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Cells and Viruses with Mutations Affecting Viral Entry Are Selected during Persistent Infections of L Cells with Mammalian Reoviruses

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Previous studies demonstrated that both cellular and viral mutants are selected during maintenance of persistent infections established in murine L cells with high-passage stocks of mammalian reoviruses. In particular, when one culture was cured of persistent infection, the resulting cells were found to support the growth of viruses isolated from persistently infected cultures (termed PI viruses here) better than that of wild-type (wt) viruses (R. Ahmed, W. M. Canning, R. S. Kauffman, A. H. Sharpe, J. V. Hallum, and B. N. Fields, Cell 25:325–332, 1981). To address the nature of cellular and viral mutations selected during maintenance of persistent reovirus infections, we established independent, persistently infected cultures with L cells and high-passage stocks of wt reovirus. These cultures served as sources of new PI viruses and cured cells for study. We found that although wt viruses grew poorly in cured cells when infection was initiated with intact virions, they grew well in cured cells when infection was initiated with infectious subvirion particles generated from virions by in vitro treatment with chymotrypsin. This finding indicates that the block to growth of wt viruses in cured cells involves an early step that is unique to infection by virions, such as proteolytic processing in an endocytic compartment. We also found that PI viruses grew better than wt viruses in L cells treated with ammonium chloride, a weak base that inhibits the pH decrease in endosomes and lysosomes. Because ammonium chloride blocks an early step in infection by intact virions, probably the proteolytic processing of viral outer capsid proteins by acid-dependent cellular proteases in late endosomes or lysosomes, this finding indicates that PI viruses differ from wt viruses with respect to viral entry into cells. Therefore, these results indicate that both cells and viruses evolve mutations that affect one or more early steps in the viral growth cycle during maintenance of L-cell cultures persistently infected with reoviruses.

Many animal viruses can establish and maintain noncytolytic, persistent infections in cell culture (4). At a fundamental level, the mechanisms used in the establishment and maintenance of persistent infections are likely to involve modulation of the type or degree of injury to cells that results from viral infection. Characterization of these mechanisms should provide information about important sites of virus-cell interaction. In several instances, mutations in both viruses and cells are selected during the maintenance of persistence. Coevolution of virus and persistently infected cells was first documented in studies with mammalian reoviruses, in which viruses selected during maintenance of persistent infection were shown to grow better than wild-type (wt) viruses in cells cured of persistent infection (1, 14). Both viral and cellular changes also have been observed in persistent infections by other RNA-containing viruses, such as foot-and-mouth disease virus (10) and poliovirus (6), and by DNA-containing viruses, such as lymphotropic minute virus of mice (23). However, the mechanisms by which these changes are selected and exert their influence on the maintenance of persistent infections are largely undefined.

Persistent infections of murine L cells can be readily established with defective, high-passage (hp) stocks of some strains of mammalian reoviruses (1, 3, 7). Such persistently infected cultures produce high titers of infectious virus over long periods of time, and most cells in culture show evidence of infection (1–3). Previous studies of viruses isolated from these cultures indicate that mutations in specific viral genes play distinct roles in persistent infection. Changes in the S4 gene are important for establishment of persistence (2); changes in the S1 gene (in addition to cellular changes) are important for its maintenance (14). Since the S4 gene (which encodes the σ3 protein, a major outer capsid protein of virions [19, 21]) also determines the relative capacities of different reovirus strains to inhibit cellular RNA and/or protein synthesis (27) and since the S1 gene (which encodes both the cell attachment protein σ1 [19, 21] and the nonstructural protein σ1s [11, 13, 24]) also determines the relative capacities of different viral strains to inhibit cellular DNA synthesis (26), changes in S4 and S1 might contribute to establishment and maintenance of persistence by reoviruses through alterations in their effects on cellular macromolecular synthesis (7).

Previous findings suggest that changes in viruses and cells that affect early steps in infection might also contribute to maintenance of persistent infection. The most interesting of these concern the accumulation of large numbers of vacuoles containing lysosomal enzymes in persistently infected cells as well as in cells that were cured of persistent infection (1, 9, 28). Current understanding of the early steps in infection by virions of reoviruses (also referred to as steps in viral entry) includes attachment of an intact virion to receptors on the cell surface via the σ1 protein; receptor-mediated endo-

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cytosis; proteolytic processing of viral outer capsid proteins σ3 and μ1/μ1C by cellular proteases within a vacuolar, endocytic compartment; penetration of the vacuolar membrane by the proteolytically processed form of the viral particle, which is similar to infectious subvirion particles (ISVPs) generated from virions by in vitro treatment with chymotrypsin or other proteases; and activation of the viral transcriptase contained within the viral core to permit synthesis and transport of viral mRNAs into the cytoplasm of the infected cell (for a review, see reference 22). Any of these early steps might represent targets for mutations in viruses and cells during persistent infections by reoviruses.

This report describes the nature of cellular and viral mutants that are selected during the maintenance of L-cell cultures persistently infected with reoviruses. We established eight independent, persistently infected cultures with L cells and hp stocks of reovirus strain type 3 Dearing (T3D) and maintained each of them for more than 500 days. Cells cured of persistent infection were found to support good growth of wt viruses only if ISVPs, generated from virions by chymotrypsin treatment in vitro, were used to initiate infection. Viruses isolated from the persistently infected cultures were found to grow in the presence of ammonium chloride, a weak base that blocks infection by wt virus through an inhibition of the proteolytic processing of viral outer capsid proteins by acid-dependent endocytic proteases. These results, which indicate that mutations in both cells and viruses affect early steps in infection, suggest that virus-cell coevolution with respect to viral entry is an important mechanism for maintenance of L-cell cultures persistently infected with reoviruses.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted mouse L cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) that was supplemented to contain 5% fetal calf serum (HyClone Laboratories, Logan, Utah), 2 mM L-glutamine, 1 U of penicillin per ml, and 1 μg of streptomycin per ml (Irvine Scientific). Reovirus strains type 1 Lang (T1L) and T3D are laboratory stocks; the L/C virus was isolated from the persistently infected L/C cell culture which was initiated with a ninth-passage stock of T3D temperature-sensitive mutant *ts*C447 (1, 3, 14). Purified virion preparations were made by using second- and third-passage L-cell lysate stocks of twice-plaque-purified reovirus (12). T3D ISVPs were prepared by digestion of purified T3D virions with chymotrypsin (30).

