Infectious Rotavirus Enters Cells by Direct Cell Membrane Penetration, Not by Endocytosis

KAAREL T. KALJOT,^{1,2†} ROBERT D. SHAW,^{1,2} DONALD H. RUBIN,³ AND HARRY B. GREENBERG^{1,2*}

Division of Gastroenterology, Department of Medicine, Stanford University School of Medicine, Stanford, California 95305¹; Palo Alto Veterans Administration Medical Center, Palo Alto, California 94304²; and Departments of Medicine and Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104³*

Received 9 March 1987/Accepted 16 December 1987

Rotaviruses are icosahedral viruses with a segmented, double-stranded RNA genome. They are the major cause of severe infantile infectious diarrhea. Rotavirus growth in tissue culture is markedly enhanced by pretreatment of virus with trypsin. Trypsin activation is associated with cleavage of the viral hemagglutinin (viral protein 3 [VP3]; 88 kilodaltons) into two fragments (60 and 28 kilodaltons). The mechanism by which proteolytic cleavage leads to enhanced growth is unknown. Cleavage of VP3 does not alter viral binding to cell monolayers. In previous electron microscopic studies of infected cell cultures, it has been demonstrated that rotavirus particles enter cells by both endocytosis and direct cell membrane penetration. To determine whether trypsin treatment affected rotavirus internalization, we studied the kinetics of entry of infectious rhesus rotavirus (RRV) into MA104 cells. Trypsin-activated RRV was internalized with a half-time of 3 to 5 min, while nonactivated virus disappeared from the cell surface with a half-time of 30 to 50 min. In contrast to trypsin-activated RRV, loss of nonactivated RRV from the cell surface did not result in the appearance of infection, as measured by plaque formation. Endocytosis inhibitors (sodium azide, dinitrophenol) and lysosomotropic agents (ammonium chloride, chloroquine) had a limited effect on the entry of infectious virus into cells. Purified trypsin-activated RRV added to cell monolayers at pH 7.4 mediated ⁵¹Cr, [¹⁴C]choline, and [³H]inositol released from prelabeled MA104 cells. This release could be specifically blocked by neutralizing antibodies to VP3. These results suggest that MA104 cell infection follows the rapid entry of trypsin-activated RRV by direct cell membrane penetration. Cell membrane penetration of infectious RRV is initiated by trypsin cleavage of VP3. Neutralizing antibodies can inhibit this direct membrane penetration.

Rotaviruses are the major cause of severe infectious diarrhea in children under 2 years of age. They also cause disease in elderly and immunocompromised individuals, as well as in a variety of domesticated and wild animals. The viruses are members of the *Reoviridae*, a family of icosahedral (nonenveloped) viruses with a double-shelled protein capsid and a segmented, double-stranded RNA genome (20). Detailed study of human rotaviruses was initially delayed because of the difficulty in growing them in vitro. Improved cultivation of rotavirus resulted from the identification of a highly permissive cell line, MA104 (36); the discovery that fastidious strains grow better in a roller rather than a stationary culture (4); and the observation that viral replication is markedly enhanced by the presence of trypsin (1, 3, 20, 51). Presumably, this last condition occurs during host infection in the intestinal lumen, when rotavirus is exposed to pancreatic secretions.

The effect of trypsin on viral infectivity is mediated by alteration of the virion rather than the cell surface (5, 19). The outer shell of the rotavirus capsid is formed by two proteins, viral proteins 3 and 7 (VP3 and VP7, respectively), which are the products of genes 4 and 9, respectively (35). In genetic studies in which rotavirus reassortants have been used, the gene 4 product VP3 has been identified as the protein that is correlated with trypsin-enhanced growth in tissue culture (24). Trypsin activation of rotavirus is associated with cleavage of the viral hemagglutinin VP3 (88 kilo-

daltons) into 60- and 28-kilodalton fragments (11, 14, 15). The protein sequence of VP3 has recently been determined from a cDNA copy of genomic RNA (24a, 27). Two closely spaced arginines that would yield the appropriately sized cleavage fragments were identified in the sequence. N-terminal amino acid sequencing of the 60-kilodalton cleavage fragment derived from trypsin-treated virus showed that cleavage could occur at either of these arginines and confirmed that this location was the cleavage region. These two sites have subsequently been shown to be highly conserved among rotaviruses of different serotypes and host species (18, 28).

The mechanism by which this proteolytic cleavage leads to enhanced growth is unknown. In a previous study (11) it was revealed that trypsin treatment does not alter radiolabeled virus binding to cell monolayers and it was suggested that viral uncoating, measured as the conversion of double-shelled to single-shelled particles, was augmented by trypsin. More recently, a novel effect of trypsin on viral entry has been proposed. Early electron microscopic studies of rotavirus-infected cell cultures demonstrated rotavirus in coated pits and a variety of vesicles, signifying entry by endocytosis (40, 41). Electron micrographs by Suzuki et al. (48), however, appear to show that trypsin treatment of rotavirus leads to formation of a viral subpopulation which enters MA104 cell monolayers by direct penetration of the plasma membrane. They proposed that this is the mode of entry for infectious particles and that the nonactivated particles are removed from the surface by endocytosis and do not lead to infection.

In this study we present evidence for the direct penetration model by showing that (i) trypsin treatment converts

* Corresponding author.

† Present address: Molecular Pathobiology Section, Laboratory of Pathobiology, Rocky Mountain Laboratories, Hamilton, MT 59840.

potentially infectious particles into infectious particles; (ii) these two different particles can both enter cells, but they do so with markedly different rates and, thus, by presumably different pathways; (iii) rotavirus infection in tissue culture is not substantially reduced by endocytosis inhibitors or by alkalization of endosomes with weak bases at concentrations of these agents which significantly reduce reovirus type 1 and 3 infection; and (iv) infectious particles increase cell membrane permeability, as measured by ^{51}Cr , $[^{14}\text{C}]$ choline, and $[^3\text{H}]$ inositol release from prelabeled cells.

MATERIALS AND METHODS

Materials. All chemicals used in this study were of reagent grade.

Cell culture. MA104 cells were propagated in medium 199 (catalog no. 9466; Irvine Scientific, Santa Ana, Calif.) supplemented with 7.5% fetal calf serum (catalog no. 200-6140; GIBCO Laboratories, Chagrin Falls, Ohio)-glutamine (2 mM)-penicillin G (50 U/ml)-streptomycin (10 $\mu\text{g}/\text{ml}$)-gentamicin (5 $\mu\text{g}/\text{ml}$) in 5% CO_2 . Bovine aortic endothelial cells (generous gift from Dale Leitman) were grown as described previously (25).

