Genetic Mapping Indicates that VP4 Is the Rotavirus Cell Attachment Protein In Vitro and In Vivo

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To identify the rotavirus protein which mediates attachment to cells in culture, viral reassortants between the simian rotavirus strain RRV and the murine strains EHP and EW or between the simian strain SA-11 and the human strain DS-1 were isolated. These parental strains differ in the requirement for sialic acid to bind and infect cells in culture. Infectivity and binding assays with the parental and reassortant rotaviruses indicate that gene 4 encodes the rotavirus protein which mediates attachment to cells in culture for both sialic acid-dependent and -independent strains. Using ligated intestinal segments of newborn mice and reassortants obtained between the murine strain EW and RRV, we developed an in vivo infectivity assay. In this system, the infectivity of EW was not affected by prior treatment of the enterocytes with neuraminidase, while neuraminidase treatment reduced the infectivity of a reassortant carrying gene 4 from RRV on an EW background more than 80% relative to the controls. Thus, VP4 appears to function as the cell attachment protein in vivo as well as in vitro.

The rotavirus virion is composed of three concentric layers of proteins and 11 segments of double-stranded RNA (4). The outer layer is composed of two proteins, VP4 and VP7. VP4 forms dimeric spikes that project from the surface of the virus (33, 37). VP7 is a structural glycoprotein, the major constituent of the outer protein layer (4), and it probably forms trimers on the viral surface. VP4 is the product of gene 4, while VP7 is the product of gene 7, 8, or 9, depending on the viral strain. In the presence of trypsin, VP4 is cleaved into two polypeptides, VP5* and VP8*. This proteolytic cleavage is associated with an increase in infectivity and penetration of the virus into the cell cytoplasm. However, viral binding is not dependent on VP4 cleavage (12, 17). In addition, VP4 is responsible for the hemagglutination activity of rotavirus and has been associated with viral stability and virulence (6, 11, 20, 25). Both VP4 and VP7 induce neutralizing and protective antibodies (13, 22).

Identification of the rotavirus protein which mediates cell attachment has been controversial. Earlier reports suggested that VP7 is the major cell attachment protein of rotavirus (17, 23, 30). However, recent indirect evidence strongly suggests that VP4 is the viral attachment protein. Fukudome et al. (16) found that most animal rotaviruses depend on the presence of sialic acid on the target cells for hemagglutination, cell binding and infectivity. Since the hemagglutination activity has been mapped to VP8* (15), this peptide has been assigned a role in cell binding. Furthermore, monoclonal antibodies directed at VP8* have been shown to prevent binding of rhesus rotavirus to cells in culture more efficiently than antibodies directed at VP5* or VP7 (29). In other studies, baculovirus-expressed VP4 competed with rotavirus particles for binding sites on murine enterocytes in a viral overlay protein blot assay (2). More recently, animal rotavirus mutants whose infectivity is no longer dependent on the presence of sialic acid on the cell surface have been isolated, and the mutation has been mapped to gene 4 (24). Finally, Crawford et al. (14) have shown that the binding of virus-like particles to MA-104 cells requires the presence of VP4. Although each of these studies provides indirect support for the notion that VP4 mediates cell attachment, direct proof of this possibility is not yet available.

On the basis of the ability of rotavirus to form reassortants in vitro and the finding that binding and infectivity of most animal rotaviruses depend on the presence of sialic acid on the cell surface, while those of humans and some animals isolates do not (16), we took a genetic approach to directly identify the rotavirus cell attachment protein. In this communication, we report that gene 4 codes for the rotavirus protein which mediates attachment to cells in culture for both sialic acid-dependent and -independent rotavirus strains. Furthermore, we present evidence supporting the conclusion that VP4 is also the rotavirus attachment protein in vivo.