Establishment and maintenance of persistently infected L-cell cultures. Six plaque-purified isolates of reovirus T3D that were passaged serially at high multiplicity (7) were used to establish independent persistently infected L-cell cultures. Persistent reovirus infection was established by infecting confluent L-cell monolayers (6×10^6 cells) in T25 flasks (Corning Scientific, Corning, N.Y.) with 0.5 ml of one of the six hp stocks at the eighth or ninth passage (Table 1). The cultures were maintained at 37°C, and cells were allowed to grow to confluence before being trypsinized and diluted into fresh medium. Fresh medium was added to the cultures every fourth day if the cell density was not sufficient to permit passage. Cell culture supernatants were collected at each passage for further analysis.

Determination of virus titer in persistently infected cell culture supernatants. The virus titer in the cell culture supernatants was determined by the plaque assay technique (31). Cell culture supernatants were collected from each passage, and samples were frozen and thawed (−70 and 37°C) three times prior to assay. Samples were serially diluted 10-fold and were used to infect L-cell monolayers in duplicate in six-well plates (Costar, Cambridge, Mass.). The L cells were then overlaid with medium 199 (Irvine Scientific) supplemented to contain 2.5% fetal calf serum, 2 mM L-glutamine, 1 U of penicillin per ml, 1 μg of streptomycin per ml, and 1% agar (Difco, Detroit, Mich.). Plaques were counted on day 7 after staining with neutral red (Fisher Scientific, Pittsburgh, Pa.).

TABLE 1. Prototype PI viruses isolated from independent persistently infected L-cell cultures established with hp stocks of reovirus T3D

hp stock of reovirus T3D[a]	Persistently infected cell culture	PI virus strain[b]	Day of harvest[c]	LX/L ratio[d]	AC/L ratio[e]
T3D-1 P8	L/T3D-1A	PI 1A1	151	0.14	0.13
T3D-1 P8	L/T3D-1B	PI 1B1	91	0.34	0.80
T3D-2 P8	L/T3D-2A	PI 2A1	151	3.07	0.53
T3D-2 P8	L/T3D-2B	PI 2B1	181	1.53	0.25
T3D-3 P8	L/T3D-3	PI 3-1	178	0.59	0.46
T3D-5 P9	L/T3D-5	PI 5-1	89	0.15	0.22
T3D-6 P8	L/T3D-6	PI 6-1	90	0.16	0.73
T3D-7 P8	L/T3D-7	PI 7-1	118	0.39	1.22

[a] Independent twice-plaque-purified stocks of T3D were passaged serially in L cells (7). Eight- or ninth-passage stocks (P8 or P9, respectively) were used to establish independent persistently infected L-cell cultures.

[b] Each virus strain was plaque purified twice from the supernatant of a persistently infected cell culture. A PI virus strain is defined as an isolate from a persistently infected culture that grows better than wt virus in cured cells. The prototype PI viruses are single isolates from each of the eight persistently infected cultures that resulted in the highest yield when grown in cured cells in comparison with wt virus.

[c] Day of passage of the persistently infected culture that the PI virus strain was isolated.

[d] The mean viral yield after 72 h of growth in LX-2 cells was divided by that in L cells (LX/L ratio) for each PI virus shown; the LX/L ratio for wt reovirus strain T3D was 0.034.

[e] The mean viral yield after 24 h of growth in L cells treated with ammonium chloride was divided by that in untreated L cells (AC/L ratio) for each PI virus shown; the AC/L ratio for T3D was 0.0022.

Generation of cells cured of persistent reovirus infection. Variant L cells (designated LX) were rendered virus free by maintenance of persistently infected L-cell cultures in medium supplemented to contain 1% rabbit anti-reovirus antiserum (provided by E. Brown) for 30 days according to the method described by Ahmed et al. (1). The antiserum was heat inactivated by incubation at 56°C for 60 min prior to use. Cured-cell clones were obtained by limiting dilution into 96-well plates (Costar). Individual wells were inspected visually, and only those containing a single cell were used. Cell clones presumed to be virus free were assessed by plaque assay of cell culture supernatants (31), infectious center assay (3), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16) of reovirus genomic double-stranded RNA prepared from cell culture supernatants (29), and immunocytochemical staining with rabbit anti-reovirus antiserum and the VECTASTAIN immunocytochemistry kit (Vector, Burlingame, Calif.).

Growth of viruses in L cells and in cells cured of persistent infection. Viral growth in L cells and LX cells was determined by a modification of the technique described by Ahmed et al. (1). Monolayers of L and LX cells (2×10^5 cells per ml) were infected with viral isolates in 24-well plates (Costar). After a 1-h adsorption period at 4°C, the inoculum

was removed, 1.0 ml of fresh medium was added, and the plates were incubated at 37°C for defined intervals. Cells were frozen and thawed three times prior to being titrated on L-cell monolayers by the plaque assay technique (31).

Growth of viruses in L cells maintained in medium supplemented with ammonium chloride. Viral growth in L cells in the presence and absence of ammonium chloride was determined by a modification of the technique described by Sturzenbecker et al. (30). Monolayers of L cells (5×10^5 cells per ml) were infected with viral isolates in 24-well plates. After a 1-h adsorption period at 4°C, the inoculum was removed, 1.0 ml of fresh medium supplemented to contain 0 to 40 mM ammonium chloride was added, and the plates were incubated at 37°C for defined intervals. Cells were frozen and thawed three times prior to being titrated on L-cell monolayers by the plaque assay technique (31).

RESULTS

Characterization of independently established and maintained persistently infected cultures. In order to extend previous findings about persistent infections by reoviruses, we first established eight independent, persistently infected cultures with murine L cells and hp stocks of wt reovirus T3D (Table 1). Titers of infectious virus in the supernatants of each culture were found to vary between 10^6 and 10^9 PFU/ml during the first 500 days of their maintenance, and a large percentage of cells were infected at any given time as determined by infectious center assays and immunocytochemical staining (data not shown). These findings are in concordance with previous studies of persistent infections established with hp stocks of temperature-sensitive mutants derived from T3D (1, 3). In anticipation of experiments with viruses obtained from persistent infections established with hp stocks of reovirus T3D, we isolated viral clones from each of the eight persistently infected cultures at approximately 30-day intervals between days 30 and 180 of their maintenance.

