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Role of VP3 in Human Rotavirus Internalization after Target Cell Attachment via VP7

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A cell lysate prepared from MA104 cells that had been infected with human rotavirus KUN strain (HRV-KUN) contained a 35-kilodalton protein capable of binding to MA104 cells. The binding of the 35-kilodalton protein was inhibited by a serotype 2-specific antiserum but not by antisera to other serotypes. Not only trypsin-treated, infectious HRV-KUN but also untreated, noninfectious virions effectively competed with the 35-kilodalton protein for the same cell surface binding sites. One monoclonal anti-VP7 (AH6) absorbed the 35-kilodalton protein from the HRV-KUN-infected cell lysate, whereas another monoclonal anti-VP7 (S2-2G10) inhibited the virions to compete with the 35-kilodalton protein for the cell surface binding sites. Both anti-VP7 (S2-2G10) and anti-VP3 (K-1532, K-376) monoclonal antibodies had the virus-neutralization activity, but only anti-VP7 inhibited virus adsorption. On the other hand, anti-VP3 monoclonal antibodies were capable of completely inhibiting the infection of preadsorbed HRV-KUN as long as virions were not yet internalized. Subsequent studies with [^{35}S]methionine-labeled and purified HRV-KUN showed that not only trypsin-treated, infectious virions but also untreated, noninfectious virions were capable of efficient target cell binding and internalization. The internalization modes of these two HRV-KUN preparations were, however, quite different. Only the components of the inner capsid were internalized from trypsin-treated virions, whereas no such selective internalization was seen with untreated virions. Furthermore, anti-VP3 inhibited this selective internalization of the inner capsid from the infectious virions. From these results we conclude that VP7 is the HRV-KUN cell attachment protein and that adsorption of HRV-KUN via VP7 is independent of trypsin treatment, whereas the limited cleavage of VP3 by trypsin, which is essential for the development of HRV-KUN infectivity, is needed for the selective internalization of the inner capsid components, a process that is apparently essential for HRV-KUN infection.

Rotaviruses, new members of the family *Reoviridae*, are now known as major etiologic agents of nonbacterial gastroenteritis in a wide variety of species, including humans. The virus particles have a nonenveloped double-capsid structure and contain 11 segments of double-stranded RNA. The outer capsid has a major 35- to 41-kilodalton (kDa) component, VP7, and a minor 80- to 88-kDa component VP3, (for a review see reference 13). Studies employing isolation of various gene reassortants and/or preparation of highly specific reagents such as monoclonal antibodies have established that VP7 and VP3 both function as independent neutralization antigens (7, 10, 11, 14–16, 28, 30, 33, 35, 40–42). Sabara et al. showed that free-form VP7 of bovine rotavirus was capable of binding to cells and that monoclonal antibodies to VP7 could inhibit cell attachment of bovine rotavirus (33). Therefore, it is likely that VP7 is the rotavirus cell attachment protein. On the other hand, VP3, which has hemagglutinin activity (13), has been shown to be associated with in vitro growth restriction (10) and in vivo virulence (29). Furthermore, trypsin cleavage of VP3 enhances the infectivity of bovine and simian rotaviruses (5, 9) and is essential for human rotavirus infectivity (17, 18). Nevertheless, the exact role of VP3 in rotavirus infection is largely unknown.

Previously we showed that human rotavirus (HRV) KUN strain (HRV-KUN) was absolutely dependent on trypsin for propagation in vitro (17, 18). The mild trypsin treatment specifically cleaved VP3, with a concomitant loss of the hemagglutinin activity that was demonstrable only by using 1-day-old chicken erythrocytes (18). Subsequently, we found from electron microscopic studies that trypsin-treated, infectious HRV-KUN apparently entered cells by a direct penetration through the cell membrane, whereas untreated HRV-KUN, although noninfectious, was also taken up into cells by the process of endocytosis (36, 37). The independence of HRV-KUN infection from endocytosis and low-pH vesicles was further demonstrated by the finding that HRV-KUN infection was quite resistant to various lysosomotropic weak bases and cytochalasin B (8). In sum, the trypsin cleavage of VP3 might be needed for the unique internalization process of HRV-KUN after attachment to the target cell. On the other hand, the possibility exists that the virus-cell attachment is not dependent on the trypsin cleavage of VP3. We have also described monoclonal antibodies that inhibited the hemagglutinin activity of untreated HRV-KUN, neutralized trypsin-treated HRV-KUN, and specifically bound to VP3 and one of its trypsin-cleaved fragments (16).

In the present study, we showed first that the lysate of HRV-KUN-infected cells contained a 35-kDa protein capable of binding to MA104 cells. The binding of the 35-kDa protein was inhibited only by a serotype 2-specific antiserum. Furthermore, one monoclonal anti-VP7 could absorbed the 35-kDa protein before the binding assay, whereas another monoclonal anti-VP7 could block HRV-KUN virions to compete with the 35-kDa protein for the same cell surface binding sites. From these results and from the size of the protein, we concluded that the 35-kDa cell-binding protein was HRV-KUN VP7. Second, we found that anti-VP3 monoclonal antibodies neither inhibited the binding of free

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VP7 or whole HRV-KUN virions to target cells nor affected the virions to complete with VP7 for the same cell surface binding sites. Third, monoclonal anti-VP3s could block the infection of preadsorbed HRV-KUN as long as virions were not yet internalized. Fourth, we found that trypsin-treated HRV-KUN had only its inner capsid components internalized into target cells, whereas untreated, noninfectious HRV-KUN was internalized into target cells without such selectivity. Finally, we demonstrated that anti-VP3 suppressed the selective internalization of the inner capsid from trypsin-treated HRV-KUN after its adsorption to target cells.

MATERIALS AND METHODS

Cells and viruses. A fetal rhesus monkey kidney line, MA104, was maintained in Eagle minimum essential medium (MEM) supplemented with 10% fetal bovine serum. Three strains of HRV were used: KUN (serotype 2, subgroup 1) (21), Wa (serotype 1, subgroup 2) (44); MO (serotype 3, subgroup 2) (21). HRV was prepared as described previously (18). In brief, MA104 cells in serum-free MEM were inoculated with HRV at a multiplicity of infection (MOI) of 0.1 and cultured with or without 0.5 μg of trypsin per ml for 1 to 2 days. The whole culture was rapidly frozen and thawed twice, and cell debris was removed by centrifugation. The supernatant containing HRV was stored at −80°C until use. The HRV titer, expressed as fluorescent cell-forming units (FCFU) per ml, was determined by indirect immunofluorescence staining employing hyperimmune guinea pig anti-HRV sera (see below) as the first antibody and fluorescein isothiocyanate-conjugated goat anti-guinea pig immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) as the second antibody as described previously (18).

Virus purification. HRV supernatant (about 500 ml) was mixed with ¼ volume of trichlorotrifluoroethane and the mixture was homogenized in a Dounce-type homogenizer. The aqueous phase was collected by low-speed centrifugation and further centrifuged at 7,000 × *g* for 30 min. The supernatant was layered on a 20% sucrose cushion and centrifuged at 90,000 × *g* for 3 h. The pellet was suspended in a minimum volume of phosphate-buffered saline (PBS), layered on a linear sucrose gradient (20 to 60%), and centrifuged at 100,000 × *g* for 2.5 h. Fractions were collected by dripping from the tube bottom, and the HRV titer was determined by the immunofluorescence staining method (see above). The fractions containing HRV were pooled and stored at −80°C until use. [^{35}S]methionine-labeled HRV-KUN virions were prepared as follows. MA104 cells (2×10^6 cells in 25-cm^2 culture flasks) were infected with trypsin-treated HRV-KUN at an MOI of 1 and cultured for 4.5 h. After cells were washed with methionine-free MEM, they were cultured in methionine-free MEM containing 25 μCi of L-[^{35}S]methionine (>800 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. After 1 h, cold methionine was added to the culture at a final concentration of 0.75 μg/ml. At 24 h postinfection, the whole culture was rapidly frozen and thawed twice, and cell debris was removed by centrifugation. HRV in the supernatant was purified as described above and stored at −80°C until use. Some samples of purified virions were further treated with 2 μg of trypsin per ml at 37°C for 10 min, added to 2 mM phenylmethylsulfonyl fluoride, and stored at −80°C until use.

