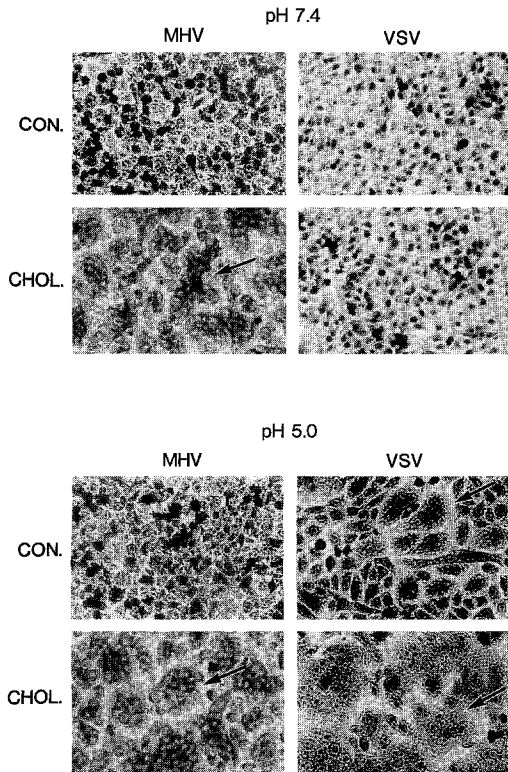MHV   VSV

CON.

CHOL.

FIG. 7. Effect of pH on fusion from without by MHV and VSV. MHV or VSV was adsorbed at 4° to unsupplemented or cholesterol-supplemented L-2 or Vero cells, respectively. After removal of the inocula, the cells were treated with warm medium, pH 7.4 or pH 5.0, for 1 min. The medium was removed and warm unsupplemented medium or cholesterol-supplemented medium was added and the cells incubated at 37° for 1 hr at pH 7.4. Arrows indicate syncytia.

pH 5.0

MHV   VSV

CON.

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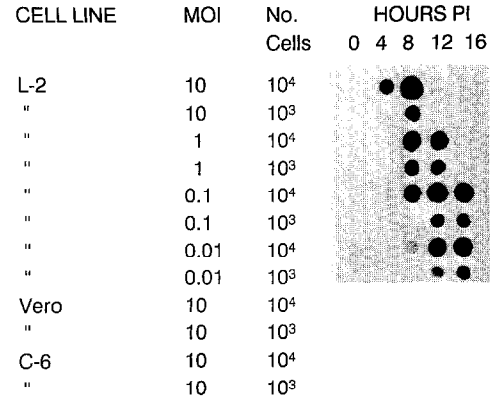| CELL LINE	MOI	No. Cells	HOURS PI 0 4 8 12 16
L-2	10	10^4	
"	10	10^3	
"	1	10^4	
"	1	10^3	
"	0.1	10^4	
"	0.1	10^3	
"	0.01	10^4	
"	0.01	10^3	
Vero	10	10^4	
"	10	10^3	
C-6	10	10^4	
"	10	10^3	

FIG. 8. MHV-RNA synthesis in L-2, C-6, and Vero cells. Monolayer cultures (35-mm dishes) were inoculated with MHV at the indicated m.o.i. RNA was extracted from the cultures at the times indicated and aliquots corresponding to 10^4 or 10^3 cells were dot-blotted onto nitrocellulose. MHV-RNA was detected by hybridization with a ^{32}P-labeled cDNA probe made against MHV nucleocapsid protein mRNA and subsequent autoradiography.

enter cells by fusion at the plasma membrane or with early vesicles in the endocytic pathway.

Internalization of MHV into susceptible versus resistant cells

A number of cell lines show resistance to MHV infection at an early stage. We chose to study two such cell lines, the C-6 astrocytoma cell line which is defective in MHV internalization (Van Dinter and Flintoff, 1987) and the monkey kidney cell line (Vero). Cultures of L-2, C-6, and Vero cells were inoculated and adsorbed with MHV, incubated at 37°, and harvested for RNA extraction at various times. Dot-blot hybridization of the extracted RNA using an MHV-specific cDNA probe demonstrated the absence of MHV-encoded RNA in either Vero or C-6 cells (Fig. 8). It is thus clear that both Vero and C-6 cells are resistant to MHV infection at a stage prior to viral RNA synthesis.