Isolation of cells cured of persistent infection and studies of their capacity to support growth of viruses from persistently infected cultures. Maintenance of persistent infections by reoviruses is characterized by selection of both cellular and viral mutants within the persistently infected cultures (1, 14). Mutant L cells (designated LR cells) were previously obtained by curing the persistently infected L/C cell culture (initiated with an hp stock of T3D-derived temperature-sensitive mutant *ts*C447 [3]) with a neutralizing anti-reovirus antiserum (1). LR cells were found to support the growth of viruses isolated from persistently infected cultures better than that of wt viruses (1). For the current study, we derived new lines of cured cells by maintaining day-166 subcultures of the persistently infected L/T3D-1A and L/T3D-2A cultures in medium containing a heat-inactivated, neutralizing rabbit anti-reovirus antiserum. Antiserum treatment was maintained for a 30-day period, during which the viral titer was found to decrease to <10 PFU/ml of supernatant in each culture (the lower limit of detection). Cloned cells were obtained from the cured L/T3D-1A culture (designated LX-1) and the L/T3D-2A culture (designated LX-2) by two cycles of limiting dilution in 96-well plates. Subclones from the LX-1 and LX-2 cell lines were confirmed to be virus free by negative infectious center assays, the absence of viral genomic double-stranded RNA in cytoplasmic extracts of cells infected with supernatants from each subclone, and the failure to demonstrate viral antigens by immunocytochemical staining (data not shown).

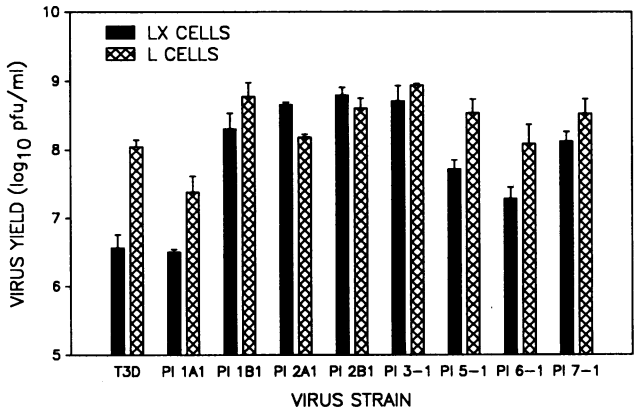

FIG. 1. Growth of T3D and PI viruses in cured LX-2 cells and normal L cells. Monolayers of LX-2 and L cells (2×10^5 cells per ml) were infected with either T3D or the PI viruses at a multiplicity of infection of 0.1 PFU per cell in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium was added, and the plates were incubated at 37°C for 72 h. Cells were frozen and thawed three times prior to being titrated on L-cell monolayers by the plaque assay technique. The results are presented as the mean viral yield (± standard deviation) determined from three independent experiments for each virus in LX-2 and L cells.

We wished to determine whether viruses isolated in the current study from persistently infected cultures established with hp stocks of reovirus T3D could grow better than wt viruses in cells cured of persistent infection. Cell lysate stocks of viruses isolated from the persistently infected cultures were therefore used to infect either L cells or cured LX cells. In each case, viruses isolated from the persistently infected cultures after approximately day 90 of maintenance grew significantly better than wt T3D in cured cells, even though the two types of viruses grew comparably in L cells. We refer to viruses from persistently infected cultures that exhibit better growth than wt viruses in cured cells as PI viruses. The growth of a single PI virus from each of the eight persistently infected cultures that yielded the highest titer when grown in cured cells was compared with that of T3D in cured LX-2 cells and in normal L cells (Fig. 1). In these experiments, the mean yield of wt virus was found to be 3.7×10^6 PFU/ml in cured cells and 1.1×10^8 PFU/ml in L cells, whereas the mean yields of the eight PI viruses were 2.5×10^8 PFU/ml in cured cells and 3.5×10^8 PFU/ml in L cells. To assess the efficiency of growth of the PI viruses relative to T3D in cured cells, we divided the viral yield in LX-2 cells by that in L cells (LX/L ratio) for each PI virus and T3D. The LX/L ratio was 0.034 for T3D and varied from 0.14 (PI 1A1) to 3.07 (PI 2A1) for the PI viruses (Table 1). Given that PI and wt viruses grew comparably in L cells, these results indicate that the PI viruses grew in cured LX-2 cells 4.1 (PI 1A1) to 90 (PI 2A1) times better than T3D.

To determine more precisely when in the course of persistent infection the capacity of viruses to grow well in cured cells evolved, we measured viral growth in LX-2 cells after infection with supernatants collected over time from the eight persistently infected cultures. As anticipated by the observations with cloned viral isolates, we found that the capacity for efficient growth in cured cells was established by about passage day 100 in each culture (data not shown); furthermore, the capacity to grow well in cured cells developed gradually, as was observed by Kauffman et al. (14) in

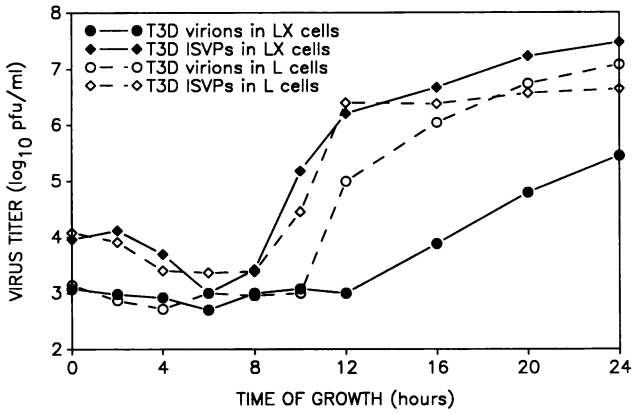

FIG. 2. Growth of T3D virions and ISVPs in cured LX-2 cells and normal L cells. Monolayers of LX-2 and L cells (2×10^5 cells per ml) were infected with either T3D virions or ISVPs at a multiplicity of infection of 1 PFU per cell in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium was added, and the plates were incubated at 37°C for the times shown.