Antibodies. Guinea pig hyperimmune antisera to three HRV strains (KUN, Wa, MO) were prepared as described previously (38). The neutralization titer of each serum was about 5×10^4 U/ml against the immunized HRV strain and less than 200 U/ml against unrelated HRV strains. The isolation and characterization of murine monoclonal antibodies against VP3 (K-376, K-1532) and against VP6 (K-381) of HRV-KUN were described previously (16). AH6 and S2-2G10 are two murine monoclonal antibodies to human rotavirus VP7. AH6 (immunoglobulin G3) was shown to react with human rotavirus of any serotype in an enzyme-linked immunosorbent assay, precipitate VP7, but not neutralize viral infection (N. Ikegami and K. Akatani, *in Proceedings of the First International Conference on the Impact of Viral Diseases on the Development of Asian Countries*, in press). AH6 was kindly provided by N. Ikegami, Osaka National Hospital, Osaka, Japan. S2-2G10 (immunoglobulin G2a) is a relatively weak neutralization antibody against HRV serotype 2. S2-2G10 could not precipitate any viral polypeptide but was shown to neutralize only an HRV reassortant having serotype 2 VP7 (41, 42). S2-2G10 was kindly provided by S. Urasawa, Sapporo Medical College, Sapporo, Japan. Immunoglobulin G was purified from ascites fluids with Affi-Gel Protein A (Bio-Rad Laboratories, Richmond, Calif.). Virus neutralization titers of purified anti-VP3 (K-376 and K-1532) and anti-VP7 (S2-2G10), determined as described previously (16), were about 10^5 and 10^4 U/mg, respectively. Therefore, anti-VP7 was used at a 10-fold-higher concentration than anti-VP3s throughout the following experiments to ensure the same level of neutralization activity unless otherwise stated. The VP6 binding activity of K-381 was determined by an enzyme-linked immunosorbent assay (16).

Cell-binding assay of viral polypeptides. MA104 cells (2×10^6 cells in each 25-cm^2 culture flask) were mock infected or infected with trypsin-treated (infectious) HRV-KUN at an MOI of 1 and cultured for 4.5 h. After cells were washed with methionine-free MEM, they were cultured in methionine-free MEM containing 25 μCi of L-[^{35}S]methionine per ml. At 8 h postinfection, the cells were washed with MEM, rapidly frozen and thawed twice, and lysed at 0°C for 5 min in 1 ml of a buffer (10 mM NaCl, 10 mM Tris hydrochloride [pH 7.4], 1.5 mM $MgCl_2$) containing 1 mM phenylmethylsulfonyl fluoride unless otherwise required. An equal volume of 2× MEM was then added to the lysate, and nuclei were removed by low-speed centrifugation. Cell debris was further removed by centrifugation at 150,000 × *g* for 2.5 h, and the supernatant was stored at −80°C until use.

The binding activity of free-form viral polypeptides to intact cells was examined essentially as described by Lee et al. (23). In brief, 200 μl of the [^{35}S]methionine-labeled cell lysate prepared from mock-infected or HRV-infected MA104 cells was added to cells in 24-well plates (3×10^5 cells per well), and the incubation was carried out at 4°C for 1.5 h with intermittent mixing. The cells were then washed three times with ice-cold Earle balanced salt solution (pH 7.4) and lysed with 50 μl of 1% Triton X-100 in PBS on ice for 10 min. The cell lysates were mixed with 25 μl of 3× Laemmli sample buffer, boiled for 1 min, and subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (22). After the marker proteins (Bio-Rad) were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.), the gels were soaked in Enlightning (New England Nuclear), dried, and then allowed to expose Kodak X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) at −80°C for 24 to 48 h.

The effects of guinea pig anti-HRV sera on the cell binding of viral polypeptides were examined by incubating 200 μl of the HRV-infected and [^{35}S]methionine-labeled cell lysate

with 200 μl of each serum (diluted 1:100 with PBS) at 37°C for 1 h prior to the binding assay. The competition of intact HRV-KUN virions with the free viral polypeptides for the cell-binding sites was determined by incubating 200 μl of the HRV-infected, [^{35}S]methionine-labeled cell lysate at 37°C for 1 h with 200 μl of PBS containing various amounts of purified HRV-KUN virions. The effects of monoclonal antibodies to VP3 (K-1532) or to VP7 (S2-2G10) on the cell binding of viral polypeptides were determined by incubation of 200 μl of the HRV-infected, [^{35}S]methionine-labeled cell lysate with monoclonal antibodies (K-1532, 10 μg/ml; S2-2G10, 100 μg/ml) at 37°C for 1 h prior to the binding assay. Various amounts of purified, trypsin-treated (infectious) HRV-KUN in 250 μl of PBS pretreated with anti-VP3 (K-1532, 10 μg/ml) or anti-VP7 (S2-2G10, 100 μg/ml) at 37°C for 1 h were also used for the competition assay. In some experiments, 1 ml of the HRV-infected, [^{35}S]methionine-labeled cell lysate was added to 250 μl of 50% Affi-Gel protein A (Bio-Rad) in PBS and incubated at 4°C for 1 h with constant rotation. After centrifugation, 200 μl of the supernatant was mixed with 10 μg of anti-VP3 (K-1532), anti-VP7 (AH6), or anti-VP6 (K-381) monoclonal antibody. After incubation at 37°C for 1 h, 50 μl of 50% Affi-Gel protein A was added, and incubation was carried out at 4°C for 1 h with constant rotation. After centrifugation, the supernatants were used for the cell-binding assay as described above. In some experiments, after the binding assay, the cells were lysed with 1 ml of 1% Triton X-100 in PBS, and mixed with 5 ml of Atomlight (New England Nuclear), and the radioactivity was measured in a scintillation counter.

Effects of anti-VP7 and anti-VP3 on HRV-KUN adsorption. Purified, trypsin-treated, [^{35}S]methionine-labeled HRV-KUN virions (4×10^5 FCFU in 200 μl of MEM, 0.13 pCi/FCFU) were mixed with 200 μl of PBS or PBS containing various amounts of anti-VP7 (S2-2G10), anti-VP3 (K-1532), or anti-VP6 (K-381). After incubation at 37°C for 1 h, the virions were adsorbed to MA104 cells (10^6) at 4°C for 2 h. After cells were washed with Earle balanced salt solution twice, the radioactivities bound to the cells were measured in a scintillation counter (see above).

Neutralization of preadsorbed HRV-KUN with anti-VP3 and anti-VP7 monoclonal antibodies. Trypsin-treated HRV-KUN was added to MA104 cells in eight-well Lab-Tek chamber slides (Miles Laboratories, Inc., Naperville, Ill.) (10^5 cells per well) at an MOI of 0.05, and the incubation was carried out at 4°C for 1.5 h. After cells were washed, they were supplied with 100 μl of fresh MEM, and the temperature was raised to 37°C. At various time points, 2 μg of anti-VP3 antibodies (K-376, K-1532) or anti-VP6 (K-381), or 20 μg of anti-VP7 (S2-2G10) in 100 μl of MEM was added to the cultures. After 20 h at 37°C, cells that were producing viral antigens were counted by the immunofluorescence staining method (see above).

Trypsin digestion of cell-bound HRV-KUN. Trypsin-treated (infectious) HRV-KUN was added to MA104 cells in 25-cm^2 culture flasks at an MOI of 0.1, and incubation was carried out at 4°C for 1.5 h. After cells were washed twice with ice-cold MEM, they were placed in fresh MEM and incubated at 37°C. At various time points, the cells were treated with 0.05% trypsin–0.05% EDTA at 37°C for 10 min. This treatment digested surface-bound virions and also released MA104 cells from the plastic surface. The cells were washed twice in MEM supplemented with 10% fetal bovine serum, and viable cells were counted by trypan blue dye exclusion. The cells were then cultured in eight-well Lab-Tek chamber slides (1.5×10^5 cell per well) at 37°C for 20 h, and cells that were producing viral antigens were counted by the immunofluorescence staining method (see above).

Effects of anti-VP3 and anti-VP7 on HRV-KUN internalization. [^{35}S]methionine-labeled, trypsin-treated HRV-KUN (0.13 pCi/FCFU) was added to MA104 cells (1.6×10^6 cells per dish) at an MOI of 0.5, and the incubation was carried out at 4°C for 2 h. After cultures were washed twice with ice-cold MEM, some of them were further incubated at 37°C for 1 h in fresh medium with or without 10 μg of anti-VP3 (K-1532) or anti-VP6 (K-381) per ml or 100 μg of anti-VP7 (S2-2G10) per ml. The cells were harvested either by a cell scraper (for analysis of total cell-associated viral components) or by treatment with 0.05% trypsin–0.05% EDTA (for analysis of internalized viral components) and were washed twice in ice-cold MEM by a low-speed centrifugation. Cell pellets were lysed with 50 μl of 1% Triton X-100 in PBS on ice for 10 min. The cell lysates were mixed with 3× Laemmli sample buffer and subjected to SDS-PAGE (22). Viral polypeptides were visualized by fluorography. Samples obtained from a parallel set of experiments were used to determine the radioactivities associated with the cells in a scintillation counter (see above).