Internalization of MHV by the permissive L-2 cell and the resistant Vero and C-6 cells was compared. As

shown in Fig. 9, all cell lines displayed evidence of virus uptake during the first 20–30 min of 37° incubation. The increase in cell-associated virus infectivity during this time can only be explained by a process of virion internalization in which surface-bound MHV becomes progressively more resistant to proteinase K treatment as a result of membrane envelopment during the internalization process. Surprisingly, the nonpermissive Vero and C-6 cells showed higher levels of internalized infectious MHV than the permissive L-2 cell (Fig. 9). It is thus apparent that the resistant Vero and C-6 cells do not block endocytic internalization of MHV.
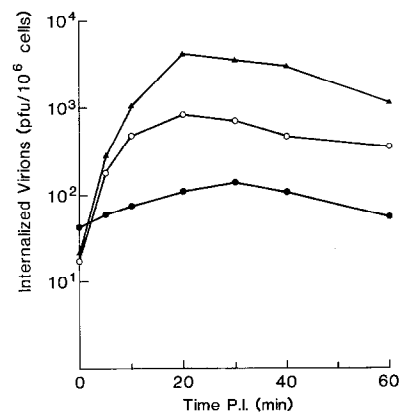
FIG. 9. Time-course study of MHV internalization into nonpermissive (Vero, ▲, and C-6, ○) and permissive (L-2, ●) cells. Cultures were inoculated with MHV and allowed to adsorb for 30 min at 4°C. They were then washed and incubated at 37° for various times to allow virus internalization. Noninternalized virus was removed by proteinase K treatment and the amount of internalized virus was quantitated by plaque assay of disrupted cells.

A. Contact-transfer of cell-associated MHV to L-2 cells
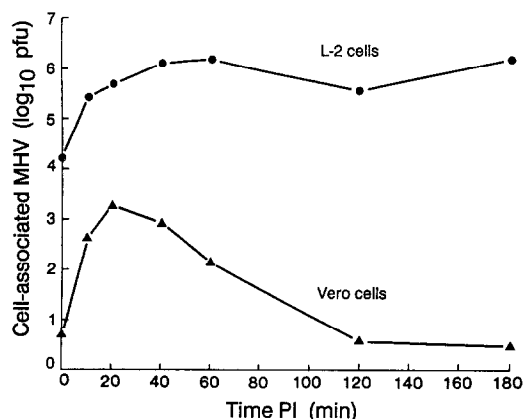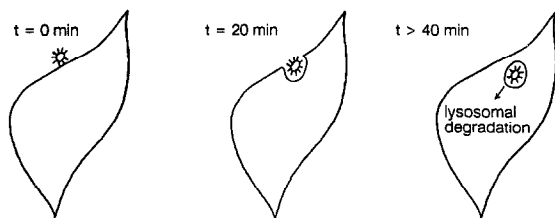

B. Postulated fate of MHV in Vero cells

FIG. 10. (A) Contact transfer of Vero cell-associated MHV to L-2 cells. MHV (m.o.i. = 10) was adsorbed to monolayer cultures (35-mm dishes) of L-2 (●) and Vero (▲) cells for 30 min at 4°. Cells were incubated at 37° to permit virus internalization, washed with cold MEM, and treated with proteinase K to remove uninternalized virus. After removal of proteinase K, cells were plated onto L-2 cell monolayers and monitored for infectious center formation. (B) Postulated fate of MHV in Vero cells. At 4° (t = 0) virus is adsorbed to cell surface. Upon incubation at 37° (t = 20) virus is internalized into the Vero cells. At later times (t > 40), MHV undergoes lysosomal degradation and thus leads to a nonproductive infection.

Contact transfer of cell-associated MHV to L-2 cells

Verification that MHV undergoes envelopment by the surface membrane of the nonpermissive Vero cell was obtained by an independent method. Cultures of Vero and L-2 cells were inoculated with MHV and incubated at 37° to permit virus internalization. Proteinase K was then employed to remove cell-surface-associated virions from the cell surface. Proteinase K-treated cells were then seeded onto L-2 cell monolayers for assay of infectious centers. As shown in Fig. 10a, MHV-inoculated L-2 cells gave rise to increasing numbers of infectious centers, with a maximum at 40 60 min, presumably reflecting the length of time necessary for virions to become partially internalized by the plasma membrane and thus protected from proteinase

K digestion. After this 40- to 60-min interval, the numbers of infectious centers remained constant, indicative of the MHV-inoculated L-2 cells being productively infected.