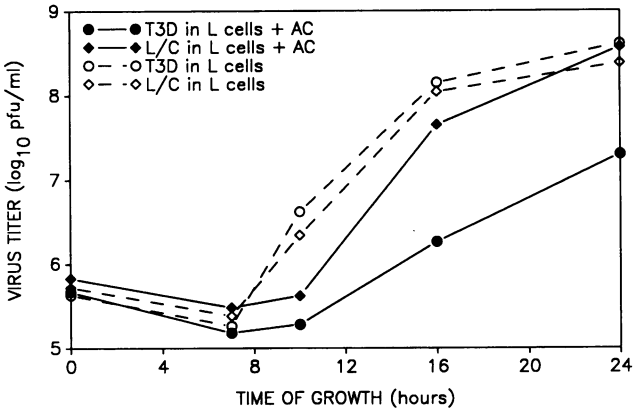

FIG. 3. Growth of T3D and L/C viruses in L cells maintained in the presence and absence of 10 mM ammonium chloride. Monolayers of L cells (5×10^5 cells per ml) were infected with either T3D or L/C virus at a multiplicity of infection of 2 PFU per cell in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium was added (with or without 10 mM ammonium chloride), and the plates were incubated at 37°C for the times shown.

studies of viruses from the persistently infected L/C culture and cured LR cells. These results indicate that during the second and third months of maintenance of persistent infections established with hp stocks of reovirus T3D, viral mutants which have the capacity to grow well in cells cured of persistent infection were selected. In addition, the capacity of cured cells to limit the growth of wt viruses indicates that, at least by day 166, when curing of the cultures began, cellular mutants were also selected. These findings support the notion of virus-cell coevolution during maintenance of persistent infection by reoviruses (1).

Growth of wt viruses in cured cells after infection with ISVPs. We next undertook studies to identify the change in cured cells that is involved in limiting the growth of wt viruses. In order for infection to proceed, virions of reoviruses appear to require proteolytic processing of outer capsid proteins σ3 and μ1/μ1C in late endosomes or lysosomes to which they are delivered after attachment and endocytosis; in contrast, ISVPs, which have already undergone processing by chymotrypsin in vitro, do not require this step (30). This difference in requirement for processing appears to determine the capacity of ISVPs to initiate infection and grow efficiently in L cells treated with ammonium chloride (30), a weak base that raises the pH in acidic compartments of the central vacuolar system (15), including lysosomes (18). Previous data identifying an accumulation of lysosome-like vacuoles in persistently infected and cured cells suggested that an early step in infection involving this cellular compartment, e.g., proteolytic processing, might be affected during persistent infections with reoviruses (1, 9, 28). We therefore decided to test whether wt ISVPs, in contrast to wt virions, could initiate infection in cured cells as a probe for whether early steps in infection by reoviruses are affected in these cells. In these experiments, the duration of the eclipse phase for T3D ISVPs in L cells was found to be approximately 2 h less than that for T3D virions (Fig. 2), as has been previously noted (5). More important, T3D ISVPs were found to provide yields of viral progeny 240 times higher than those of T3D virions after a 24-h period of growth in LX-2 cells; moreover, the eclipse and other phases of growth by wt ISVPs were nearly identical in cured LX-2 and L cells. These findings indicate that cured cells showed no discernible capacity to affect either initiation of infection or subsequent growth by wt ISVPs, which contrasts with the slower growth and lower final yield of viral progeny obtained after infection by wt virions. In additional experiments, we found that ISVPs generated from PI viruses PI 1A1 and PI 2A1 grew as well in cured cells as wt ISVPs (data not shown). Thus, wt ISVPs are similar to virions and ISVPs of PI viruses in that each can initiate infection and grow in cells cured of persistent infection. These findings suggest that the change that has evolved in cured cells affects an early step in the viral growth cycle for which wt virions and ISVPs have different requirements.

Growth of PI viruses in L cells treated with ammonium chloride. Results from the preceding experiments showed that cured cells are similar to L cells treated with ammonium chloride in that they block infection by wt virions but permit infection and growth by wt ISVPs. Because PI viruses can infect and grow normally in cured cells, we reasoned that they might also be capable of infection and growth in ammonium chloride-treated L cells. We pursued this question initially by determining whether T3D and the PI virus L/C differed in their capacities to grow in L cells treated with 10 mM ammonium chloride. As shown in Fig. 3, a dramatic difference was noted between the two viruses: the yield of T3D was significantly decreased by ammonium chloride, as previously noted (8); however, the yields of L/C were nearly equivalent at 0 and 10 mM ammonium chloride. In addition, the rate of L/C growth was only slightly slowed in L cells treated with 10 mM ammonium chloride. Thus, L/C virus is significantly less sensitive than wt virus to the effects of 10 mM ammonium chloride, suggesting that PI viruses contain mutations that affect an early, ammonium chloride-sensitive step in the viral infectious cycle.

We next determined whether the capacity to grow in L cells treated with ammonium chloride is a general property of PI viruses by growing the eight prototype PI viruses (Table 1) in L cells maintained in the presence and absence of 10 mM ammonium chloride. Yields of viral progeny in treated and untreated cells for T3D and the PI viruses are

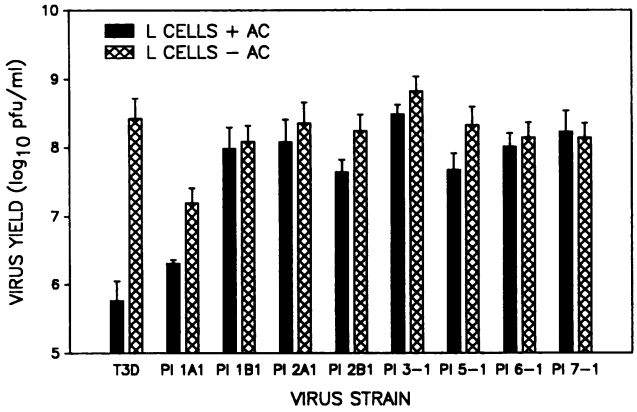

FIG. 4. Growth of T3D and PI viruses in L cells maintained in the presence and absence of 10 mM ammonium chloride. Monolayers of L cells (5×10^5 cells per ml) were infected with either T3D or the PI viruses at a multiplicity of infection of 2 PFU per cell in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium was added (with or without 10 mM ammonium chloride), and the plates were incubated at 37°C for 24 h. The results are presented as the mean viral yield (± standard deviation) determined from four independent experiments for each virus in ammonium chloride-treated and untreated L cells.