RESULTS

Identification of VP7 as the cell attachment protein of HRV-KUN. The cell attachment proteins of reovirus (σ1) and of bovine rotavirus (VP7) were identified from their capability to bind to target cells even in free protein forms in the lysates of virus-infected cells (23, 33). We employed the same method to identify the cell attachment proteins of HRV-KUN. Mock-infected or HRV-KUN-infected MA104 cells were labeled with [^{35}S]methionine, and the cell lysates were prepared 8 h postinfection. The lysates were then incubated with MA104 cells at 4°C for 1.5 h. The cells were washed, and [^{35}S]methionine-labeled proteins bound to the cells were analyzed with SDS-PAGE. A band of 35 kDa was detected when MA104 cells were incubated with the lysate of HRV-KUN-infected cells (Fig. 1, lane B); no such band was seen with the mock-infected cell lysate (lane H). The binding of the 35-kDa protein to MA104 cells was completely inhibited by an antiserum to HRV-KUN (serotype 2) (lane C) but not by a preimmune serum (lane F), an antiserum to Wa (serotype 1) (lane D), or an antiserum to MO (serotype 3) (lane E).

Infectious (i.e., mildly treated with trypsin) HRV-KUN blocked the binding of 35-kDa protein to MA104 cells in a dose-dependent fashion (Fig. 2A). Interestingly, even untreated, noninfectious HRV-KUN could similarly compete with the 35-kDa protein for cell-binding sites. This strongly suggested that not only trypsin-treated, infectious HRV-KUN but also untreated, noninfectious virions had the full capacity to bind to the target cells (see below for further evidence). The quantitative data obtained from a parallel set of experiments are shown in Fig. 2B.

These results clearly demonstrated that the 35-kDa protein was the HRV-KUN product and, at least in the present experimental conditions, the only viral protein capable of binding to the surface of MA104 cells. It also had the KUN strain serotype specificity and competed with intact viral particles for the same cell-binding sites, suggesting that it was the cell attachment protein of HRV-KUN. Preliminary experiments also confirmed that the size of the cell-binding protein in the lysate was shifted from 35 to 29 kDa by the simultaneous incubation of HRV-KUN-infected cells with 5 μg of tunicamycin per ml (data not shown). The molecular

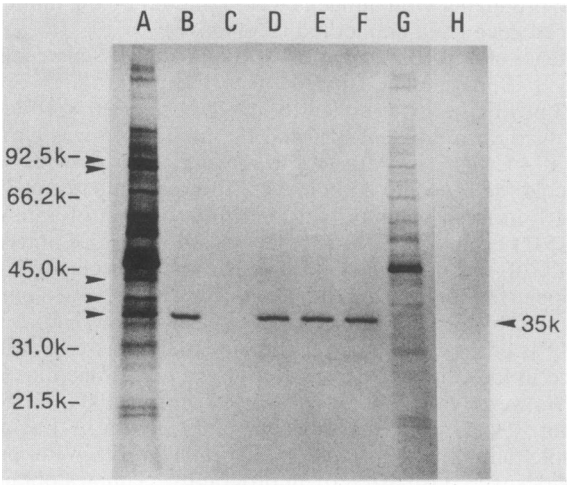

FIG. 1. Binding of free viral polypeptides to MA104 cells. MA104 cells (3×10^5 cells per well) were incubated at 4°C for 1.5 h with 200 μl of an [^{35}S]methionine-labeled lysate prepared from mock-infected MA104 cells (5.8×10^5 cpm/200 μl) or HRV-KUN-infected MA104 cells (6.7×10^5 cpm/200 μl). [^{35}S]methionine-labeled proteins bound to MA104 cells were visualized with fluorography after SDS-PAGE. The binding experiment was carried out with the HRV-KUN-infected cell lysate (lane B) or with the mock-infected cell lysate (lane H). The binding experiment with the HRV-KUN-infected cell lysate was also carried out in the presence of anti-KUN serum (lane C), anti-Wa serum (lane D), anti-MO serum (lane E), or preimmune serum (lane F). Total [^{35}S]methionine-labeled polypeptides in the HRV-infected and the mock-infected cell lysates are shown in lane A and lane G, respectively. Viral proteins, indicated by arrowheads at the side of lane A, are VP2, VP3, VP6, and VP7 (two bands) (from top to bottom). For details, see Materials and Methods. k, Kilodaltons.

size of 35 kDa and its shift to 29 kDa by tunicamycin (34, 39) and the presence of KUN serotype determinants strongly suggested that the 35-kDa protein was the HRV-KUN major outer capsid protein VP7.

To further confirm that the 35-kDa cell-binding protein in the HRV-KUN-infected cell lysate was indeed VP7, we next examined the effects of two monoclonal antibodies that were supposed to recognize HRV-KUN VP7, AH6 (Ikegami and Akatani, in press), and S2-2G10 (41, 42) on the 35-kDa protein binding to MA104 cells. AH6 reacts with rotavirus of many different serotypes and specifically precipitates VP7, but it cannot neutralize viral infection (Ikegami and Akatani, in press; our own results). Therefore, even though AH6 is directed to an antigenic determinant common to all VP7 from different serotypes, it is likely that its binding to VP7 does not block the function of VP7. Another monoclonal anti-VP7, S2-2G10, is specific for serotype 2 HRV and is also capable of neutralizing virus infection. By using an HRV reassortant between serotype 2 and serotype 1, it was concluded that S2-2G10 was directed to the serotype 2 VP7 (41, 42). However, the direct binding of S2-2G10 to VP7 cannot be demonstrated (41, 42; our own results). Therefore, S2-2G10 seems to recognize VP7 only on intact viral particles and not in the free protein form. Because of these limitations, it was not surprising that the direct addition of either AH6 or S2-2G10 in the cell-binding assay did not affect the binding of the 35-kDa protein to MA104 cells at all (data not shown) (Fig. 3B, lane F). Therefore, we next tested whether AH6 (the antibody capable of binding to free VP7) could absorb the 35-kDa protein in the HRV-KUN-infected cell lysate prior to the binding assay. AH6 indeed absorbed the 35-kDa protein in the HRV-KUN-infected cell lysate (Fig. 3A, lane B), whereas anti-VP3 (K-1532) or anti-VP6 (K-381) had no such effect (lanes C and D). We also tested whether precoating of HRV-KUN virions with S2-2G10 (the antibody only capable of binding to virion VP7) could block the capability of the virions to compete with the 35-kDa protein for the same binding sites on MA104 cells (Fig. 2). HRV-KUN virions precoated with S2-2G10 were indeed incapable of blocking 35-kDa protein binding to MA104 cells (Fig. 3B, lanes G to I), whereas HRV-KUN virions precoated with anti-VP3 (K-1532) inhibited 35-kDa protein binding to MA104 cells in a dose-dependent manner (lanes C to E). These results, obtained by using two different types of monoclonal anti-VP7, provided further evidence that the 35-kDa cell-binding protein was HRV-KUN VP7.