Virus cultivation and purification. For nonactivated virus, rhesus rotavirus (RRV) was activated with 5 μg of trypsin (type IX, no. T-0134; Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 1 h. Washed MA104 cell monolayers were inoculated with activated RRV at a multiplicity of infection (MOI) of 1 to 5. After 2 h the inoculum was aspirated, the flasks were washed twice and refed with medium 199 without trypsin, and the cells were grown until a 2 to 3+ cytopathic effect was visible, usually within 24 h. Flasks were frozen at -70°C. For trypsin-activated virus, washed MA104 cell monolayers were inoculated with trypsin-activated virus at an MOI of 0.1 to 1. The inoculum was not removed, and 0.5 μg of trypsin per ml was included in the medium. Flasks were frozen at -70°C after a complete cytopathic effect appeared. Virus was purified by freeze-thawing twice, trichlorotrifluoroethane (Baron, Blakeslee, Inc., San Francisco, Calif.) extraction, and sucrose gradient rate zonal centrifugation at 25,000 rpm for 75 min (20 to 40% [wt/vol] sucrose gradient; SW 28 rotor; Beckman Instruments, Inc., Fullerton, Calif.). Double- and single-shelled particle-containing fractions were identified by enzyme-linked immunosorbent assay and hemagglutination. Reovirus was grown and purified by the method of Joklik (22).

Monoclonal antibodies. The neutralizing RRV anti-VP3 and anti-VP7 and the nonneutralizing anti-VP6 monoclonal antibodies used in this study have been described previously (47).

Plaque titration. RRV-inoculated MA104 cell monolayers were washed twice with medium 199 and overlaid with medium 199 containing 0.55% agarose (SeaKem ME agarose; FMC Corp., Philadelphia, Pa.) and 0.5 μg of trypsin per ml, and then incubated for 3 to 5 days. Monolayers were stained with neutral red solution (catalog no. 630-5330; GIBCO) diluted 1:20 with medium 199, and plaques were counted. Reovirus plaque titration was performed as described previously (43).

Immunohistochemical antigen detection assay. Following overnight growth of infected MA104 cell monolayers, the cells were washed twice with phosphate-buffered saline and fixed in cold methanol. The fixed monolayers were washed with phosphate-buffered saline and incubated with guinea pig anti-rotavirus hyperimmune serum (1:2,000 dilution in phosphate-buffered saline-1% bovine serum albumin) for 30

min at 37°C. The monolayers were then washed and incubated with peroxidase-conjugated rabbit anti-guinea pig immunoglobulin (Dako immunoglobulins a/s; Accurate Chemicals, Westbury, N.Y.) diluted 1:500 in phosphate-buffered saline-1% bovine serum albumin for 30 min at 37°C. The test was developed with 4 mg of 3-amino-9-ethylcarbazole (no. A-5754; Sigma) per ml in *N,N*-dimethylformamide (no. D-4254; Sigma) mixed with 0.05 M sodium acetate buffer and hydrogen peroxide (1.5 ml, 3.5 ml, and 5 μl , respectively). Infected cells were counted with an inverted microscope.

Kinetics of trypsin-activated RRV entry into MA104 cells. Trypsin- (5 $\mu\text{g}/\text{ml}$) or nontrypsin-treated RRV was incubated with MA104 cell monolayers washed twice in medium 199-20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5) at 4°C for 1 h. Unbound virus was aspirated, medium 199 warmed to 37°C was added, and the plates were incubated at 37°C. Neutralizing monoclonal antibody 159 (anti-VP7; 1:2000 dilution in medium 199) was added at the specified times after warming. After 30 min the wells were washed twice to remove the antibody and overlaid with trypsin-containing agar. The viral entry rate was calculated by measuring the rate at which virus escaped neutralization at the cell surface. The half-time of entry ($t_{1/2}$) is defined as the period required for half of the surface-bound infectious virions to be transported to a cellular compartment characterized by resistance to neutralizing antibody in the medium. This could be determined directly from linear plots of the data by measuring the time required for the plaque number to double. The fastest and slowest possible times were chosen as the limits of the range.

Kinetics of nonactivated RRV entry into MA104 cells. Nonactivated RRV was incubated with twice-washed MA104 cell monolayers in medium 199-20 mM HEPES (pH 7.5) at 4°C for 1 h. Unbound virus was aspirated, medium 199 warmed to 37°C was added, and the plates were incubated at 37°C. At the specified times a trypsin-containing agar overlay was applied. The viral entry rate of these nonactivated particles was calculated by measuring the rate at which surface-bound virus lost the ability to be activated by trypsin. The half-time of entry of these particles is defined as the period required for half of the surface-bound potentially infectious virus particles to be transported to a cellular compartment characterized by resistance to activation by trypsin in the medium. This rate was also derived from linear graphs of the data which allowed direct determination of the time required for the plaque number to decrease by half. The fastest and slowest rates determined the reported range.

Effect of weak bases on RRV infectivity. MA104 cell monolayers were preincubated with 230 μM chloroquine or 30 mM NH_4Cl for 30 min at 37°C. Trypsin-activated RRV (MOI, 0.1 to 1) was added for 1 h, and then neutralizing monoclonal antibody 159 (anti-VP7) was applied. Control monolayers received either antibody and no weak base or weak base added with the antibody following the 1-h incubation of RRV. Following overnight growth (16 h) the monolayers were washed, fixed with methanol, and stained. Alternatively, the plates were freeze-thawed three times, and the viral titers were determined by the plaque assay described above. Inhibitors and antibody were present overnight. Positive controls for the effect of weak bases on intravesicular pH were provided by infection of treated MA104 cell monolayers with reovirus types 1 and 3 (MOI, 5). After 24 h the reovirus-infected monolayers were freeze-thawed three times, and viral titers were determined by plaque assay.

Effect of metabolic inhibitors on RRV infectivity. Sodium

azide (10 mM) and dinitrophenol (1 mM) were tested for their effect on RRV infectivity. MA104 cell monolayers were preincubated with inhibitors for 30 min at 37°C. RRV (MOI, 1) was then added for 30 min. After the unbound virus and inhibitor were aspirated, neutralizing monoclonal antibody was added for 60 min. Control wells received no inhibitor or inhibitor during the antibody incubation period only. Inhibitors were present only for 60 min to reduce cytotoxic effects. Infection was determined by either plaque or immunohistochemical antigen detection assay. Treated cell monolayers infected with reovirus type 1 or 3 served as positive controls.