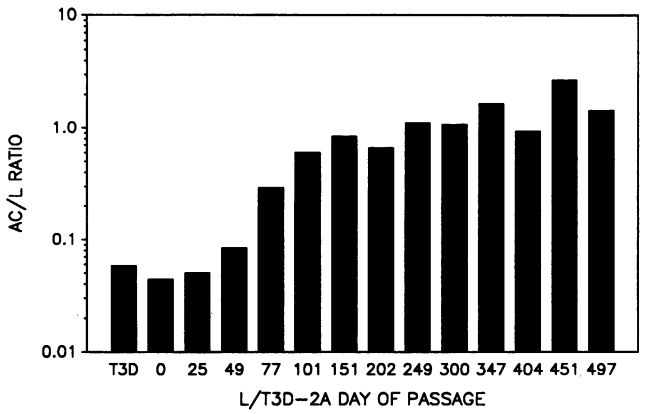

FIG. 5. Effect of day of cell culture maintenance on the capacity of viruses to grow in the presence of ammonium chloride. Monolayers of L cells (5×10^5 cells per ml) were infected with either T3D or cell culture supernatants obtained from the persistently infected L/T3D-2A cell culture in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium was added (with or without 10 mM ammonium chloride), and the plates were incubated at 37°C for 24 h. The results are presented as the yield of the L/T3D-2A cell culture supernatants in ammonium chloride-treated cells divided by the yield in untreated L cells (AC/L ratio).

shown in Fig. 4. In these experiments, the mean yield of T3D was found to be 5.8×10^5 PFU/ml in L cells treated with ammonium chloride and 2.6×10^8 PFU/ml in untreated L cells, whereas the mean yields of the eight PI viruses were 1.1×10^8 PFU/ml in treated L cells and 2.1×10^8 PFU/ml in untreated L cells. To determine the efficiency of growth of the PI viruses relative to T3D in ammonium chloride-treated L cells, we divided the yield of virus in treated L cells by that in untreated L cells (AC/L ratio) for each PI virus and T3D. The AC/L ratio was 0.0022 for T3D and varied from 0.13 (PI 1A1) to 1.22 (PI 7-1) for the PI viruses (Table 1). Given that PI and wt viruses grew comparably in untreated L cells, these results indicate that the PI viruses grew 59 (PI 1A1) to 550 (PI 7-1) times better than wt virus in L cells treated with 10 mM ammonium chloride. Therefore, viruses isolated from persistently infected cultures that grow better than wt virus in cured cells also grow better than wt virus in ammonium chloride-treated L cells.

Using an approach similar to our experiments to study the evolution of growth in cured cells, we conducted experiments to determine when in the time course of persistent infection the capacity of viruses to grow in ammonium chloride-treated L cells evolved. In these experiments, we measured viral growth in the presence and absence of 10 mM ammonium chloride after infection with cell culture supernatant stocks collected over time from the persistently infected L/T3D-1A (data not shown) and L/T3D-2A (Fig. 5) cell lines. We found that the capacity of viruses to grow in L cells treated with ammonium chloride was not a property of the hp stocks used to establish persistent infection but instead was gradually selected in these cultures by passage day 100. Thus, among viruses present in persistently infected cultures established with hp stocks of T3D, evolution of the capacity to grow in L cells treated with ammonium chloride occurs concomitantly with the capacity to grow in cured cells.

To investigate further the difference between wt and PI viruses with respect to growth in ammonium chloride-treated L cells, we determined the effect of ammonium chloride concentrations between 0 and 40 mM on the growth of wt T3D (virions and ISVPs), and two PI viruses, PI 5-1 and PI 5-2. As shown in Fig. 6, a significant difference was again observed in the yields of progeny after infection with T3D virions and the PI viruses in L cells treated with 10 mM ammonium chloride. However, increasing concentrations of ammonium chloride resulted in a decreased yield of PI viruses: the PI viruses grew somewhat less well in 20 mM than in 10 mM and grew poorly in 40 mM ammonium chloride. Thus, PI viruses are like ISVPs of wt strains T1L

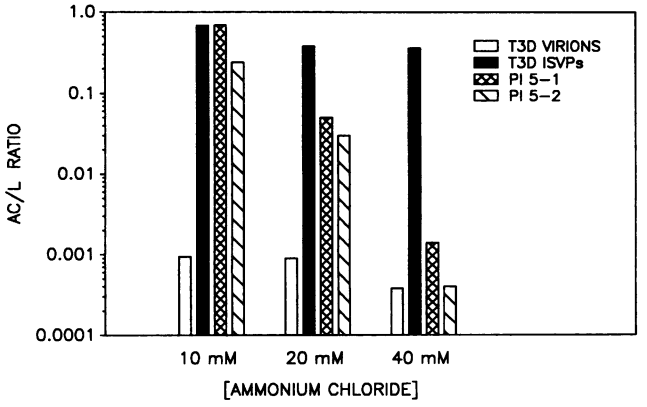

FIG. 6. Effect of ammonium chloride concentration on the growth of T3D and PI viruses. Monolayers of L cells (5×10^5 cells per ml) were infected with T3D (virions or ISVPs), PI 5-1, or PI 5-2 at a multiplicity of infection of 2 PFU per cell in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium was added (with or without 10, 20, or 40 mM ammonium chloride), and the plates were incubated at 37°C for 24 h. The results are presented as the yield of virus in L cells grown in the presence of ammonium chloride divided by the yield in L cells grown in the absence of ammonium chloride (AC/L ratio).

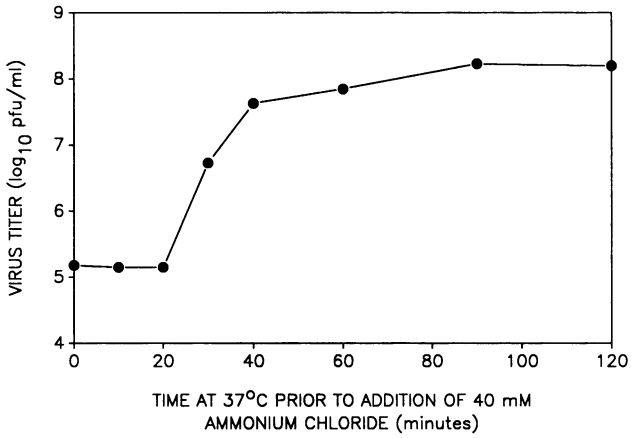

FIG. 7. Effect of time of ammonium chloride addition after virus adsorption on the growth of L/C virus. Monolayers of L cells (5×10^5 cells per ml) were infected with L/C virus at a multiplicity of infection of 2 PFU per cell in 24-well plates. After a 1-h adsorption period, the inoculum was removed, 1.0 ml of fresh medium supplemented with 40 mM ammonium chloride was added to the culture at the indicated times, and the plates were incubated at 37°C for 24 h. The results are presented as the yield of virus after a 24-h period of growth.

(30) and T3D in that they grow better than wt virions in dilute concentrations of ammonium chloride but are unlike ISVPs in that the yield of the PI viruses is decreased to the same extent as that of wt virions at higher ammonium chloride concentrations.