Effects of anti-VP3 and anti-VP7 on HRV-KUN adsorption to MA104 cells. It has been demonstrated that an antiserum

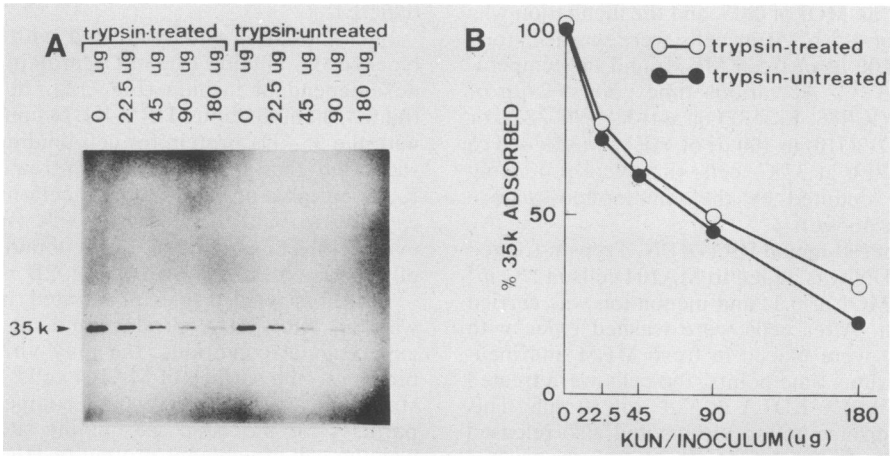

FIG. 2. Inhibition of the binding of the 35-kDa (k) protein to MA104 cells by HRV-KUN virions treated with or without trypsin. Samples of an [^{35}S]methionine-labeled lysate (200 μl, 6.7×10^5 cpm) prepared from HRV-KUN-infected MA104 cells were each mixed with indicated amounts of trypsin-treated or untreated HRV-KUN and then subjected to incubation with MA104 cells (3×10^5 cells per well) at 4°C for 1.5 h. [^{35}S]methionine-labeled proteins bound to MA104 cells were either visualized by fluorography after SDS-PAGE (A) or measured with a scintillation counter (B). The measurement was done in duplicate, and 100% was 3,492 cpm. For details, see Materials and Methods.

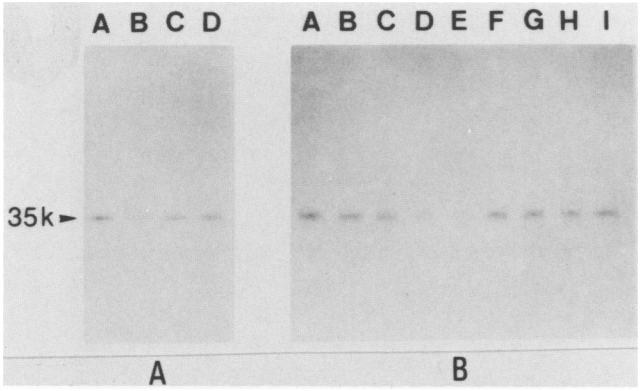

FIG. 3. (A) Absorption of HRV-KUN 35-kDa (k) cell-binding protein by monoclonal anti-VP7 (AH6) and (B) effects of monoclonal anti-VP3 (K-1532) and anti-VP7 (S2-2G10) on the competition of HRV-KUN virions with the 35-kDa cell-binding protein. (A) [^{35}S]methionine-labeled lysate (1 ml; 4.2×10^6 cpm/ml) prepared from HRV-KUN-infected cells was mixed with 250 μl of 50% Affi-Gel protein A in PBS and incubated at 4°C for 1 h with constant rotation. After centrifugation, 200 μl of the precleaned lysate was mixed with 10 μg of anti-VP7 (AH6), anti-VP3 (K-1532), or anti-VP6 (K-381). After incubation at 37°C for 1 h, 50 μl of 50% Affi-Gel protein A in PBS was added, and incubation was carried out at 4°C for 1 h with constant rotation. After centrifugation, the supernatants were used for the cell-binding assay as described in Materials and Methods. Lanes: A, no antibody; B, anti-VP7 (AH6); C, anti-VP3 (K-1532); D, anti-VP6 (K-381). (B) [^{35}S]methionine-labeled lysate (200 μl; 8.4×10^5 cpm) prepared from HRV-KUN-infected cells was mixed with 250 μl of anti-VP3 (K-1532) (10 μg/ml in MEM) or anti-VP7 (S2-2G10) (100 μg/ml in MEM) or with various amounts of trypsin-treated HRV-KUN virions in 250 μl of MEM preincubated at 37°C for 1 h with 10 μg of anti-VP3 (K-1532) per ml or 100 μg of anti-VP7 (S2-2G10) per ml, and the cell-binding assay was carried out as described in Materials and Methods. Cell-bound viral proteins were visualized with fluorography after SDS-PAGE. The concentration of anti-VP7 employed was 10 times higher than that of anti-VP3 because the virus neutralization activity of anti-VP7 was about 1/10 of that of anti-VP3 (Table 1). Lanes: A, no antibody nor virus; B, anti-VP3 only; C, 45 μg of virus pretreated with anti-VP3; D, 90 μg of virus pretreated with anti-VP3; E, 180 μg of virus pretreated with anti-VP3; F, anti-VP7 only; G, 45 μg of virus pretreated with anti-VP7; H, 90 μg of virus pretreated with anti-VP7; I, 180 μg of virus pretreated with anti-VP7. For details, see Materials and Methods.

monospecific to VP7 or monoclonal antibodies to VP7 with virus-neutralization activity inhibit virus adsorption (27, 33). Recently, it has been reported that some monoclonal antibodies to VP3 also have virus-neutralization activity (11, 16, 33, 35, 40–42). The inhibitory mechanism of rotavirus infection by anti-VP3 is not known. Therefore, we next compared the effects of anti-VP3 and anti-VP7 on cell adsorption of [^{35}S]methionine-labeled HRV-KUN virions. Both anti-VP3 (K-1532) and anti-VP7 (S2-2G10) could neutralize HRV-KUN infection (Table 1). Control anti-VP6 (K-381) had no inhibitory effect on virus infection. Anti-VP7 also inhibited HRV-KUN adsorption in a dose-dependent manner. This result is consistent with our conclusion that the cell-attachment protein of HRV-KUN is VP7 (see above). On the other hand, anti-VP3, like anti-VP6, did not affect virus adsorption at all. Therefore, anti-VP3 seemed to inhibit HRV-KUN infection after virus adsorption.

Anti-VP3 monoclonal antibodies inhibit HRV-KUN infection after cell adsorption. The above results suggested that anti-VP3 antibodies might block an infectious process of HRV-KUN after its cell adsorption. Therefore, we examined their effects on preadsorbed HRV-KUN virions. Trypsin-treated HRV-KUN virions were incubated with MA104 cells at 4°C for 1.5 h. After cells were washed, the temperature was raised to 37°C. Simultaneously or at various time points after the temperature rise, monoclonal antibodies to VP3, VP7, or VP6 were added to the cells. Anti-VP3 antibodies K-376 and K-1532 were clearly capable of blocking infection of preadsorbed virions (Fig. 4A). Complete inhibition was obtained when anti-VP3 antibodies were added simultaneously or soon after the temperature rise. The inhibitory effects were decreased when the addition of anti-VP3 antibodies was further delayed, and no inhibition was seen when anti-VP3 antibodies were added 2 h after 37°C. Unexpectedly, anti-VP7 (S2-2G10) also had some inhibitory effect even on preadsorbed HRV-KUN. However, the inhibitory effect was much less than that of anti-VP3 antibodies, and no significant inhibition was seen when anti-VP7 was added 1 h after 37°C. Anti-VP6 (K-381) had no inhibitory effect on HRV-KUN infection.

TABLE 1. Effects of anti-VP7 and anti-VP3 on HRV-KUN adsorption

Antibody[a] and concn (μg/ml)	% Adsorption[b]	% Infectivity[c]
Anti-VP7		
10	26	0
1	45	41
0.1	96	93
Anti-VP3		
1	85	0
0.1	89	22
0.01	96	110
Anti-VP6		
1	88	97
0.1	95	82
0.01	96	100

[a] [^{35}S]methionine-labeled, trypsin-treated HRV-KUN (200 μl; 4×10^5 FCFU, 0.13 pCi/FCFU) was first incubated with 200 μl of PBS or PBS containing anti-VP7 (S2-2G10), anti-VP3 (K-1532), or anti-VP6 (K-381) at the indicated concentrations at 37°C for 1 h and then incubated with 10^6 MA104 cells at 4°C for 2 h.

[b] Virus adsorption was measured by determining the radioactivity associated with MA104 cells after washing. The assay was done in duplicate, and the mean 100% was 13,358 cpm. The nonspecific level of binding, i.e., the radioactivity that could not be removed by trypsin digestion, was 20%.

[c] Virus infectivity was determined by culturing the virus-adsorbed cells in Lab-Tek chambers (10^5 cells per well) and staining the cells producing viral antigens after 20 h. The assay was done in duplicate, and the mean 100% infectivity was 10^6 FCFU/ml.