⁵¹Cr, [¹⁴C]choline, and [³H]inositol release assays. For the ⁵¹Cr release assay, twice-washed MA104 cell monolayers (10⁵ cells per monolayer) in 24-well plates were incubated overnight with ⁵¹Cr (10 µCi/ml; 250 to 500 mCi/mg of Cr; Amersham Corp., Arlington Heights, Ill.). The cells were washed four times and incubated with sucrose gradient-purified RRV (10¹⁰ PFU/ml; 250 µl per well) for 30 min at 37°C. Medium (750 µl) was then added and the incubation was continued for an additional 90 min. The medium was aspirated and centrifuged (microfuge B; Beckman) for 1 min to remove the cells that were lost from the monolayer. The supernatant from the ⁵¹Cr-labeled cells was then counted (Gamma 300 counter; Beckman). To determine the time course, pH optimum, and effect of neutralizing monoclonal antibodies on RRV-mediated ⁵¹Cr release, RRV was incubated with prelabeled cells at 4°C for 1 to 2 h. Medium warmed to 37°C and containing monoclonal antibody (ascites 1:100 dilution) or with appropriately adjusted pH was then added. The incubation was continued at 37°C for 60 to 90 min, and the ⁵¹Cr released from the MA104 cells was then determined as described above.

For the [¹⁴C]choline release assay, twice-washed MA104 cell monolayers in 24-well plates were incubated with [¹⁴C]choline chloride (1 µCi/ml; 50 to 60 mCi/mmol; Amersham) in medium 199 at 37°C for 30 min. The cells were washed and incubated with virus, and the supernatant was collected as described above for the ⁵¹Cr release experiments. [¹⁴C]choline release was determined by scintillation counting.

For the [³H]inositol release assay, twice-washed MA104 cell monolayers in 24-well plates were incubated with myo[³H]inositol (5 µCi/ml; 10-20 Ci/mmol; Amersham) in medium 199 at 37°C for 5 h. The cells were washed four times with medium 199 containing 0.1% fatty acid-free bovine serum albumin. Rotavirus (250 µl) and medium 199 (250 µl) with 0.1% fatty acid-free bovine serum albumin were added to the monolayers, and the cells were incubated at 37°C. Portions of 50 µl were removed at 5, 15, 30, 60, and 90 min after virus addition and added to 4 ml of ACS (Amersham); and [³H]inositol release was determined by scintillation counting.

RESULTS

Effect of trypsin preactivation of RRV on binding to cell monolayers. Trypsin activation did not alter rotavirus binding to cell monolayers. Nonactivated virus received either 5 µg of trypsin per ml or an equal volume of phosphate-buffered saline for 1 h at 37°C. When these preparations were bound to monolayers at 37°C for 1 h, followed by removal of the unbound virus fraction and addition of trypsin-containing agarose, there was no difference in plaque titer: $1,700 \times 10^5$ versus $1,620 \times 10^5$ PFU/ml, respectively. If, however, after the unbound virus was removed and then neutralizing anti-

FIG. 1. Kinetics of internalization of trypsin-activated RRV. Nonactivated RRV was treated with 5 µg of trypsin per ml at 37°C for 1 h. Serial log₁₀ dilutions were made. Virus (diluted 1:10² or 1:10³) was bound to MA104 cell monolayers at 4°C for 1 h, and entry was synchronized by warming the cells to 37°C. Neutralizing monoclonal antibody 159 (anti-VP7) was added to the cell monolayers at the specified times after warming. The number of infected cells was then determined by either immunohistochemical antigen detection or plaque assay, as described in the text.

body was added to the monolayers for 30 min and washed away before the trypsin overlay was applied, a dramatic difference between the two types of virus was observed. While the titer of the trypsin-treated RRV was reduced to 590×10^5 PFU/ml, reduction in the titer of the nonactivated virus was almost 300-fold greater (2×10^5 PFU/ml). This implies that during the 1 h of incubation, trypsin-activated virus was almost completely removed from the cell surface, as measured by resistance to neutralization by antibody. The nonactivated, but potentially infectious, virus, in contrast, remained on the cell surface, where it was available either for activation by trypsin in the overlay or neutralization by monoclonal antibody ($1,620 \times 10^5$ versus 2×10^5 PFU/ml). This nonactivated RRV preparation and the trypsin-treated virus derived from it were then used in the following entry experiments.

Internalization kinetics of trypsin-activated infectious RRV. To characterize further the internalization differences between trypsin-treated and nontreated rotavirus, the rate of infectious virus entry was studied. The entry of trypsin-activated virus was measured by determining the level of escape from neutralizing antibody (Fig. 1). Surface-bound virus was almost completely neutralized when antibody was added at time zero, indicating that less than 1% of the bound infectious virus entered the cells at 4°C. Viral entry was initiated by warming the cell monolayers to 37°C. Entry of trypsin-activated rotavirus had a half-time of 3 to 5 min and appeared to be most consistent with first-order kinetics.

Disappearance kinetics of nonactivated, potentially infectious RRV. The entry rate of nonactivated but potentially infectious virus was determined by exploiting its potential for enhanced infectivity in the presence of trypsin. Nonactivated virus bound to MA104 cell monolayers was activated by the addition of a trypsin-containing overlay at various times (Fig. 2). In this experiment, the loss of potentially infectious virus from the cell surface was measured. These potentially infectious particles were not released into the supernatant since the supernatant contained less than 10² PFU when 10³ PFU was associated with the monolayer. Virus not treated with trypsin also disappeared from the cell surface by first-order kinetics, but the half-time was 30 to 50 min.

Internalization kinetics of the infectious virus fraction associated with nonactivated RRV. There was always some

FIG. 2. Kinetics of nontrypsin-activated RRV internalization. Nonactivated RRV (diluted $1:10^5$, $1:10^6$, or $1:10^7$) was bound to MA104 cell monolayers at 4°C for 1 h. Following warming to 37°C , a trypsin-containing agar overlay was applied at the specified times (0, 15, 40, 70, 120, 170, and 200 min), and the plaque number was determined.

infectious virus in a nonactivated rotavirus pool. These were the particles which were not neutralized by the addition of neutralizing antibody (2×10^5 PFU/ml) in the experiments described above. This population of infectious virus represented less than 1% of the total infectivity present after the pool was treated with trypsin ($1,620 \times 10^5$ PFU/ml; the overlay was applied without pretreatment of the monolayer with neutralizing antibody). We found that the half-time of internalization of this preexisting infectious virus fraction present in the nonactivated pool was also 3 to 5 min, and it, too, disappeared from the cell surface in a log-linear manner (Fig. 3). Results of these experiments showed that (i) trypsin-treated virus enters the cells rapidly ($t_{1/2} = 3$ to 5 min) and led to infection (titer, 590×10^5 PFU/ml); (ii) virus not exposed to trypsin disappeared from the cell surface 10 times more slowly ($t_{1/2} = 30$ to 50 min) and was substantially less infectious (titer, 2×10^5 PFU/ml); and (iii) the small fraction of infectious activity associated with the nonactivated virus pool (2×10^5 PFU/ml) entered the cells at the same rate ($t_{1/2} = 3$ to 5 min) as the trypsin-treated rotavirus.