The finding that PI viruses produce minimal yields in the presence of high concentrations of ammonium chloride allowed us to determine the stage in reovirus replication at which ammonium chloride acts to inhibit the growth of PI viruses. In the experiments described above, ammonium chloride was added to cells immediately after virus adsorption and prior to incubation at 37°C in order to allow a 24-h period of viral growth in ammonium chloride-treated L cells. In these experiments, after adsorption of a PI virus strain to L cells, the cells were incubated at 37°C for increasing periods of time prior to the addition of ammonium chloride. We found that the susceptibility of L/C virus to growth inhibition by ammonium chloride decreased logarithmically when ammonium chloride was added 20 to 40 min after adsorption (Fig. 7), which is consistent with the observations of Sturzenbecker et al. (30) with wt T1L. In additional experiments, PI viruses PI 5-1 and PI 5-2 and wt T3D showed similar results (data not shown). Therefore, the block to PI virus growth mediated by high ammonium chloride concentrations most likely involves the same early step in reovirus replication as suggested for wt virus (30).

DISCUSSION

Early steps in the replication cycle of mammalian reoviruses are altered during persistent infection of L cells. In previous studies, Ahmed et al. (1) and Kauffman et al. (14) provided evidence that both cellular and viral mutants are selected during maintenance of L-cell cultures persistently infected with mammalian reoviruses. In the present report, we provide evidence that cells and viruses selected during persistent infection contain mutations that specifically affect one or more early steps in the reovirus replication cycle. The properties of these mutants are consistent with the hypothesis that cells and viruses undergo coevolution with respect to steps in viral entry during maintenance of persistent infections of L cells by reoviruses.

We found that one mutation in cells cured of persistent infection affects the growth of wt viruses after infection by intact virions but not after infection by ISVPs. The replication cycle of reoviruses after infection by virions differs from that after infection by ISVPs only with regard to several early steps (22). Therefore, the normal growth of wt viruses in cured cells after infection by ISVPs indicates that a mutation in these cells is likely to affect a cellular structure or function involved in one or more of these early steps unique to the replication cycle of virions. Moreover, our results indicate that the phenotype that evolves in cells during maintenance of at least some persistent infections by reoviruses is similar to the L-cell phenotype induced by treatment with ammonium chloride. These phenotypes have very similar effects on the growth of wt or PI viruses after infection with either intact virions or ISVPs. Thus, the findings suggest that the two alterations affect either the same cellular structure or function or different ones that are nonetheless closely related in terms of their effects on viral growth. Ammonium chloride, a weak base, is known to raise pH in normally acidic organelles of the central vacuolar system, including lysosomes (18). The specific mutation in cured cells that results in a phenotype similar to that caused by treatment with ammonium chloride might affect acidification of organelles in the central vacuolar system, such as has been selected in Chinese hamster ovary cells by using diphtheria toxin (20). Acidification defects could result in the same effects on viral growth through decreased activity of acid-dependent proteases in lysosomes or prelysosomes, through decreased transport of virions from the cell surface to the organelle in which proteolytic processing occurs, or through decreased transport of virions from the processing organelle to the organelle from which penetration occurs. Future studies will more directly address the biochemical basis of mutations in cured cells that arise during maintenance of persistent infection.

Our experiments with a large collection of viruses isolated from persistently infected cultures show that one mutation common to the PI viruses studied here is such that, in contrast to wt viruses, they exhibit normal infection and growth in L cells treated with ammonium chloride. The capacities of viruses isolated from persistently infected cultures to grow normally in cured LX-2 cells and in L cells treated with low concentrations of ammonium chloride appear at approximately the same time during maintenance of persistent infection and are not independent of each other in any of the PI viruses studied to date. These findings suggest that the two properties are based on the same or related viral mutations. Ammonium chloride is known to block infection by wt reoviruses at an early step in the replication cycle of virions but not ISVPs (30). In our current understanding of reovirus replication, the specific step in infection by virions with which ammonium chloride interferes is the proteolytic processing of viral outer capsid proteins σ3 and μ1/μ1C by cellular proteases in lysosomes or prelysosomal organelles (30). This processing is thought to be required for the viral particle to penetrate the vacuolar membrane and to begin the intracytoplasmic steps in replication (22). Increased pH due to ammonium chloride in these vacuolar compartments might inhibit proteolytic processing of viral proteins either through decreased activity of acid-dependent proteases contained there or through decreased transport of virions to the organelle in which processing occurs (25). The consistently

decreased sensitivity of PI viruses to the effects of ammonium chloride suggests that they contain mutations affecting early steps in the viral growth cycle that relate to proteolytic processing. Kauffman et al. (14) identified the viral S1 gene as the primary genetic determinant of the capacity of PI virus L/C to grow in cured LR-7 cells. This finding seems likely to implicate a function of the cell attachment protein σ1 encoded by S1 in this property. Other outer capsid proteins that are cleaved by proteases (σ3 and μ1/μ1C) are additional candidates for sites of pertinent mutations in PI viruses.

Previous studies with reoviruses (1, 2) and polioviruses (6) have indicated that the establishment and maintenance phases of a persistent infection have distinct features. The present study addresses features of the maintenance phase of persistent infections with reoviruses: viral mutants with capacities to initiate infection and grow well in cured cells and in L cells treated with ammonium chloride were not selected until the second and third months of maintenance, suggesting that their selection is not associated with establishment of the persistently infected culture. In addition, cured cells with the capacity to interfere with infection by wt virions were obtained in this study beginning only after 166 days of maintenance of the persistently infected cultures. How soon after establishment of the persistent infections these cellular mutants were selected was not addressed here, but it is likely that cellular mutants were selected in parallel with viral mutants. Interestingly, treatment of L cells with ammonium chloride was noted previously to favor the establishment of persistent infections with low-passage stocks of wt reoviruses (8). The relationship of that finding to findings in this report regarding the effects of ammonium chloride is unknown. However, it seems reasonable to conclude that pH-sensitive steps in the reovirus growth cycle might be important in determining cellular injury and influencing the nature of virus-cell interactions that favor both the establishment and maintenance of persistent infections.