We also determined in parallel the kinetics of HRV-KUN internalization after the temperature was raised to 37°C. At various time points after the temperature rise, virions remaining on the cell surface were digested with trypsin to determine the kinetics of virus internalization (Fig. 4B). By comparing Fig. 4A and B, we can clearly see that the kinetics of development of virus resistance to anti-VP3 antibodies and that of virus internalization were essentially identical after the temperature rise to 37°C. On the other hand, the kinetics of development of virus resistance to anti-VP7 was much faster than that of virus internalization. These results indicate that anti-VP3 antibodies can inhibit HRV-KUN infection even after virus adsorption as long as internalization has not yet been accomplished.

Selective internalization of inner capsid components from trypsin-treated HRV-KUN. To characterize further the inhibitory mechanism of anti-VP3s on HRV-KUN infection, we

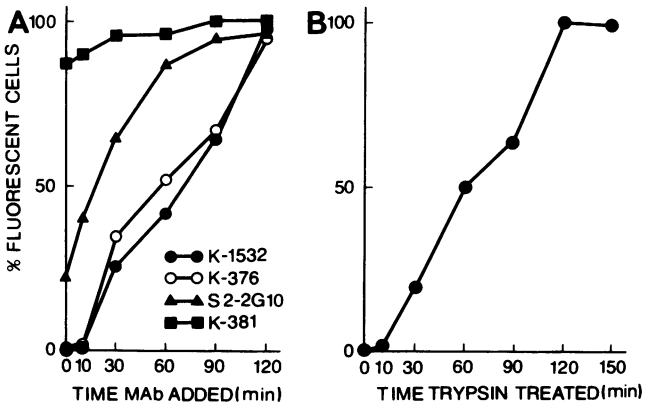

FIG. 4. (A) Effects of anti-VP3 and anti-VP7 on trypsin-treated HRV-KUN preadsorbed to MA104 cells and (B) kinetics of internalization of trypsin-treated HRV-KUN preadsorbed to MA104 cells. (A) Trypsin-treated HRV-KUN was added to MA104 cells in eight-well Lab-Tek chamber slides at an MOI of 0.05, and incubation was carried out at 4°C for 1.5 h. After slides were washed, the temperature was raised to 37°C, and at indicated time points purified anti-VP3 (K-1532 and K-376), anti-VP7 (S2-2G10), or anti-VP6 (K-381) was added at a final concentration of 10 μg/ml (anti-VP3 and anti-VP6) or 100 μg/ml (anti-VP7). The concentration of anti-VP7 employed was 10 times higher than that of anti-VP3 because the virus-neutralization activity of anti-VP7 was about 1/10 of that of anti-VP3 (Table 1). After 20 h, cells producing viral antigens were counted by indirect immunofluorescence staining. The assay was done in duplicate, and 100% was 225 cells per well. For details, see Materials and Methods. (B) Trypsin-treated HRV-KUN was added to MA104 cells at an MOI of 0.05 and the incubation was carried out at 4°C for 1.5 h. After cells were washed, the temperature was raised to 37°C, and at the indicated time points the cells were treated with 0.05% trypsin–0.05% EDTA. The suspended cells were seeded in eight-well Lab-Tek chamber slides at 1.5×10^5 cells per well and cultured at 37°C for 20 h. Cells producing viral antigens were then counted by indirect immunofluorescence staining. The assay was done in duplicate, and 100% was 217 cells per well. For details, see Materials and Methods.

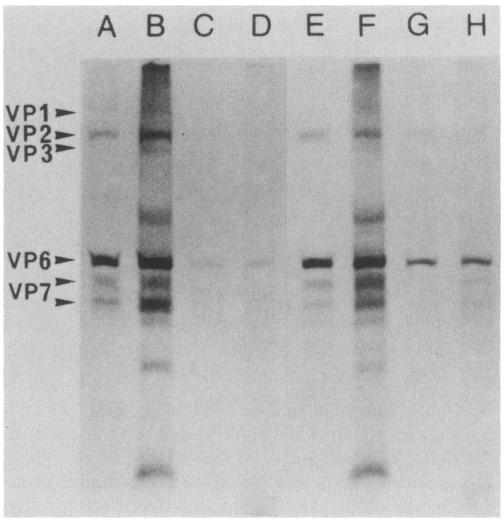

FIG. 5. Binding and internalization of trypsin-treated and untreated HRV-KUN. Purified [^{35}S]methionine-labeled HRV-KUN virions (0.1 pCi/FCFU) treated with trypsin or left untreated were added to MA104 cells (6×10^4 cpm per 1.6×10^6 cells), and incubation was carried out at 4°C for 2 h. After cultures were washed, some of them were further incubated at 37°C for 1 h. The cells were harvested either mechanically with a cell scraper for the analysis of total (surface-bound and internalized) viral polypeptides or by digestion with 0.05% trypsin–0.05% EDTA for the analysis of internalized viral polypeptides. Cell pellets were then lysed and subjected to SDS-PAGE. Viral polypeptides were visualized with fluorography. Lanes: A, viral polypeptides from trypsin-treated HRV-KUN associated with MA104 cells at 4°C; B, viral polypeptides from untreated HRV-KUN associated with MA104 cells at 4°C; C, viral polypeptides from trypsin-treated HRV-KUN internalized into MA104 cells at 4°C; D, viral polypeptides from untreated HRV-KUN internalized into MA104 cells at 4°C; E, viral polypeptides from trypsin-treated HRV-KUN associated with MA104 cells at 37°C; F, viral polypeptides from untreated HRV-KUN associated with MA104 cells at 37°C; G, viral polypeptides from trypsin-treated HRV-KUN internalized into MA104 cells at 37°C; H, viral polypeptides from untreated HRV-KUN internalized into MA104 cells at 37°C. For details, see Materials and Methods.

proceeded to examine their effects on HRV-KUN internalization. First, we studied the cell adsorption and subsequent internalization of trypsin-treated (infectious) and untreated (noninfectious) HRV-KUN. [^{35}S]methionine-labeled HRV-KUN virions were purified and treated with trypsin or left untreated. Virions were then adsorbed to MA104 cells at 4°C for 2 h. After cell cultures were washed, some of them were further incubated at 37°C for 1 h. Viral polypeptides associated with the cells were analyzed by SDS-PAGE, and their internalization was demonstrated by resistance to trypsin digestion (representative results are shown in Fig. 5). After incubation at 4°C for 2 h, not only virions treated with trypsin (lane A) but also the untreated virions (lane B) were efficiently adsorbed to the cells as demonstrated by cell association of the viral components. These were VP1 (faint), VP2, VP3 (faint and only in lane B), VP6, and VP7 (two bands). It should be noted that VP3 was missing in trypsin-treated virions (lane A). Several other bands seen in lane B were apparently contaminants that were easily destroyed during the mild trypsin treatment of virions (compare lanes A and B). These cell-associated viral polypeptides were quite sensitive to trypsin digestion (lanes C and D), indicating that adsorbed virions remained on the cell surface at 4°C (Fig. 4B). After incubation at 37°C for 1 h, similar sets of viral polypeptides were again demonstrated to be associated with the cells (Fig. 5, lanes E and F). Some of these polypeptides were found to be resistant to trypsin digestion (lanes G and H). It was notable that not only trypsin-treated, infectious virions but also untreated, noninfectious virions were internalized with similar efficiencies. The mode of internalization, however, seemed to be quite different between these two preparations of HRV-KUN. With trypsin-treated virions, only the components of the inner capsid (see VP2 and VP6) were apparently internalized (Fig. 5, lane G), whereas with untreated virions not only the inner capsid components (VP2 and VP6) but also those of the outer capsid (see VP7) were clearly internalized (lane H). These results first of all confirmed that not only trypsin-treated, infectious HRV-KUN virions but also untreated, noninfectious virions were able to bind to MA104 cells with similar efficiencies. Second, trypsin-treated virions showed a selective internalization of the inner capsid components, whereas in untreated virions all viral components were taken up into the cells.