The discovery of two different rates of virus removal from the cell surface, only one of which appeared to be associated with infection, implied to us that trypsin-treated and non-treated rotavirus might enter the cell by different pathways. These observations were compatible with the model of

FIG. 3. Kinetics of internalization of the residual infectious fraction present in nonactivated RRV. Nonactivated RRV was bound to MA104 cell monolayers at 4°C for 1 h. Dilutions ($1:10$, $1:10^2$, and $1:10^3$) were selected to ensure detectable infectious virus in the assay. Following warming to 37°C , neutralizing antibody was added to the monolayers at the specified times. The number of infected cells was determined by plaque assay.

FIG. 4. Effect of lysosomotropic agents (NH_4Cl and chloroquine) on RRV infection. For the control, cells were incubated with serially diluted trypsin-activated RRV for 1 h at 37°C , the inoculum was removed, and the cells were then incubated overnight with neutralizing monoclonal antibody 159. (A) Cells were incubated as described above, but the indicated lysosomotropic agents were added, along with neutralizing monoclonal antibody, after the viral inoculum was aspirated, and both agents were present during the overnight incubation. (B) The indicated lysosomotropic agents were added for 30 min prior to virus inoculation and were present throughout the experiment. The viral inoculum was removed, and neutralizing antibody was added 1 h after infection, as described for panel A. After an 18-h incubation, the cells were washed three times and fixed with cold methanol. Infected cells were stained with anti-rotavirus hyperimmune guinea pig serum, followed by peroxidase-conjugated rabbit anti-guinea pig immunoglobulin G antiserum, and counted under an inverted microscope. To determine the effect of energy inhibitors (sodium azide and dinitrophenol (DNP)) on RRV infection, control cells were incubated with serially diluted, trypsin-activated RRV for 30 min at 37°C . The inoculum was aspirated, and the cells were incubated for 60 min with neutralizing monoclonal antibody 159 before they were washed and overlaid with trypsin-containing agar. (A) The cells were incubated with virus as described above. After aspiration of the inoculum, the indicated metabolic inhibitors were added, along with neutralizing antibody, for 60 min. The monolayers were washed and then overlaid with trypsin-containing agar. (B) The indicated metabolic inhibitors were added 30 min prior to virus inoculation and were present during the 30-min viral incubation period, after which the monolayers were washed and neutralizing antibody was added for 60 min. The monolayers were then washed again, and the overlay was added. Plaques were counted after 4 days. The numbers above the columns are the percent reduction of treated cells compared with those of controls, which were not treated with the agents.

Suzuki et al. (48, 49), which proposes that infectious (i.e., trypsin-treated) rotavirus enters MA104 cells rapidly by direct plasma membrane penetration, while nontreated (non-infectious) virus enters via the relatively slower endocytic pathway. We sought to test several of the predictions of this model. If the model were correct, infectious would not be affected by inhibitors of endocytosis or by alkalization of endosomes by use of weak bases. In addition, passage of a 70-nm virus particle through a 5-nm membrane might create a pore or injury which could then be detected as an alteration in cell permeability.

Effect of weak bases on RRV infection. Preincubation of MA104 cell monolayers with 30 mM NH_4Cl or 230 μM chloroquine reduced the number of infectious centers by approximately 50%. However, this reduction occurred whether the monolayers were preincubated with the weak bases for 30 min before RRV inoculation (Fig. 4B) or were not added until 1 h after viral inoculation (Fig. 4A), when neutralizing antibody was applied. Weak bases did, how-

ever, reduce reovirus type 1 and 3 output by infected MA104 cells at least 100-fold compared with that by untreated cell monolayers. NH_4Cl (20 mM) pretreatment reduced reovirus type 1 output from the control level of 1.5×10^5 PFU/ml to 2.0×10^2 PFU/ml and reduced reovirus type 3 output from 2×10^4 to $<1 \times 10^2$ PFU/ml. Monolayers treated in parallel and infected with RRV showed less than 20% reduction at this concentration of NH_4Cl . Chloroquine (200 μM) pretreatment reduced reovirus type 1 output from the control level of 2.6×10^5 PFU/ml to 2.0×10^2 PFU/ml and reovirus type 3 from 8.5×10^4 to $<1 \times 10^2$ PFU/ml. Again, RRV output was reduced by less than 20% at this chloroquine concentration.

Effect of energy inhibitors on RRV infection. Since endocytosis is an energy-requiring process, the energy inhibitors sodium azide (10 mM) and dinitrophenol (1 mM) were tested for their ability to protect cells from infection. The number of infected cells was reduced by 30% if these agents were added after the time of viral entry (Fig. 4A). The number was further reduced if they were present before and during virus addition (Fig. 4B). Thus, the difference (10 to 20% reduction) represents the impact during the time of entry. In contrast, reovirus type 3 was reduced 84% by sodium azide treatment (7.7×10^3 to 1.2×10^3 PFU/ml), and reovirus type 1 was reduced 98% by dinitrophenol (1.5×10^5 to 3.8×10^3 PFU/ml).

RRV-induced permeability alterations in MA104 plasma membranes. To study the effect of input on the cell plasma membrane, MA104 cells were preincubated with ^{51}Cr as described above and then exposed to increasing amounts of sucrose gradient-purified rotavirus at 37°C for 2 h. Trypsin-treated RRV mediated ^{51}Cr release in a dose-dependent fashion (Fig. 5). The spontaneous release of Cr was 5% of total cell-associated radioactivity, and the maximum virus-associated release was five times the spontaneous release. In similar experiments, trypsin-treated rotavirus also mediated the release of radiolabeled choline and inositol from prelabeled MA104 cells. For [^{14}C]choline-labeled cells, spontaneous release was 978 cpm and maximum RRV-mediated release was 2,876 cpm. For [^3H]inositol-labeled cells, spontaneous release was 149 cpm and maximum RRV-mediated release was 814 cpm. To determine the time course of virus-mediated ^{51}Cr release, prelabeled cells were incubated

FIG. 5. RRV-mediated ^{51}Cr release from MA104 cells. MA104 cell monolayers (10^5 cells per monolayer) were incubated overnight with $10 \mu\text{Ci}$ of ^{51}Cr per ml. The cells were washed four times and incubated with increasing amounts of sucrose gradient-purified, double-shelled RRV (10^{10} PFU/ml) which was activated with $5 \mu\text{g}$ of trypsin per ml for 1 h. Control cells were incubated with equivalent amounts of sucrose and trypsin. The medium was aspirated after a 2-h incubation at 37°C , centrifuged to remove cells, and counted to determine the amount of ^{51}Cr release, as described in the text.