MATERIALS AND METHODS

Cells and viruses. MA-104 cells were grown in medium 199 with 7% fetal calf serum. CaCo-2 cells, a continuous line of polarized epithelium originally derived from a human colon cancer, were grown in RPMI 1640 with 10% fetal calf serum. Both media were supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. Cells were used 3 to 4 days after seeding. All parental virus strains and reassortants strains (see below) used in this study were propagated in MA-104 cells in the presence of trypsin as previously described (19).

Reassortants from two parental crosses, RRV × EHP and SA11 × DS-1, were derived by coinfection of MA-104 cells according to standard techniques. The progeny reassortant were plaque purified twice, and the parental origin of each gene was determined by electrophoresis in polyacrylamide gels as previously described (5). The production and characterization of reassortants between EW and RRV have been previously described (5).

Infectivity assays. Each well of confluent monolayers of MA-104 or CaCo-2 cells grown in 96-well plates was treated with 50 μl of a solution containing 100 mU of neuraminidase from *Arthrobacter ureafaciens* or *Vibrio cholerae* per ml for 1 to 2 h at 37°C or, as a control, phosphate-buffered saline (PBS). Previous experiments had shown that both neuraminidases inhibited RRV infectivity approximately 80 to 90% under these conditions, while the infectivity of the human strain Wa (included as a control) and the murine strains EHP and EW

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remained unaffected (data not shown). After neuraminidase treatment, cells were washed with PBS and inoculated with serial 10-fold viral dilutions. The next day, the cells were fixed with methanol and stained for focus-forming units (FFU) according to a standard protocol (1). Infectivity in neuraminidase-treated cells was expressed as a percentage of the infectivity obtained in the control cells treated with PBS.

Binding assays. Confluent monolayers of MA-104 or CaCo-2 cells, grown in 24-well plates, were treated with 400 µl of neuraminidase solution or PBS per well. After treatment with the enzyme, the cells were washed with ice-cold PBS and incubated with viruses (multiplicities of infection were 100 for RRV, 0.1 for EHP, 20 for SA-11, and 10 for DS-1) at 400 µl per well for 2 h at 4°C. The viruses were previously treated with trypsin (2.5 µg/ml) for 30 min at 37°C; after trypsin treatment, bovine serum albumin (BSA) and phenylmethylsulfonyl fluoride were added to the viruses to give final concentrations of 1% and 1 mM, respectively (16). Following incubation with the viruses, cells were extensively washed with ice-cold PBS, and 0.2 ml of medium 199 was added to the dry wells. Cells were immediately freeze-thawed three times and scraped from the dish. The supernatant recovered was clarified in a microcentrifuge, and the virus titer was determined on new MA-104 cells, using an FFU assay (1). Under the conditions used, the amount of RRV bound to control cells was between 1 and 10% of the total input virus amount.

In vivo infectivity assay. Eight to ten-day-old BALB/c mouse pups from dams seronegative for rotavirus were anesthetized by intraperitoneal injection with equivalent volumes of xylazine (20 mg/kg) and ketamine (100 mg/kg) diluted in sterile physiologic saline. Supplemental anesthesia was administered with 2-bromo-2-chloro-1,1,1,-trifluorethane (Halothene) inhaled via the open-drop method. A 2- to 3-cm midline incision was made to expose the distal ileum and cecum. An intestinal loop was formed by placing a ligature (7.0 ophthalmic vicryl; Ethicon, Somerville, N.J.) about 1 to 2 cm from the ileocecal juncture, which was used as a reference point to ensure consistency of loop placement. The loops were 1 to 2 cm in length.