Inhibition of HRV-KUN internalization by anti-VP3. On the basis of these observations, we next examined the effect of anti-VP3 on HRV-KUN internalization. [^{35}S]methionine-labeled, trypsin-treated HRV-KUN virions were adsorbed to MA104 cells at 4°C for 2 h. After cultures were washed, some of them were incubated at 37°C for 1 h in fresh medium

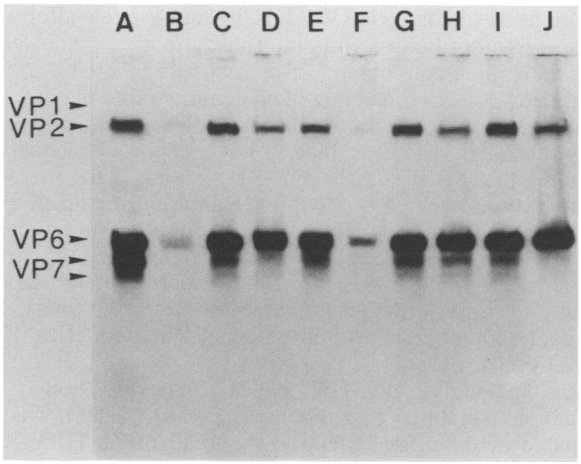

FIG. 6. Effects of anti-VP3 and anti-VP7 on HRV-KUN internalization. [^{35}S]methionine-labeled HRV-KUN virions purified and treated with trypsin (0.13 pCi/FCFU) were incubated with MA104 cells (7.8×10^4 cpm per 1.6×10^6 cells) at 4°C for 2 h. After washing, some cultures were further incubated at 37°C with or without anti-VP3 (K-1532, 10 μg/ml), anti-VP7 (S2-2G10, 100 μg/ml) or anti-VP6 (K-381, 10 μg/ml). The concentration of anti-VP7 employed was 10 times higher than that of anti-VP3 because the virus-neutralization activity of anti-VP7 was about 1/10 of that of anti-VP3 (Table 1). The cells were harvested either mechanically with a cell-scraper for the analysis of total viral polypeptides associated with MA104 cells or by digestion with 0.05% trypsin–0.05% EDTA for the analysis of viral polypeptides internalized into MA104 cells. The cell pellets were lysed and subjected to SDS-PAGE. Viral polypeptides were visualized with fluorography. Lanes: A, viral polypeptides associated with MA104 cells at 4°C; B, viral polypeptides internalized into MA104 cells at 4°C; C, viral polypeptides associated with MA104 cells at 37°C; D, viral polypeptides internalized into MA104 cells at 37°C; E, viral polypeptides associated with MA104 cells at 37°C in the presence of anti-VP3; F, viral polypeptides internalized into MA104 cells at 37°C in the presence of anti-VP3; G, viral polypeptides associated with MA104 cells at 37°C in the presence of anti-VP7; H, viral polypeptides internalized into MA104 cells at 37°C in the presence of anti-VP7; I, viral polypeptides associated with MA104 cells at 37°C in the presence of anti-VP6; J, viral polypeptides internalized into MA104 cells at 37°C in the presence of anti-VP6. For details, see Materials and Methods.

with or without either anti-VP3 (K-1532), anti-VP7 (S2-2G10), or anti-VP6 (K-381). At least four viral components were clearly demonstrated after adsorption at 4°C: VP1 (faint), VP2, VP6, and VP7 (two bands) (Fig. 6, lane A). These viral polypeptides were confirmed to be sensitive to trypsin digestion (lane B). Viral polypeptides associated with the cells after incubation at 37°C for 1 h were next shown in lane C. Among these, the inner capsid components were resistant to trypsin (only VP2 and VP6 were clearly seen), whereas the outer capsid component VP7 was digested with trypsin (lane D). The presence of anti-VP3 (K-1532) during the incubation at 37°C did not affect the association of these viral components with the cells (lane E). The viral polypeptides, however, remained mostly sensitive to trypsin digestion (compare lanes F and D), indicating that virus internalization was inhibited by anti-VP3. Anti-VP7 (S2-2G10) and anti-VP6 (K-381) also did not affect virus association with the cells (lanes G and I) or inhibit virus internalization (lanes H and J). However, there was a notable difference in the mode of virus internalization in the presence of anti-VP7 or anti-VP6. In the presence of anti-VP7, not only inner capsid components but also some VP7 proteins were clearly internalized (compare lanes D and H), whereas in the presence of anti-VP6 only inner capsid components were selectively internalized (lane J). Therefore, in the presence of anti-VP7 (S2-2G10), a large fraction of trypsin-treated HRV-KUN virions seemed to be internalized through the nonselective route (endocytosis) as untreated, noninfectious HRV-KUN (Fig. 5). The quantitative data obtained from a parallel set of assays are shown in Table 2. The background trypsin-resistant radioactivity was 20% (sample 1). The incubation at 37°C for 1 h increased the trypsin-resistant radioactivity to 76% (sample 2). Anti-VP3 clearly inhibited this increase (sample 3). On the other hand, anti-VP7 and anti-VP6 did not affect the increase in the trypsin-resistant radioactivity during the incubation at 37°C for 1 h (samples 4 and 5).

TABLE 2. Effects of anti-VP3 and anti-VP7 on HRV-KUN internalization

Sample no.	Incubation[a] at 37°C	Antibody[b]	Trypsin digestion[c]	cpm[d]	% Resistant[e]
1	−		−	15,830	
	−		+	3,180	20
2	+		−	16,580	
	+		+	12,650	76
3	+	Anti-VP3	−	15,598	
	+	Anti-VP3	+	5,893	38
4	+	Anti-VP7	−	16,723	
	+	Anti-VP7	+	12,660	76
5	+	Anti-VP6	−	15,067	
	+	Anti-VP6	+	11,896	79

[a] After adsorption of [^{35}S]methionine-labeled, trypsin-treated HRV-KUN (8×10^5 FCFU, 0.13 pCi/FCFU) to MA104 cells (1.6×10^6) at 4°C for 2 h, the washed cultures were incubated at 37°C for 1 h.

[b] Anti-VP3 (K-1532), anti-VP7 (S2-2G10), or anti-VP6 (K-381) was added to the cultures at 10 μg/ml (anti-VP3 and anti-VP6) or 100 μg/ml (anti-VP7) at the start of incubation at 37°C for 1 h. The concentration of anti-VP7 employed was 10 times higher than that of anti-VP3 because the virus-neutralization activity of anti-VP7 was about 1/10 of that of anti-VP3 (Table 1).

[c] MA104 cells were treated with 0.05% trypsin–0.05% EDTA.

[d] The mean of duplicate determinations is shown.

[e] Percentage of the trypsin-resistant radioactivity in the total cell-associated radioactivity.

DISCUSSION

In the present study, we first determined the cell attachment protein of HRV-KUN by using the method of Lee et al. (23). The same procedure was also employed by Sabara et al. to demonstrate that VP7 is the cell attachment protein of bovine rotavirus (33). The lysate of HRV-KUN-infected cells but not that of mock-infected cells contained 35-kDa proteins capable of binding to the surface of MA104 cells (Fig. 1). The binding of the 35-kDa protein was inhibited by a serotype-specific anti-HRV serum (Fig. 1) and competed with not only trypsin-treated, infectious HRV-KUN but also with untreated, noninfectious virions with the same efficiency (Fig. 2). The latter findings strongly suggested that the cell-binding capacity of HRV-KUN was independent of trypsin treatment. Furthermore, one monoclonal anti-VP7, AH6 (Ikegami and Akatani, in press), could absorb the 35-kDa protein (Fig. 3A), and another monoclonal anti-VP7, S2-2G10 (41, 42), could block the HRV-KUN virions to compete with the 35-kDa protein for the same binding sites on MA104 cells (Fig. 3B). We also confirmed that the size of the cell-binding protein was shifted from 35 to 29 kDa by the treatment with tunicamycin (data not shown) (34, 39). These results strongly suggested that the cell attachment protein of HRV-KUN was also VP7, like that of bovine rotavirus (33).

We could not demonstrate any cell-binding activity of other viral components, especially of VP3, even though we carried out the binding experiment at a wide range of pHs (4 to 9) or with HRV-infected cell lysates pretreated with trypsin at a wide range of concentrations (0.3 to 5 μg/ml) and for various lengths of time (0.5 to 5 min) (data not shown). We also confirmed the specific cleavage of VP3 in the cell lysate at optimal conditions (for example, 1 μg of trypsin per ml at 37°C for 5 min) from the immunoprecipitation of the cleavage product with the expected size of 24 kDa (16) (data not shown). In all of these experiments, 35-kDa VP7 was the only virus-specific protein that bound to the cells. Therefore, VP3 or its trypsin-cleaved fragments might not have cell-binding activity, at least in the free protein form. However, since VP3 is a relatively minor component of the outer capsid, the amount of VP3 or its trypsin-cleaved fragments associated with the cells might be too small to be clearly demonstrated in the present experimental conditions. Since HA activity of HRV-KUN is demonstrable with 1-day-old chicken erythrocytes (18), we also carried out the viral polypeptide binding assay with 1-day-old chicken erythrocytes as target cells. We were disappointed to find out that it was impossible to detect the binding of any viral polypeptide, including VP3, to chicken erythrocytes, since so many cellular proteins were also tightly bound to these cells (data not shown).