FIG. 6. Kinetics of ^{51}Cr release from MA104 cell monolayers. Replicate wells of MA104 cells (10^5 cells per monolayer) were incubated overnight with $10 \mu\text{Ci}$ of ^{51}Cr per ml. The cells were washed four times and incubated with $250 \mu\text{l}$ of activated RRV (125 PFU per cell) in medium 199–20 mM HEPES (pH 7.4) at 4°C for 150 min or in medium 199 alone. Warmed medium (1 ml) was then added, and the incubation was continued at 37°C . At the specified times the medium was aspirated and centrifuged to remove cells, and the supernatant was counted to determine the amount of ^{51}Cr release. The solid line represents virus-specific release obtained by subtracting release with medium alone from release with medium and RRV.

with trypsin-treated virus at 4°C for 150 min and then warmed to 37°C . The virus-mediated ^{51}Cr release was detected within 10 min after warming and was maximal by 60 min (Fig. 6). RRV failed to mediate ^{51}Cr release from bovine aortic endothelial cells (Fig. 7). These cells were at least 100- to 1,000-fold less sensitive to RRV infection than were MA104 cells, but were able to bind RRV (K. T. Kaljot and H. B. Greenberg, manuscript in preparation). Treatment of RRV with 10 mM EDTA, which removes VP3 and VP7, before viral addition to the MA104 cell monolayer, completely abolished the RRV-mediated release of ^{51}Cr : RRV-mediated release, 956 cpm; EDTA-treated RRV-mediated release, 449 cpm; spontaneous release, 428 cpm. There was no RRV-mediated ^{51}Cr release between pH 5.5 and 6.5; maximum release occurred at pH 8 (data not shown). Selected monoclonal antibodies also inhibited RRV-mediated ^{51}Cr release from MA104 cells (Fig. 8). Antibody directed at VP6 or a nonneutralizing epitope of VP7 had no effect on

FIG. 7. RRV-mediated ^{51}Cr release from MA104 cells and bovine aortic endothelial (BAE) cells. Cell monolayers (10^5 cells per monolayer) were incubated overnight with $10 \mu\text{Ci}$ of ^{51}Cr per ml. The cells were washed four times and incubated with increasing amounts of RRV (10^{10} PFU/ml) for 4 h. The medium was aspirated, centrifuged to remove cells, and counted to determine the amount of ^{51}Cr release. Total cell-associated ^{51}Cr counts per minute were as follows: MA104 cells, 30,963; bovine aortic endothelial cells, 32,063.

FIG. 8. Inhibition of RRV-mediated ^{51}Cr release by neutralizing monoclonal antibodies (mAb). RRV was incubated at 4°C for 1 h with MA104 cells prelabeled with ^{51}Cr , as described in the legend to Fig. 6. Warm medium containing monoclonal antibodies was added, and the incubation was continued for an additional 2 h at 37°C (22). The amount of ^{51}Cr release was determined as described in the legend to Fig. 5. 255/60 and 631/9 are monoclonal antibodies directed at nonneutralizing domains of internal capsid rotavirus protein VP6. 60 is a monoclonal antibody directed at a nonneutralizing domain of VP7. 2G4 and 1A9 are monoclonal antibodies directed to distinct neutralizing domains of VP3. 255/60 without RRV is the ^{51}Cr release from cells treated with monoclonal antibody 255/60 but no RRV.

^{51}Cr release. However, two antibodies directed at neutralizing domains on VP3 completely prevented ^{51}Cr release.

DISCUSSION

The earliest events in the viral infection of cells are attachment, penetration, and uncoating (13). Following binding to the cell surface, the virus or its genetic material must pass through at least one cellular lipid bilayer and disassemble so that the viral genome becomes available for transcription and replication. While detailed information regarding the entry of many enveloped viruses is now available (32, 34, 37), the mechanism by which icosahedral (nonenveloped) viruses penetrate cells and uncoat is less well understood.

The uncoating step for enveloped viruses, so far as it involves only shedding of the lipid envelope of the virus, is also the mechanism of cell membrane penetration. Viral fusion proteins mediate apposition and fusion of the viral and cellular membranes, allowing the viral genome to pass into the cytoplasm. Whether fusion occurs on the cell surface (neutral pH) or in an acidic endosomal vesicle is determined by the pH optimum of the viral fusion protein (26, 53). Thus, among enveloped viruses the mechanism of viral penetration, i.e., membrane fusion, is conserved; but the compartment in which this entry occurs (plasma membrane or vesicle membrane) can vary.

Nonenveloped viruses must also cross cellular membranes and deliver their genomes to an intracellular location in an appropriately uncoated form for infection to occur. For the most part, it is thought that infectious, nonenveloped virions are internalized by receptor-mediated endocytosis and that penetration subsequently occurs in an acidic vesicle (13). The evidence for localizing the penetration step to an intracellular compartment is based on (i) the finding of virions in coated pits, coated vesicles, and a variety of smooth vesicles in electron microscopic studies of infected cells and (ii) inhibition of infection with weak bases or metabolic inhibi-

tors applied at times and with concentrations which inhibit enveloped virus infectivity by raising intravesicular pH or inhibiting endocytosis. Reduced infectivity after treatment with weak bases not only identifies a compartment but also implies that acidic pH is necessary for some aspect of membrane penetration. Adenovirus is a well-studied example of this model. It can be found in vesicles (12, 16); inhibitors of endocytosis and weak bases reduce infectivity if they are applied early in infection (50); cell plasma membranes can be made permeable when they are exposed to adenovirus at acid pH, presumably mimicking the enhanced permeability of acidic endosomal vesicles with enclosed adenovirus (44); and finally, acid-dependent hydrophobicity of adenovirus surface proteins has been demonstrated, providing both a potential mechanism of membrane penetration and an analog to the acid-dependent fusion proteins of enveloped viruses (45). Similar sensitivity to weak bases has recently been described for members of two other families of nonenveloped viruses: *Reoviridae* (reovirus type 3) and *Picornaviridae* (poliovirus, rhinovirus, and foot-and-mouth disease virus) (7, 9, 30, 31, 39, 54).

There are also, however, reports questioning a common, endocytosis-dependent entry pathway for the nonenveloped viruses. Some electron microscopic studies have reported direct cell membrane penetration by nonenveloped viruses (38), and not all nonenveloped viruses have reduced infectivity in the presence of weak bases (39). Results of this study suggest that infectious rotavirus does indeed employ such a novel entry strategy: protease-dependent direct cell membrane penetration.

We used the different behaviors of trypsin-treated and nontreated RRV to study the early events of rotavirus infection. Treated and nontreated rotavirus bound to cells equally well, indicating that the conformational changes associated with growth-enhancing VP3 cleavage can be separated from the capsid domain(s) mediating RRV binding to the MA104 cell surface. This cleavage does, however, alter virus entry. Trypsin-activated RRV was infectious and entered cells rapidly ($t_{1/2} = 3$ to 5 min). RRV with uncleaved VP3 also appeared to enter cells, but at a 10-fold slower rate. This virus was $<1\%$ as infectious as the same preparation following trypsin treatment. The non-trypsin-activated RRV, which had a low level of infectivity had an entry rate identical to that of the trypsin-treated virus. The identity of these two rates suggests that the presence of a small pool of RRV with cleaved VP3 within the non-trypsin-treated RRV population accounts for the residual infectivity. This seems a more likely explanation than postulating a markedly lower efficiency of infectivity for the other pathway employed by the more slowly entering nontreated particles. Presumably, MA104 cell proteases can activate a small fraction of the progeny virions when the virus is grown without trypsin in the medium. Thus, proteolytic cleavage of VP3 determines which of two alternate entry pathways ($t_{1/2} = 3$ to 5 min or $t_{1/2} = 30$ to 50 min) rotavirus follows and is absolutely essential for infectious virus entry.