In contrast, MHV-inoculated Vero cells, when seeded onto L-2 cell monolayers, gave rise to rapidly decreasing numbers of infectious centers between 20 and 120 min (Fig. 10a). Note that, since Vero cells are not infectable by MHV (cf. Fig. 8), the infectious centers recorded in Fig. 10a are derived from the co-cultured L-2 cells to which virus has been transferred by cell–cell contact. The maximum at 20 min suggests that MHV virions are being internalized by Vero cells and/or are not being completely removed by the protease treatment. However, internalization by Vero cells does not ultimately result in a productive infection (Fig. 8) as indicated by the subsequent decline in infectious centers (Fig. 10a; 20–120 min). A likely explanation of the maximum at 20 min p.i. is that these infectious centers represent virus being partially internalized such that part of the virus including envelope spike (S) glycoproteins are exposed to the cell surface (Fig. 10b; t = 20 min). [The S glycoprotein is the one responsible for cell binding and fusion (Collins et al., 1982)]. Virus is thus protected from proteinase K treatment but can still initiate infection when placed in contact with a permissive L-2 cell. This assay demonstrates the ability of Vero cell-bound virus to initiate infection on an "indicator" L-2 cell with which it is placed in contact. The results thus demonstrate a progressive protection of Vero cell-bound MHV against external protease while preserving the virus in a surface-exposed state consistent with its ability to infect neighboring L-2 cells. We can only explain these results by a process of virus-mediated invagination of the Vero cell membrane which protects against protease but permits the subsequent interdigitation of L-2 cell membrane processes (thereby facilitating infection of the latter). However, at increasing times of infection (Fig. 10b; >40 min), the virus is internalized further (by the Vero cell) such that it is no longer exposed at the cell surface. Even though virus is fully internalized by 120 min (cf. Fig. 9), an infectious center is not formed, indicating either that there is a postinternalization block to MHV infection in Vero cells or that MHV must enter Vero cells by an alternate pathway in order to be infectious.

Effect of NH₄Cl on PEG-mediated MHV infection of C-6 and Vero cells

MHV was unable to cause fusion from without in either Vero or C-6 cells (cholesterol-supplemented or not, at either pH 5.0 or 7.4) in contrast to that observed

in L-2 cells (data not shown, cf. Fig. 7). This result clearly implicates the membrane fusion event as being blocked in Vero and C-6 cells, since virus binding (Van Dinter and Flintoff, 1987; Kooi *et al.*, 1988) and internalization (Fig. 9) are not lacking, relative to that seen in permissive L-2 cells.

The dependence on cell surface fusion for productive MHV infection was further supported by the following study. Cultures of C-6 and Vero cells were treated in medium containing 0 or 20 mM ammonium chloride for 60 min at 4°, MHV at an m.o.i. of 5 was adsorbed during this time for 30 min. Cultures were washed and treated with PEG as described and the cultures were incubated at 37° in the absence or presence of 20 mM NH$_4$Cl. MHV which does not normally replicate in Vero and C-6 cells (Figs. 11A and 11B) does undergo replication when fused with such cells in the presence of PEG (Figs. 11C and 11D). This is consistent with the findings of Van Dinter and Flintoff (1987), who demonstrated PEG-mediated MHV infection of C-6 cells and concluded that MHV replication in C-6 cells was blocked at the internalization step. From this and our present results which demonstrate endocytic internalization of MHV into C-6 and Vero cells, we conclude that PEG-mediated fusion may overcome the block in replication by allowing MHV virions to enter the C-6 and Vero cells by an alternate (nonendocytic) pathway. Such a pathway would be expected to be resistant to the effects of ammonium chloride, which was in fact found to be the case (Figs. 11C and 11D). These results suggest that PEG mediates direct virion envelope fusion with the cell plasma membrane, thereby overcoming the normal host-imposed restriction present in Vero and C-6 cells.