Our previous work demonstrated that HRV-KUN VP7 was composed of two molecular species of 35 and 37 kDa in the lysate of infected MA104 cells as well as in mature viral particles (34, 39) (Fig. 5 and 6). Both forms were reduced to 29 kDa in the presence of tunicamycin, suggesting that they were derived from a single 29-kDa precursor through differential glycosylation (34, 39). Such heterogeneities of VP7 have also been reported for simian and bovine rotaviruses (4, 19, 20). In the present study a clear-cut cell-binding activity was demonstrated only for the 35-kDa form of VP7 (Fig. 1 through 3). The reason why the 37-kDa form of VP7 does not bind to MA104 cells is not known at present, but our observation suggests that the two VP7 glycoproteins are functionally distinct. Even though a small difference in the amino-terminal sequence may be present as reported by Chan et al. (4), the major difference between 35- and 37-kDa VP7 proteins seems to be glycosylation (34, 39). Therefore, our observation suggests the importance of the structure of the carbohydrate moiety for the cell-binding activity of VP7.

We next examined the effects of neutralizing monoclonal anti-VP3 (K-1532) and anti-VP7 (S2-2G10) on HRV-KUN adsorption to MA104 cells. Anti-VP3 did not affect HRV-KUN adsorption, whereas anti-VP7 inhibited HRV-KUN adsorption (Table 1). The latter result further supports our conclusion that VP7 functions as the HRV-KUN cell attachment protein. Other workers also reported that an antiserum monospecific to VP7 or monoclonal antibodies to VP7 could inhibit adsorption of respective rotaviruses (27, 33).

We next showed that anti-VP3 antibodies were capable of inhibiting HRV-KUN infection even when applied after full adsorption of virions to target cells (Fig. 4A). HRV-KUN internalization is apparently dependent on temperature, since the majority of HRV-KUN virions adsorbed at 4°C remained sensitive to trypsin digestion. When the temperature was raised to 37°C, virions became progressively resistant to trypsin digestion with a half time of about 60 min (Fig. 4B). This, then, is the kinetics of virus internalization. The inhibitory effect of anti-VP3s on infection of preadsorbed HRV-KUN decreased with exactly the same kinetics (Fig. 4A). This clearly indicates that anti-VP3 antibodies are capable of blocking HRV-KUN infection even after virus adsorption but before virus internalization.

The studies employing [^{35}S]methionine-labeled and purified HRV-KUN virions first of all demonstrated that not only trypsin-treated, infectious HRV-KUN but also untreated, noninfectious virions seemed to have similar efficiencies for binding to the target cells (Fig. 5). Second, these two virus preparations seemed to have quite different modes of internalization. Infectious virions had only their inner capsid components internalized, whereas noninfectious virions were taken up into the cells with no such selective process (Fig. 5). Third, anti-VP3 inhibited this selective internalization of the inner capsid from trypsin-treated HRV-KUN virions (Fig. 6).

Unexpectedly, anti-VP7 (S2-2G10), even though less effective than anti-VP3 antibodies (K-1532, K-376), was also capable of inhibiting infection of preadsorbed HRV-KUN virions (Fig. 4A). This suggests that some preadsorbed virions were temporarily dissociated from the cells by the sudden temperature rise to 37°C, or that anti-VP7 removed some portion of preadsorbed visions from the cells. It is also possible that anti-VP7, even though less effective than anti-VP3 antibodies, was capable of inhibiting the internalization of preadsorbed HRV-KUN. On the other hand, the experiments with purified [^{35}S]methionine-labeled HRV-KUN virions showed that anti-VP7 (S2-2G10) could inhibit HRV-KUN adsorption (Table 1) but could not inhibit internalization of preadsorbed virions (Table 2). A clue to understand the apparent inconsistency of the effect of anti-VP7 (S2-2G10) on infection versus internalization of preadsorbed HRV-KUN was provided from the analysis of the viral components internalized in the presence of anti-VP7 (Fig. 6). Even though anti-VP7 (S2-2G10) did not inhibit the internalization of preadsorbed HRV-KUN, the main mode of internalization turned out to be nonselective in the presence of anti-VP7, as seen with untreated, noninfectious HRV-KUN (Fig. 5), instead of the selective internalization of the inner capsid components demonstrated with trypsin-treated, infectious HRV-KUN (Fig. 5). Therefore, even though anti-VP7 did not reduce the overall level of virus internalization, a large fraction of virions was internalized into the cells through the noninfectious route in the presence of anti-VP7. These results may suggest that not only VP3 but also VP7 is involved in the infectious mode of internalization of HRV-KUN. However, we would also like to point out a special problem of this particular anti-VP7 (S2-2G10). S2-2G10 was concluded to be specific for serotype 2 VP7 by using an HRV reassortant between serotype 2 and serotype 1 (41, 42). However, the direct binding of S2-2G10 to serotype 2 VP7 protein itself could not be demonstrated (41, 42; our own results). Therefore, S2-2G10 seems to recognize VP7 only on intact viral particles. The unique reactivity of this monoclonal antibody may mean that S2-2G10 recognizes an antigenic structure composed of VP7 from serotype 2 and another outer capsid component, VP3, from any serotype. In that case, S2-2G10 not only would block the function of VP7 but also would affect the function of VP3. Therefore, it may be too early to draw a conclusion concerning the function of VP7 in the virus internalization from the results obtained only with this particular monoclonal antibody. Nevertheless, the shift from selective to nonselective internalization for infectious HRV-KUN in the presence of anti-VP7 was surprising (Fig. 6). It also put forward another puzzle, i.e., the almost complete absence of endocytotic (nonselective) internalization of infectious virions in the face of inhibition of the selective internalization of the inner capsid compo-

nents by anti-VP3 (Fig. 6). At present we cannot provide a plausible explanation for these observations, but isolation of anti-VP7 monoclonal antibodies that can neutralize infection and also precipitate VP7 protein may help to clarify these puzzles.

The present findings were quite consistent with our previous electron microscopic observations that trypsin-treated, infectious HRV-KUN entered MA104 cells by a direct penetration through the plasma membrane, whereas untreated virions, even though noninfectious, also efficiently entered the cells by the endocytotic pathway (36, 37). Endocytosis-independent infection of HRV-KUN was further supported by its resistance to various lysosomotropic amines and cytochalasin B (8). On the other hand, Petri et al. and Quan et al. concluded from electron microscopic observations that the entry pathway of simian rotavirus SA11 was endocytosis (31, 32). Ludert et al., also from electron microscopic analyses, reported that porcine rotavirus OSU entered the target cells through endocytosis, even though they also found that OSU was quite resistant to some lysosomotropic agents (25). None of these authors made a close comparison of the mode of internalization of trypsin-treated versus untreated virions. However, the difference of our results obtained with HRV-KUN and those obtained with some animal rotaviruses may be due to the virus strain differences. Two modes of entry have also been described for reovirus (endocytosis and direct penetration) (3) and baculovirus (endocytosis and membrane fusion) (43).

Many virus species are known to take advantage of the cellular endocytotic activity and intralysosomal low pH for internalization into and penetration of target cells (12, 24, 26, 45). Untreated HRV-KUN virions, however, are not able to produce viral progeny, even though they are apparently taken up by endocytosis with a high efficiency. HRV virions thus internalized may not be able to penetrate the cell and therefore may be destroyed in the lysosome. The unique mode of internalization controlled by the trypsin cleavage of VP3, therefore, seems to be essential for HRV infection and to be synchronized with the virus uncoating process. Clark et al. also showed that the uncoating of bovine rotavirus in MDBK cells (i.e., the conversion from the double-shelled particle to the single-shelled particle) was greatly enhanced by trypsin cleavage of VP3 (6). The unique features of HRV internalization are in fact reasonable for its natural circumstances of infection. The integrity of HRV virions must be retained in the low-pH environment of gastric juice, i.e., the reactions such as uncoating or fusion should not be induced by low pH. The infection should be initiated only after reaching the upper small intestine, where the pancreatic juice neutralizes low pH and supplies trypsin (1, 2). The question of how the trypsin-cleaved fragments of VP3 mediate the selective internalization of the viral inner capsid remains to be answered.