A model for the maintenance of persistent infections by reoviruses in cell culture. Evidence that viruses and cells coevolve mutations that affect viral entry during maintenance of persistent infections at first seemed paradoxical and led us to contemplate how persistent reovirus infections are maintained in cell culture. Simplistically, a persistent infection in which all cells in the culture are infected and in which intracellular viral particles are efficiently distributed to daughter cells at cell division (efficient vertical transmission) would be expected to have no dependence on horizontal transmission for maintenance of persistent infection and provide no selective pressure for viruses and cells to change with respect to viral entry since entry steps are bypassed in vertical transmission. In contrast, a persistent infection exhibiting inefficient vertical transmission such that uninfected cells are continually generated in the culture because of cell division would depend on horizontal transmission for maintenance of persistent infection and provide a continuing opportunity for coevolution of viral and cellular mutations

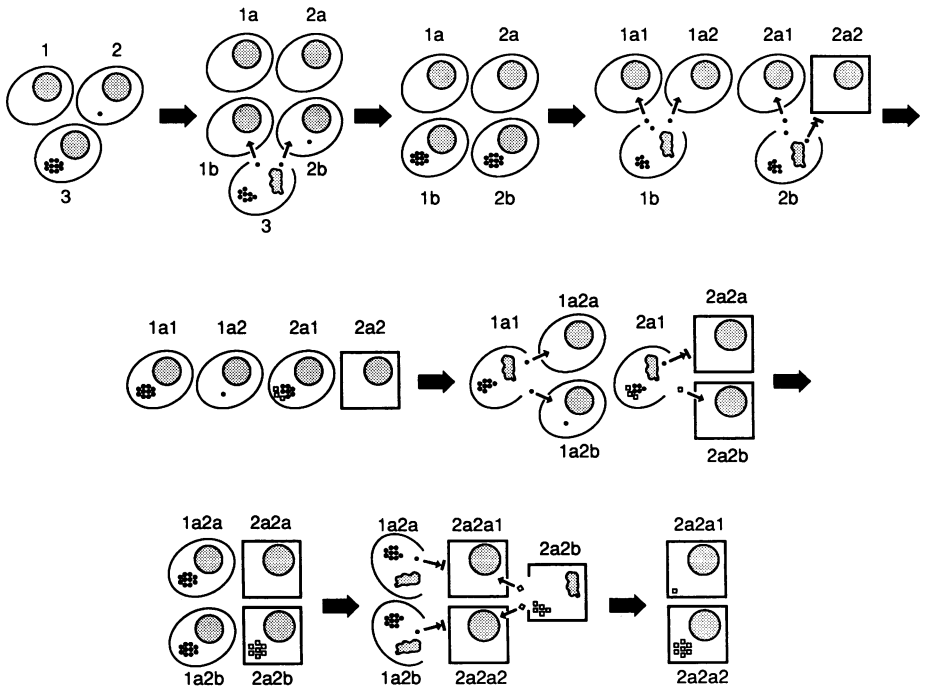

FIG. 8. Virus-cell coevolution during the maintenance of persistent reovirus infection. Cells and their progeny (large ovals and squares) and viruses and their progeny (small circles and squares) in a persistently infected culture are shown. According to this model, the culture exhibits inefficient vertical transmission of virus between cells because more severely infected cells undergo lysis (e.g., cell 3) and less severely infected cells undergo cell division (e.g., cell 2) and generate daughter cells (e.g., cells 2a and 2b) that remain susceptible to viral infection by horizontal transmission. Because of ongoing reinfection and lysis of cells within the culture, mutant cells which are more resistant to reinfection are selected (e.g., cell 2a2). In this model, mutant viruses which are more efficient than wt viruses at infecting these resistant cells are selected subsequently (e.g., virus infecting cell 2a2b). In order for the persistent infection to be maintained, cells must continue to resist lysis by mutant viruses but viruses must retain the capacity to infect mutant cells so that the potential for continuing coevolution of viruses and cells within the culture remains. The requirement for horizontal transmission of virus between cells to maintain the persistent infection provides the setting for selection of viral and cellular mutations that affect viral entry as identified in this study.

affecting viral entry since viruses must enter cells to which they are transmitted horizontally. As shown schematically in Fig. 8, persistently infected cells undergoing reinfection by viruses transmitted horizontally in the culture might be subject to greater injury or death due to increased viral load or increased cytopathic effect after reinfection. As a result, cells more resistant to reinfection (e.g., by a block to viral entry in an endocytic compartment) would be selected and account for the uninfected cells generated in the culture. Mutant viruses capable of infecting these resistant cells (e.g., by the capacity to bypass a block to replication in an endocytic compartment) would be selected since they would be able to achieve greater numbers in the culture. Maintenance of persistent infection would then depend on continuing balanced coevolution so that neither cells nor viruses became completely dominant and resulted in either a spontaneously cured culture or a lysed culture, respectively. A model of persistent infection relying on horizontal transmission of virus between cells also provides a simple explanation for curing cells of persistent infection by passage in media containing neutralizing antiviral antibodies.

If persistent infections of L cells by reoviruses are indeed maintained by horizontal rather than vertical transmission of virus in culture, we can ask whether such persistent infections fit one of the classically defined patterns of persistent infection by other viruses (17). Two types of persistent viral infections in cell culture that share features of persistent reovirus infection are (i) steady-state infections and (ii) carrier cultures. A steady-state infection is characterized as a productive persistent infection in which most or all cells in the culture are infected and which cannot be cured of virus by adding neutralizing antibodies to the culture media. A carrier culture is characterized as a productive persistent infection in which only a small number of cells in the culture are infected at a given time and which can be cured of virus by adding neutralizing antibodies to the culture media. Thus, persistent infection of L cells by reovirus is like a steady-state infection in that a large percentage of cells in the culture are infected and is like a carrier culture in that the culture can be cured of virus by passage in media containing neutralizing antibodies. With regard to transmission of viruses between cells in the persistently infected culture, a steady-state infection is characterized by efficient vertical transmission and is not dependent on horizontal transmission for maintenance whereas a carrier culture is characterized by inefficient vertical transmission and is dependent on horizontal transmission for maintenance. Therefore, persistent infection of L cells by reoviruses appears to be better described as a carrier culture in which a large percentage of cells are infected at any given time. Considering the previous arguments, we postulate that viruses and cells with mutations affecting viral entry will be found to occur in many persistent viral infections that are best described as carrier cultures. If so, persistent infections, or carrier cultures more precisely, should serve as important sources of viral and cellular mutants for the study of viral entry.