The rapid entry pathway which is associated with infectivity appears to correspond to the direct plasma membrane penetration pathway of trypsin-activated infectious rotavirus observed by Suzuki et al. (48). The failure of lysosomotropic agents and metabolic inhibitors to significantly protect cells from RRV infection suggests that infectious RRV does not use endocytosis to enter the cell. RRV-mediated ^{51}Cr , [^{14}C]choline, and [^3H]inositol release from prelabeled MA104 cells, on the other hand, provides suggestive evidence for the entry of infectious virus by plasma membrane

penetration. This activity occurs rapidly following binding and, like entry of infectious particles, does not occur at 4°C. ^{51}Cr release is only mediated by infectious double-shelled particles. EDTA treatment of RRV, which removes VP3 and VP7 from virions and abolishes infectivity, completely prevented ^{51}Cr release. In addition, release is not a nonspecific viral cytotoxic membrane effect since there was no RRV-mediated ^{51}Cr release from bovine aortic endothelial cells, which bound RRV efficiently but were 100- to 1,000-fold less permissive for rotavirus antigen synthesis than were MA104 cells. The alkaline pH optimum of RRV-mediated ^{51}Cr release also favors cell surface rather than acid endosomal or lysosomal membrane penetration.

The ability of neutralizing monoclonal antibodies to prevent RRV-mediated ^{51}Cr release indicates the specificity of the process and suggests that one mechanism of rotavirus neutralization by antibodies involves inhibition of infectious virus entry by stopping membrane penetration. Anti-penton base sera has a similar inhibitory effect on the acid-dependent ^{51}Cr release mediated by adenovirus type 2 (46). Recently, neutralizing antibodies that can inhibit viral entry by preventing membrane fusion have been described for the enveloped flavivirus West Nile virus (17). Thus, the prevention of infectious virus entry is a mechanism of antibody-mediated neutralization that is common to both enveloped and nonenveloped viruses.

In addition to the observations of Suzuki et al. (48, 49) for rotavirus, there is evidence that both a picornavirus, the encephalomyocarditis virus and another member of the *Reoviridae* can produce infection by direct plasma membrane penetration. Entry of infectious encephalomyocarditis virus does not require a low pH since lysosomotropic agents do not protect mouse L cells from infection and, indeed, low-pH exposure of cells with prebound virus protects the cells from infection (30). Furthermore, exposure to encephalomyocarditis virus leads to increased cell membrane permeability shortly after virus adsorption, implying that the site of entry is the cell membrane, where the inhibitory effect of acid pH on encephalomyocarditis virus infection is avoided (8). Borsa et al. (6) showed that chymotrypsin treatment of reovirus type 3 is associated with enhanced infectivity in mouse L cells and that this enhanced infectivity can be correlated with electron microscopic evidence of direct cell membrane penetration. Nontreated particles entered the cell by endocytosis and appeared to be less infectious. Furthermore, chymotrypsin-treated reovirus type 3 released ^{51}Cr from prelabeled L cells. It is not clear how these observations are related to the subsequent results of Canning and Fields (7) and Maratos-Flier et al. (31), who showed reduced reovirus type 3 infectivity after the pretreatment of L cells with NH_4Cl , implying that reovirus entry is through an intracellular acidic compartment (7, 31). We also noted this effect of NH_4Cl on reovirus type 3 infection of MA104 cells. These disparate observations would be resolved if chymotrypsin treatment could be shown to substitute for an acid-dependent vesicular protease in the reovirus type 3 entry process. Despite this apparent conflict, protease-dependent membrane penetration may be a common feature of the *Reoviridae*, and if so, its occurrence among the orbiviruses might also be expected.

Cleavage of VP3 with trypsin appears analogous to the proteolytic activation of ortho- and paramyxovirus fusion proteins since both events are followed by the movement of viral proteins into the lipid bilayer (53). The presence of syncytia in avian reovirus- (23) and group B rotavirus (2, 10, 52)-infected cells also supports the notion of fusion-like

activity in members of the *Reoviridae*. However, the mechanisms of these events are probably different. Cleavage of the paramyxovirus fusion protein allows exposure of a previously covered hydrophobic region (21); the new N terminus, which is extremely hydrophobic and highly conserved among the paramyxoviruses, may then be inserted into the membrane to initiate fusion since peptides homologous to the new N terminus are inhibitors of fusion protein-mediated membrane fusion (42). Unlike the cleavage of the paramyxovirus fusion proteins, trypsin activation of VP3 does not expose a long, hydrophobic, signal peptide-like sequence at the new amino terminus of the cleavage product (18, 27, 28; Kantharidis et al., in press). Oligopeptides that mimic the new N terminus do not prevent infectious virus entry (K. Kaljot, unpublished data). The VP3-cell membrane interactions may occur at hydrophobic protein regions that are distant from the cleavage site. Of note in this regard is the fact that sequencing of RRV variants selected by escape from broadly heterotypic neutralizing anti-VP3 monoclonal antibodies (including 2G4) has localized the responsible mutations to a hydrophobic region of the 60-kilodalton cleavage product VP5. Interestingly, this region is homologous to the internal fusion sites of Semliki Forest virus and Sindbis virus (28a). Thus, an antibody capable of preventing RRV-mediated ^{51}Cr release also selects an escape variant with a single amino acid substitution in a region homologous to a known internal fusion sequence.

Very recent electron microscopic studies of trypsin-treated rotavirus interactions with MA104 cells by Suzuki et al. (49) suggest that penetration of the cell membrane is not followed by transfer of the entire rotavirus particle into the cytoplasm. Rather, the virus-cell membrane interaction leads to virus capsid rearrangement, viral transcriptase activation, cell membrane pore formation, and the passage of viral RNA into the cytoplasm by a mechanism analogous to bacteriophage infection. In such a model, our observation of the antibody-mediated neutralization of prebound infectious rotavirus suggests that neutralization interferes with the VP3 and VP7 conformational changes associated with transcriptase activation and cell membrane pore formation. Conversely, once pore formation has occurred, neutralization by antibodies may no longer be possible.