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LITERATURE CITED

1. **Banfield, W. G., G. Kasnic, and J. H. Blackwell.** 1968. Further observations on the virus of epizootic diarrhoea of infant mice: an electron microscopic study. Virology **36**:411–421.
2. **Bishop, R. F., G. P. Davidson, I. H. Holmes, and B. J. Ruck.** 1973. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet **ii**: 1281–1283.
3. **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.
4. **Chan, W. K., M. E. Penaranda, S. E. Crawford, and M. K. Estes.** 1986. Two glycoproteins are produced from the rotavirus neutralization gene. Virology **151**:243–252.
5. **Clark, S. M., B. B. Barnett, and R. S. Spendlove.** 1979. Production of high-titer bovine rotavirus with trypsin. J. Clin. Microbiol. **9**:413–417.
6. **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.
7. **Flores, J., H. B. Greenberg, J. Myslinski, A. R. Kalica, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. Use of transcription probes for genotyping rotavirus reassortants. Virology **121**:288–295.
8. **Fukuhara, N., O. Yoshie, S. Kitaoka, T. Konno, and N. Ishida.** 1987. Evidence for endocytosis-independent infection by human rotavirus. Arch. Virol. **97**:93–100.
9. **Graham, D. Y., and M. K. Estes.** 1980. Proteolytic enhancement of rotavirus infectivity: biologic mechanisms. Virology **101**: 432–439.
10. **Greenberg, H. B., J. Flores, A. R. Kalica, R. G. Wyatt, and R. Jones.** 1983. Gene coding assignments for growth restriction, neutralization and subgroup specificities of the W and DS-1 strains of human rotavirus. J. Gen. Virol. **64**:313–320.
11. **Greenberg, H. B., J. Valdesuso, K. Wyke, K. Midthun, M. Walsh, V. Mcauliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47**:267–275.
12. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of semliki forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
13. **Holmes, I. H.** 1983. Rotaviruses, p. 359–423. *In* W. K. Joklik (ed.), The reoviridae. Plenum Publishing Corp., New York.
14. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82**:8701–8704.
15. **Kalica, A. R., H. B. Greenberg, R. G. Wyatt, J. Flores, M. M. Sereno, A. Z. Kapikian, and R. M. Chanock.** 1981. Genes of human (strain Wa) and bovine (strain UK) rotavirus that code for neutralization and subgroup antigens. Virology **112**:385–390.
16. **Kitaoka, S., N. Fukuhara, F. Tazawa, H. Suzuki, T. Sato, T. Konno, T. Ebina, and N. Ishida.** 1986. Characterization of monoclonal antibodies against human rotavirus hemagglutinin. J. Med. Virol. **19**:313–323.
17. **Kitaoka, S., H. Suzuki, Y. Numazaki, T. Konno, and N. Ishida.** 1986. The effect of trypsin on the growth and infectivity of human rotavirus. Tohoku J. Exp. Med. **149**:437–447.
18. **Kitaoka, S., H. Suzuki, Y. Numazaki, T. Sato, T. Konno, T. Ebina, N. Ishida, O. Nakagomi, and T. Nakagomi.** 1984. Hemagglutination by human rotavirus strains. J. Med. Virol. **13**: 215–222.
19. **Kouvelos, K., M. Petric, and P. J. Middleton.** 1984. Oligosaccharide composition of calf rotavirus. J. Gen. Virol. **65**: 1159–1164.
20. **Kouvelos, K., M. Petric, and P. J. Middleton.** 1984. Comparison of bovine, simian and human rotavirus structural glycoproteins. J. Gen. Virol. **65**:1211–1214.
21. **Kutsuzawa, T., T. Konno, H. Suzuki, A. Z. Kapikian, T. Ebina, and N. Ishida.** 1982. Isolation of human rotavirus subgroups 1 and 2 in cell culture. J. Clin. Microbiol. **16**:727–730.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

23. **Lee, P. W. K., E. C. Hayes, and W. K. Joklik.** 1981. Protein σ1 is the reovirus cell attachment protein. Virology **108**:156–163.
24. **Lenard, J., and D. K. Miller.** 1982. Uncoating of enveloped viruses. Cell **28**:5–6.
25. **Ludert, J. E., F. Michelangeli, F. Gil, F. Liprandi, and J. Esparza.** 1987. Penetration and uncoating of rotaviruses in cultured cells. Intervirology **27**:95–101.
26. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1982. Pathway of vesicular stomatitis virus entry leading to infection. J. Mol. Biol. **156**:609–631.
27. **Matsuno, S., and S. Inouye.** 1983. Purification of an outer capsid glycoprotein of neonatal calf diarrhea virus and preparation of its antisera. Infect. Immun. **39**:155–158.
28. **Offit, P. A., and G. Blavat.** 1986. Identification of the two rotavirus genes determining neutralization specificities. J. Virol. **57**:376–378.
29. **Offit, P. A., G. Blavat, H. B. Greenberg, and H. F. Clark.** 1986. Molecular basis of rotavirus virulence: role of gene segment 4. J. Virol. **57**:46–49.
30. **Offit, P. A., H. F. Clark, G. Blavat, and H. B. Greenberg.** 1986. Reassortant rotaviruses containing structural proteins vp3 and vp7 from different parents induce antibodies protective against each parental serotype. J. Virol. **60**:491–496.
31. **Petrie, B. L., D. Y. Graham, and M. K. Estes.** 1981. Identification of rotavirus particle types. Intervirology **16**:20–28.
32. **Quan, C. M., and F. W. Doane.** 1983. Ultrastructural evidence for the cellular uptake of rotavirus by endocytosis. Intervirology **20**:223–231.
33. **Sabara, M., J. Gilchrist, G. R. Hudson, and L. A. Babiuk.** 1985. Preliminary characterization of an epitope involved in neutralization and cell attachment that is located on the major bovine rotavirus glycoprotein. J. Virol. **53**:58–66.
34. **Sato, T., H. Suzuki, S. Kitaoka, T. Konno, and N. Ishida.** 1986. Patterns of polypeptide synthesis in human rotavirus infected cells. Arch. Virol. **90**:29–40.
35. **Shaw, R. D., P. T. Vo, P. A. Offit, B. S. Coulson, and H. B. Greenberg.** 1986. Antigenic mapping of the surface proteins of rhesus rotavirus. Virology **155**:434–451.
36. **Suzuki, H., S. Kitaoka, T. Konno, T. Sato, and N. Ishida.** 1985. Two modes of human rotavirus entry into MA104 cells. Arch. Virol. **85**:25–43.
37. **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.
38. **Suzuki, H., T. Konno, Y. Numazaki, S. Kitaoka, T. Sato, A. Imai, F. Tazawa, T. Nakagomi, O. Nakagomi, and N. Ishida.** 1984. Three different serotypes of human rotavirus determined using an interference test with coxsackievirus B1. J. Med. Virol. **13**:41–44.
39. **Suzuki, H., T. Sato, T. Konno, S. Kitaoka, T. Ebina, and N. Ishida.** 1984. Effect of tunicamycin on human rotavirus morphogenesis and infectivity. Arch. Virol. **81**:363–369.
40. **Taniguchi, K., Y. Morita, T. Urasawa, and S. Urasawa.** 1987. Cross-reactive neutralization epitopes on vp3 of human rotavirus: analysis with monoclonal antibodies and antigenic variants. J. Virol. **6**:1726–1730.
41. **Taniguchi, K., S. Urasawa, and T. Urasawa.** 1985. Preparation and characterization of neutralizing monoclonal antibodies with different reactivity patterns to human rotaviruses. J. Gen. Virol. **66**:1045–1053.
42. **Taniguchi, K., T. Urasawa, Y. Morita, H. B. Greenberg, and S. Urasawa.** 1987. Direct serotyping of human rotavirus in stools by an enzyme-linked immunosorbent assay using serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies to VP7. J. Infect. Dis. **155**:1159–1166.
43. **Volkman, L. E., P. A. Goldsmith, and R. T. Hess.** 1986. Alternate pathway of entry of budded *Autographa californica* nuclear polyhedrosis virus: fusion at the plasma membrane. Virology **148**:288–297.
44. **Wyatt, R. G., W. D. James, E. H. Bohl, K. W. Theil, L. J. Saif, A. R. Kalika, H. B. Greenberg, A. Z. Kapikian, and R. M. Chanock.** 1980. Human rotavirus type 2: cultivation in vitro. Science **207**:189–191.
45. **Yoshimura, A., and S. Ohnishi.** 1984. Uncoating of influenza virus in endosomes. J. Virol. **51**:497–504.