DISCUSSION

It is evident from the present study that virion internalization by an apparently endocytic mechanism can take place even in cells which are nonpermissive for MHV replication. Within a few minutes of internalization in both permissive (L-2) and nonpermissive (Vero and C-6) cells, MHV can be detected as an intracellular, infectious form, which reaches maximal levels at about 20–30 min postadsorption. In both cell types, subsequent processing results in the loss of viral infectivity, consistent with uncoating or degradative events occurring in low-pH lysosomal and/or endosomal compartments. While such results are reminiscent of conventional endocytic internalization (for viruses such as VSV, influenza, and Semliki Forest virus) there are reasons to believe that productive MHV infection may depend on a different entry mechanism.

First, MHV replication is much less affected by the lysosomotropic agents, ammonium chloride and chloroquine, than is the replication of VSV, a virus known to enter cells by a process of low pH-dependent endosomal fusion (Matlin *et al.*, 1982). In contrast, viruses such as Sendai virus and HIV which evidently enter cells by direct plasma membrane fusion are only weakly or inconsistently inhibitable by ammonium chloride (Nagai *et al.*, 1983; McClure *et al.*, 1988; Stein *et al.*, 1987).

Second, MHV replication is inhibited only slightly by dansylcadaverine, which is a very effective blocker of VSV replication. Dansylcadaverine has been used to inhibit receptor-mediated endocytosis of VSV (Schlegel *et al.*, 1982), adenovirus (Svensson, 1985), and a variety of ligands (Cheng *et al.*, 1980; Haigler *et al.*, 1980; Maxfield *et al.*, 1979). Electron microscopy of dansylcadaverine-treated cells showed that α_2-macroglobulin, *Pseudomonas* toxin, and LDL remained diffusely bound to the cell membrane and did not form clusters in coated pits. Thus the decreased rate of internalization may be due to this inhibition of clustering (Fitzgerald *et al.*, 1980; Pastan and Willingham, 1981a, b). Our own findings presented in this report suggested that dansylcadaverine has inhibitory effects both on endocytic internalization of MHV and on subsequent processing events which are also blocked by the lysosomotropic agent, ammonium chloride. Despite these inhibitory actions, the effect of dansylcadaverine treatment on MHV replication is minimal compared to its effect on the replication of VSV. The need for an intact, functioning endocytic pathway would therefore seem to be much greater for VSV than for MHV.

Third, MHV virions can induce cell fusion from without at pH 7.4 while VSV is fusogenic at a lower pH (pH 5.0), suggesting an ability for MHV to initiate fusion-dependent entry at physiological pH. A distinctive characteristic of most enveloped viruses is their ability to induce cell to cell fusion when they are added to cells at a high m.o.i. However, to observe this fusion from without the medium pH should correspond to the pH at which the virus displays optimal fusion activity. Sendai virus, which enters cells by direct plasma membrane fusion, demonstrates optimal fusion activity at pH 7.5 (Hsu *et al.*, 1982; Hoekstra *et al.*, 1985). At mildly acidic pH, fusion is reduced, suggesting that Sendai virus enters cells predominantly if not completely by direct plasma membrane fusion. The finding that VSV is fusogenic only at pH 5.0 is consistent with the notion that VSV enters cells by endocytosis and fuses with endosomal membranes following acidification (White *et al.*, 1981). In contrast, the ability of MHV to induce fusion from without at pH 7.4 suggests that MHV is able to
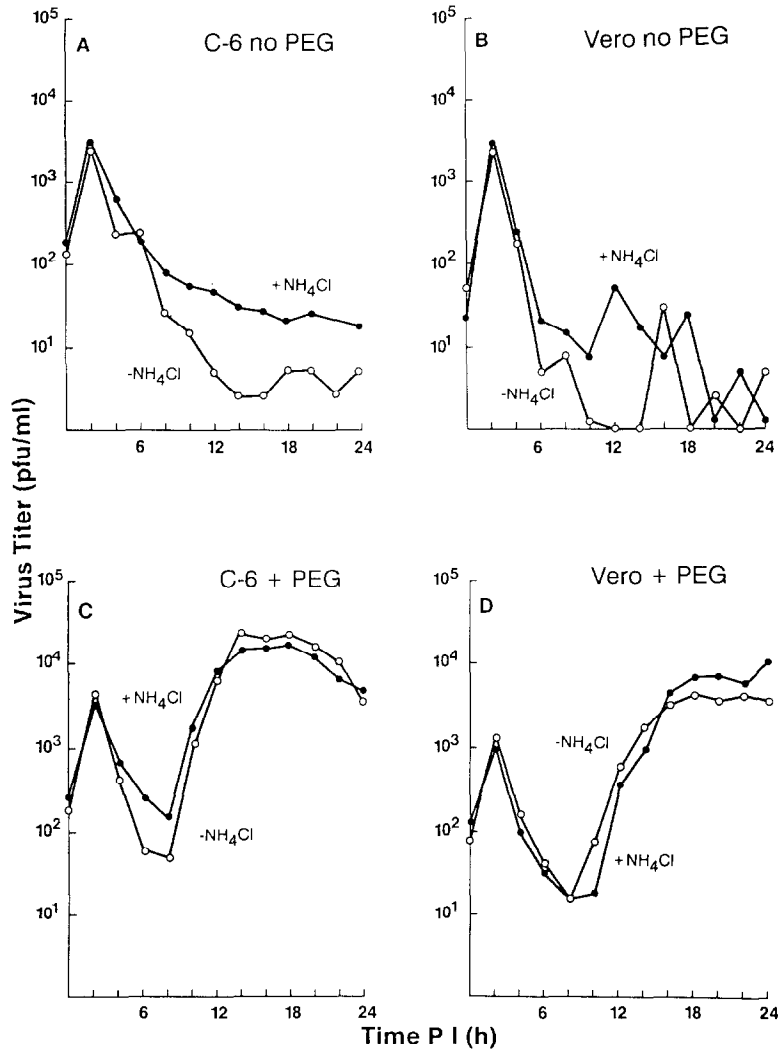
FIG. 11. Effect of ammonium chloride on PEG-mediated MHV infection of C-6 and Vero cells. MHV-adsorbed C-6 (A and C) or Vero (B and D) cultures were briefly treated with MEM in the absence (A and B) or presence of PEG (C and D). Cultures were then maintained at 37° in the absence (O) or presence (●) of 20 mM ammonium chloride and extracellular virus production was determined.