The slow internalization pathway used by noninfectious (intact VP3) rotavirus probably corresponds to the endocytosis of rotavirus detected in the electron microscopic studies. The half-time of this entry pathway (30 to 50 min) is unusually long for most reported endocytic processes. However, our warming procedure which involved the aspiration of cold media from chilled plates, replacement of this with warmed media, and placement of the cool plates in a 37°C incubator probably resulted in the slow warming of the cell monolayers. A similarly long apparent half-time (35 min) of endocytosis was observed for Semliki Forest virus entry, which has been studied under similar warming conditions (33). When the plates were warmed rapidly by total immersion in warm media, the half-time of Semliki Forest virus endocytosis was reduced to 5 to 10 min. A corresponding reduction in the rate of trypsin-treated particle entry might also be expected under these more rapid warming conditions, so that the half-time of RRV direct membrane penetration would then be 1 min or less.

Our results show that tryptic cleavage of VP3 determines which of two separate entry pathways leads to rotavirus removal from the MA104 cell surface. The rapid entry of activated particles leads to infection, while the slower internalization of particles with intact VP3 does not produce

infection. Unlike viruses that enter cells by endocytosis, the entry pathway of infectious rotavirus is not sensitive to alkalization of vesicles by weak bases. The failure of energy inhibitors, which probably interfere with endocytosis rather than the acidification of vesicles, to reduce rotavirus infection while protecting cells from reovirus infection also suggests that rotavirus employs an alternative pathway. The ability of infectious rotavirus to release ^{51}Cr , [^{14}C]choline, and [^3H]inositol from prelabeled MA104 cells is consistent with direct virus penetration of the plasma membrane. Combined with the electron microscopic observations of Suzuki et al. (49), these results strongly suggest that trypsin cleavage of VP3 initiates cell membrane penetration by infectious rotavirus. Rotavirus particles with intact VP3 are probably internalized by endocytosis and ultimately are degraded in lysosomes since they lack the ability to penetrate endosomal or lysosomal membranes. The failure of activated rotavirus to release ^{51}Cr from bovine aortic endothelial cells, even though they bind to their surface, implies that the specific cell membrane components necessary for rotavirus attachment may not be identical to those required for subsequent viral penetration of the membrane. It will be interesting to determine whether the rotavirus tissue tropism can be linked to viral entry rather than cell binding. Finally, the ability of monoclonal antibodies directed at VP3 to inhibit ^{51}Cr release and efficiently neutralize prebound virus implies that at least one mechanism of neutralization is penetration inhibition.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Phuoc Vo.

This work was supported in part by Public Health Service grant R22AI 21362-01 from the National Institutes of Health. R.D.S. was supported by an Associate Investigator Career Development award from the Veterans Administration, and K.T.K. was supported by a Substance Abuse fellowship from the Veterans Administration.

LITERATURE CITED

- Almeida, J. D., T. Hall, J. E. Banatvala, B. M. Totterdell, and I. L. Chrystie. 1978. The effect of trypsin on the growth of rotavirus. *J. Gen. Virol.* **40**:213-218.
- Askaa, J., and B. Bloch. 1984. Infection in piglets with a porcine rotavirus-like virus. Experimental inoculation and ultrastructural examination. *Arch. Virol.* **80**:291-303.
- Babiuk, L. A., K. Mohammad, L. Spence, M. Fauvel, and R. Petro. 1977. Rotavirus isolation and cultivation in the presence of trypsin. *J. Clin. Microbiol.* **6**:610-617.
- Banatvala, J. E., B. Totterdell, I. L. Chrystie, and G. N. Woode. 1975. *In vitro* detection of human rotaviruses. *Lancet* **ii**:821.
- Barnett, B. B., R. S. Spendlove, and M. L. Clark. 1979. Effect of enzymes on rotavirus infectivity. *J. Clin. Microbiol.* **10**:111-113.
- Borsa, J., B. D. Morash, M. D. Sargent, T. P. Copps, P. A. Lievaart, and J. G. Szekely. 1979. Two modes of entry of reovirus particles into L cells. *J. Gen. Virol.* **45**:161-170.
- 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.
- Carrasco, L. 1981. Modification of membrane permeability induced by animal viruses early in infection. *Virology* **113**:623-629.
- Carrillo, E. C., C. Giachetti, and R. H. Campos. 1984. Effect of lysosomotropic agents on foot-and-mouth disease virus replication. *Virology* **135**:542-545.
- Chasey, D., and J. Banks. 1986. Replication of atypical ovine rotavirus in small intestine and cell culture. *J. Gen. Virol.* **67**:567-576.
- Clark, S. M., J. R. Roth, M. L. Clark, B. B. Barnett, and R. S. Spendlove. 1981. Trypsin enhancement of rotavirus infectivity: mechanism of enhancement. *J. Virol.* **39**:816-822.
- Dales, S. 1973. Early events in cell-animal virus interactions. *Bacteriol. Rev.* **37**:103-135.
- Dimmock, N. J. 1982. Initial stages in infection with animal viruses. *J. Gen. Virol.* **59**:1-22.
- Espejo, R. T., S. Lopez, and C. Arias. 1981. Structural polypeptides of simian rotavirus and the effect of trypsin. *J. Virol.* **37**:156-160.
- Estes, M. K., D. Y. Graham, and B. B. Mason. 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. *J. Virol.* **39**:879-888.
- Fitzgerald, D. J. P., R. Padmanabhan, I. Pastan, and M. C. Willingham. 1983. Adenovirus-induced release of epidermal growth factor and Pseudomonas toxin into the cytosol of KB cells during receptor mediated endocytosis. *Cell* **32**:607-617.
- Gollins, S. W., and J. S. Porterfield. 1986. A new mechanism for the neutralization of enveloped viruses by antiviral antibody. *Nature (London)* **321**:244-246.
- Gorziglia, M., Y. Hoshino, A. Buckler-White, I. Blumentals, R. Glass, J. Flores, A. Z. Kapikian, and R. M. Chanock. 1986. Conservation of amino acid sequence of VP8 and cleavage region of 84-kDa outer capsid protein among rotaviruses recovered from asymptomatic neonatal infection. *Proc. Natl. Acad. Sci. USA* **83**:7039-7043.
- Graham, D. Y., and M. K. Estes. 1980. Proteolytic enhancement of rotavirus infectivity: biologic mechanisms. *Virology* **101**:432-439.
- Holmes, I. H. 1983. Rotaviruses, p. 359-423. In W. K. Joklik, (ed.), *The reoviridae*, Plenum Publishing Corp., New York.
- Hsu, M., A. Scheid, and P. W. Choppin. 1981. Activation of the Sendai virus fusion protein (F) involves a conformational change with exposure of a new hydrophobic region. *J. Biol. Chem.* **256**:3557-3563.
- Joklik, W. K. 1972. Studies on the effect of chymotrypsin on reovirus. *Virology* **49**:700-715.
- Joklik, W. K. 1983. The reovirus particle, p. 9-78. In W. K. Joklik, (ed.), *The reoviridae*. Plenum Publishing Corp., New York.
- Kalica, A. R., J. Flores, and H. B. Greenberg. 1983. Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. *Virology* **125**:194-205.
- Kantharidis, P., M. L. Dyall-Smith, and I. H. Holmes. 1987. Marked sequence variation between segment 4 genes of human RV-5 and simian SA11 rotaviruses. *Arch. Virol.* **93**:111-121.
- Leitman, D., R. R. Fiscus, and F. Murad. 1986. Forskolin, phosphodiesterase inhibitors, and cyclic AMP analogs inhibit proliferation of cultured bovine aortic endothelial cells. *J. Cell. Physiol.* **127**:237-243.
- Lenard, J., and D. K. Miller. 1982. Uncoating of enveloped viruses. *Cell* **28**:5-6.
- Lopez, S., C. F. Arias, J. R. Bell, J. H. Strauss, and R. T. Espejo. 1985. Primary structure of the cleavage site associated with trypsin enhancement of rotavirus SA11 infectivity. *Virology* **144**:11-19.
- Lopez, S., C. F. Arias, E. Mendez, and R. T. Espejo. 1986. Conservation in rotaviruses of the protein region containing the two sites associated with trypsin enhancement of infectivity. *Virology* **154**:224-227.
- Mackow, E., R. Shaw, S. Matsui, P. Vo, M. Dang, and H. Greenberg. 1988. Characterization of rhesus rotavirus VP3 gene: location of amino acids involved in homologous and heterologous rotavirus neutralization and identification of a putative fusion region. *Proc. Natl. Acad. Sci. USA* **85**:465-469.
- Madhus, I. H., S. Olsnes, and K. Sandvig. 1984. Different pH requirements for entry of the two picornaviruses, human rhinovirus 2 and murine encephalomyocarditis virus. *Virology* **139**:346-357.
- Madhus, I. H., S. Olsnes, and K. Sandvig. 1984. Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. *J. Cell Biol.* **98**:1194-1200.
- Maratos-Flier, E., M. J. Goodman, A. H. Murray, and C. R. Kahn. 1986. Ammonium inhibits processing and cytotoxicity of reovirus, a nonenveloped virus. *J. Clin. Invest.* **78**:1003-1007.