In this study, we addressed the nature of mutations in viruses and cells that occur during maintenance of persistent reovirus infection of L cells. We found that viruses and cells undergo coevolution at a particular site of virus-cell interaction during maintenance of persistent infection: processing of outer capsid proteins of infecting virions in an endocytic compartment of cells. These findings indicate that an early step in the infectious cycle can be a determinant of whether viruses cause lytic or persistent infections in their host cells. Our results also suggest that continuing infection or reinfection of cells in a persistently infected culture can force selection of cells that more effectively resist viral infection, presumably because continuing infection or reinfection contributes importantly to cellular injury in this setting. The observation that cells and viruses change with respect to a specific early step in the infectious cycle during maintenance of persistent infection is likely to reflect that this step is one in a series that leads to cellular injury. Moreover, the phenomenon of cellular and viral mutation at a site of virus-cell interaction involving viral entry into cells might have relevance to persistent infections with other viruses, including those associated with immunosuppression or tumorigenesis in host organisms.

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REFERENCES

1. **Ahmed, R., W. M. Canning, R. S. Kauffman, A. H. Sharpe, J. V. Hallum, and B. N. Fields.** 1981. Role of the host cell in persistent viral infection: coevolution of L cells and reovirus during persistent infection. Cell **25**:325–332.
2. **Ahmed, R., and B. N. Fields.** 1982. Role of the S4 gene in the establishment of persistent reovirus infection in L cells. Cell **28**:605–612.
3. **Ahmed, R., and A. F. Graham.** 1977. Persistent infections in L cells with temperature-sensitive mutants of reovirus. J. Virol. **23**:250–262.
4. **Ahmed, R., and J. G. Stevens.** 1990. Viral persistence, p. 241–265. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
5. **Borsa, J., M. D. Sargent, P. A. Lievaart, and T. P. Copps.** 1981. Reovirus: evidence for a second step in the intracellular uncoating and transcriptase activation process. Virology **111**:191–200.
6. **Borzakian, S., T. Couderc, Y. Barbier, G. Attal, I. Pelletier, and F. Colbere-Garapin.** 1992. Persistent poliovirus infection: establishment and maintenance involve distinct mechanisms. Virology **186**:398–408.
7. **Brown, E. G., M. L. Nibert, and B. N. Fields.** 1983. The L2 gene of reovirus serotype 3 controls the capacity to interfere, accumulate deletions and establish persistent infection, p. 275–287. *In* R. W. Compans and D. H. L. Bishop (ed.), Double-stranded RNA viruses. Elsevier Biomedical, New York.
8. **Canning, W. M., and B. N. Fields.** 1983. Ammonium chloride prevents lytic growth of reovirus and helps to establish persistent infection in mouse L cells. Science **219**:987–988.
9. **Canning, W. M., J. Robinson, and B. N. Fields.** Unpublished observations.
10. **de la Torre, J. C., E. Martínez-Salas, J. Diez, A. Villaverde, F. Gebauer, E. Rocha, M. Dávila, and E. Domingo.** 1988. Coevolution of cells and viruses in a persistent infection of foot-and-mouth disease virus in cell culture. J. Virol. **62**:2050–2058.
11. **Ernst, G., and A. J. Shatkin.** 1985. Reovirus hemagglutinin mRNA codes for two polypeptides in overlapping reading frames. Proc. Natl. Acad. Sci. USA **82**:48–52.
12. **Furlong, D. B., M. L. Nibert, and B. N. Fields.** 1988. Sigma 1 protein of mammalian reoviruses extends from the surfaces of viral particles. J. Virol. **62**:246–256.
13. **Jacobs, B. L., and C. E. Samuel.** 1985. Biosynthesis of reovirus-

specified polypeptides: the reovirus S1 mRNA encodes two primary translation products. Virology **143:**63–74.
14. **Kauffman, R. S., R. Ahmed, and B. N. Fields.** 1983. Selection of a mutant S1 gene during reovirus persistent infection of L cells: role in maintenance of the persistent state. Virology **131:**79–87.
15. **Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz.** 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. J. Cell Biol. **116:**1071–1080.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
17. **Mahy, B. W. J.** 1985. Strategies of viral persistence. Br. Med. Bull. **41:**50–55.
18. **Maxfield, F. R.** 1982. Weak bases and ionophores rapidly and reversibly raise the pH in endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. **95:**676–681.
19. **McCrae, M. A., and W. K. Joklik.** 1978. The nature of the polypeptide encoded by each of the ten double-stranded RNA segments of reovirus type 3. Virology **89:**578–593.
20. **Merion, M. L., P. Schlesinger, R. M. Brooks, J. M. Moehring, T. J. Moehring, and W. S. Sly.** 1983. Defective acidification of endosomes in Chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses. Proc. Natl. Acad. Sci. USA **80:**5315–5319.
21. **Mustoe, T. A., R. F. Ramig, A. H. Sharpe, and B. N. Fields.** 1978. Genetics of reovirus: identification of the dsRNA segments encoding the polypeptides of the λ and σ size classes. Virology **89:**594–604.
22. **Nibert, M. L., D. B. Furlong, and B. N. Fields.** 1991. Mechanisms of viral pathogenesis: distinct forms of reoviruses and their roles during replication in cells and host. J. Clin. Invest. **88:**727–734.
23. **Ron, D., and J. Tal.** 1985. Coevolution of cells and virus as a mechanism for the persistence of lymphotropic minute virus of mice in L-cells. J. Virol. **55:**424–430.
24. **Sarkar, G., J. Pelletier, R. Bassel-Duby, A. Jayasuriya, B. N. Fields, and N. Sonenberg.** 1985. Identification of a new polypeptide coded by reovirus gene S1. J. Virol. **54:**720–725.
25. **Seglen, P. O.** 1983. Inhibitors of lysosomal function. Methods Enzymol. **96:**737–764.
26. **Sharpe, A. H., and B. N. Fields.** 1981. Reovirus inhibition of cellular DNA synthesis: role of the S1 gene. J. Virol. **38:**389–392.
27. **Sharpe, A. H., and B. N. Fields.** 1982. Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene. Virology **122:**381–391.
28. **Sharpe, A. H., and B. N. Fields.** 1983. Pathogenesis of reovirus infection, p. 229–285. *In* W. K. Joklik (ed.), The reoviridae. Plenum Publishing Corp., New York.
29. **Sharpe, A. H., R. F. Ramig, T. A. Mustoe, and B. N. Fields.** 1978. A genetic map of reovirus. I. Correlations of genome RNAs between serotypes 1, 2, and 3. Virology **84:**63–74.
30. **Sturzenbecker, L. J., M. Nibert, D. Furlong, and B. N. Fields.** 1987. Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle. J. Virol. **61:**2351–2361.
31. **Virgin, H. W., IV, R. Bassel-Duby, B. N. Fields, and K. L. Tyler.** 1988. Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). J. Virol. **62:**4594–4604.