enter L-2 cells by direct fusion at neutral pH with the plasma membrane or with early vesicles in the endocytic pathway. The idea that MHV may enter cells by membrane fusion at neutral pH is also supported by studies of Sawicki and Sawicki (1986), who noted that in 17Cl1 cells MHV-induced fusion from within occurred more efficiently at pH 7.2 than at 6.8.

Fourth, the normally abortive MHV infection of Vero and C-6 cells can be rescued, in an ammonium chloride-insensitive fashion, with the known fusogen, PEG, suggesting that MHV infection can be initiated by a cell surface fusion event. In the absence of such fusion-inducing agents, MHV is apparently taken up by an endocytic pathway which does not lead to productive infection. Such endocytosis may be a relatively nonspecific event since cells are known to internalize the

equivalent of their total plasma membrane surface area within time intervals of 30 min to several hours (Steinman et al., 1976). Cell surface-bound ligands and even adventitiously bound structures, such as latex beads (Roberts and Quastel, 1963), can also be included in this overall process of membrane turnover. Our results indicate quantitatively different efficiencies of internalization of two viruses, namely MHV and VSV, into L-2 cells. Despite the quantiative differences, it is clear that some MHV is internalized by an apparently endocytic process in L-2 cells (as well as the nonproductively infected Vero and C-6 cells; Fig. 8). The results of the present work may also explain previous electron microscopic studies, demonstrating the presence of endocytically enclosed virions (David-Ferreira and Manaker, 1965; Arnheiter et al., 1982) which have been inter-

preted as supporting a role for endocytosis in MHV infection. Such observations may at least in part represent a mechanism of virus internalization which is nonproductive, in a manner similar to that found in the present study in cells such as Vero and C-6.

Early events in the coronavirus replication cycle are of increasing interest to the understanding of mechanisms of MHV pathogenesis. Considerable evidence is available which suggests that cell tropism and/or permissiveness is often decided at the virus penetration level. While the presence of a cell receptor is obviously crucial for MHV infection (Boyle et al., 1987) some cells are apparently able to bind virus while blocking infection at a subsequent postadsorption stage (Shif and Bang, 1970; Knobler et al., 1981; Arnheiter et al., 1982; Beushausen et al., 1987; Van Dinter and Flintoff, 1987; Kooi et al., 1988). In some of these cases, the virus penetration step, or a closely related event, may determine the success of infection (Beushausen et al., 1987; Van Dinter and Flintoff, 1987; Kooi et al., 1988).

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