32. Marsh, M. 1984. The entry of enveloped viruses into cells by endocytosis. *Biochem. J.* **218**:1-10.
33. Marsh, M., and A. Helenius. 1980. Absorptive endocytosis of Semliki Forest virus. *J. Mol. Biol.* **142**:439-454.
34. Marsh, M., K. Matlin, K. Simons, H. Reggio, J. White, J. Kartenbeck, and A. Helenius. 1982. Are lysosomes a site of enveloped virus penetration? Cold Spring Harbor Symp. Quant. Biol. **46**:835-843.
35. Mason, B. B., D. Y. Graham, and M. K. Estes. 1983. Biochemical mapping of the simian SA11 genome. *J. Virol.* **46**:413-423.
36. Matsuno, S., S. Inouye, and R. Kono. 1977. Plaque assay of neonatal calf diarrhea virus and the neutralizing antibody in human sera. *J. Clin. Microbiol.* **5**:1-4.
37. Miller, D. K., and J. Lenard. 1981. Antihistaminics, local anesthetics, and other amines as antiviral agents. *Proc. Natl. Acad. Sci. USA* **78**:3605-3609.
38. Morgan, C., H. S. Rosenkranz, and B. Mednis. 1969. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating. *J. Virol.* **4**:777-796.
39. Olsnes, S., I. H. Madshus, and K. Sandvig. 1986. Entry mechanism of picornaviruses, p. 171-181. In R. L. Crowell and K. Lonberg-Holm (ed.), *Virus attachment and entry into cells*. American Society for Microbiology, Washington, D.C.
40. Petrie, B. L., D. Y. Graham, and M. K. Estes. 1981. Identification of rotavirus particle types. *Intervirology* **16**:20-28.
41. Quann, C. M., and F. W. Doane. 1983. Ultrastructural evidence for the cellular uptake of rotavirus by endocytosis. *Intervirology* **20**:223-231.
42. Richardson, C. R., A. Scheid, and P. W. Choppin. 1980. Specific inhibition of Paramyxovirus and Myxovirus replication by oligopeptides with amino acid sequences similar to those at the N-termini of the F₁ or HA₂ viral polypeptides. *Virology* **105**:205-222.
43. Rubin, D. H., M. J. Kornstein, and A. O. Anderson. 1985. Reovirus serotype 1 intestinal infection: a novel replicative cycle with ileal disease. *J. Virol.* **53**:391-398.
44. Seth, P., I. Pastan, and M. C. Willingham. 1985. Adenovirus dependent increase in cell membrane permeability. *J. Biol. Chem.* **260**:9598-9602.
45. Seth, R., M. C. Willingham, and I. Pastan. 1985. Binding of adenovirus and its external proteins to Triton X-114. *J. Biol. Chem.* **260**:14431-14434.
46. Seth, P., M. C. Willingham, and I. Pastan. 1985. Adenovirus-dependent release of ⁵¹Cr from KB cells at an acidic pH. *J. Biol. Chem.* **259**:14350-14353.
47. Shaw, R. D., P. T. Vo, P. A. Offitt, B. S. Coulson, and H. B. Greenberg. 1986. Antigenic mapping of the surface proteins of rhesus rotavirus. *Virology* **155**:434-451.
48. Suzuki, H., S. Kitaoka, T. Konno, T. Sato, and N. Ishida. 1985. Two modes of human rotavirus entry into MA 104 cells. *Arch. Virol.* **85**:25-34.
49. Suzuki, H., S. Kitaoka, T. Sato, T. Konno, Y. Iwasaki, Y. Numazaki, and N. Ishida. 1986. Further investigation on the mode of entry of human rotavirus into cells. *Arch. Virol.* **91**:135-144.
50. Svensson, U. 1985. Role of vesicles during adenovirus 2 internalization into HeLa cells. *J. Virol.* **55**:442-449.
51. Thiel, K. W., E. H. Bohl, and A. G. Agnes. 1977. Cell culture propagation of porcine rotavirus (reovirus-like agent). *Am. J. Vet. Res.* **38**:1765-1768.
52. Vonderfecht, S. L., A. C. Huber, J. Eiden, L. C. Mader, and R. H. Yolken. 1984. Infectious diarrhoea of infant rats produced by a rotavirus-like agent. *J. Virol.* **52**:94-98.
53. White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* **16**:151-195.
54. Zeichardt, H., K. Wetz, P. Willingham, and K.-O. Habermehl. 1985. Entry of poliovirus type 1 and mouse Elberfeld (ME) virus into HEP-2 cells: receptor mediated endocytosis and endosomal or lysosomal uncoating. *J. Gen. Virol.* **66**:483-492.