EW virus (7×10^4 FFU/ml) or reassortant EW/R4 (3×10^7 FFU/ml) was directly inoculated into the lumen of the loop, using a 0.5-cm³ tuberculin syringe (27-gauge by 1/2-in needle) (5). The inoculum volume was 50 to 100 µl. After inoculation, the intestines were returned to the abdominal cavity, physiologic saline was administered intraperitoneally, and the incision was closed with cyanoacrylate adhesive (Krazy Glue; Borden). For neuraminidase treatment, 50 to 100 µl of a solution containing 250 mU of *V. cholerae* neuraminidase per ml diluted in PBS was inoculated directly into the lumen of the loop 30 min prior to inoculation of the virus. Because of the ligature placed on the intestine, the neuraminidase could not be washed from the lumen before virus inoculation. However, treatment of SA-11 rotavirus with neuraminidase from *V. cholerae* had no effect on hemagglutination, indicating that the effect of treatment was on the cell, not the virus (3). Similarly, treatment of EW, EW/R4, and RRV viruses with 200 mU of *V. cholerae* neuraminidase per ml for 1 h at 37°C had no effect on infectivity (data not shown). The mice were recovered and sacrificed by cervical dislocation 12 h postinfection. The ligated intestinal loops were then excised, fixed in 10% neutral formalin (Sigma Chemical Co.) overnight at 4°C, and embedded in paraffin. Following deparaffination of the sections (26), the samples were immunostained by using a Vectastain ABC kit (Vector Laboratories) as instructed by the manufacturer. A rabbit hyperimmune antiserum diluted in PBS–1% BSA was used as a primary antibody. Finally, the slides were counterstained with hematoxylin.

The number of rotavirus-infected enterocytes and the total number of enterocytes were counted in five villi per section, and three sections were counted per animal. The results were expressed as percentage of infected cells in relation to the total number of cells in the villi.

Statistical analysis. All statistical analyses were performed with Statview (Abacus Concepts, Inc., Berkeley, Calif.), a statistical program for Macintosh computers. Analysis of variance was used to examine the differences in infectivity between parental and reassortant viruses after treatment with neuraminidase for the in vitro experiments and the difference in infectivity in intestines treated with neuraminidase in relation to the control for the in vivo experiments. Fisher's protected least-significance-difference test was used for pairwise comparison among selected groups.

RESULTS

Characterization of the parental and reassortant rotaviruses. The reassortants used in this study are described in Table 1. The parental origin of each segment was determined by electrophoresis as previously described (5). Prior studies in this and other laboratories had demonstrated that RRV and SA-11 hemagglutinate human type O erythrocytes, while EHP, DS-1, and EW do not. In addition, neuraminidase treatment of MA-104 cells decreased the binding and infectivity of RRV and SA-11 but not of EHP, DS-1, or EW (16, 21, 24) (Fig. 1; see Fig. 4). Since reassortants from three different parental combinations were used in this study, reassortants are desig-

TABLE 1. Genotypes of reassortants used to map the gene segment encoding the rotavirus attachment protein

Virus	Parental origin of indicated segment[a]										
	1	2	3	4	5	6	7	8	9	10	11
Parental											
RRV (simian)	R	R	R	R	R	R	R	R	R	R	R
EHP (murine)	E	E	E	E	E	E	E	E	E	E	E
Reassortant											
RRV/E4	R	R	R	E	R	R	R	R	R	R	R
EHP/R4	E	E	E	R	E	E	E	E	E	E	E
RRV/E7	R	R	R	R	R	R	E	R	R	R	R
EHP/R7	E	E	E	E	E	E	R	E	E	E	E
Parental											
SA11 (simian)	S	S	S	S	S	S	S	S	S	S	S
DS-1 (human)	D	D	D	D	D	D	D	D	D	D	D
Reassortant											
SA-11/D4	S	S	S	D	S	S	S	S	S	S	S
DS-1/S4	D	D	D	S	D	D	D	D	D	D	D
Parental											
RRV (simian)	R	R	R	R	R	R	R	R	R	R	R
EW (murine)	W	W	W	W	W	W	W	W	W	W	W
Reassortant											
EW/R4	W	W	W	R	W	W	W	W	W	W	W

[a] R, RRV; E, EHP; D, DS-1; W, EW.

nated by the name of the virus with the most-represented genes first, followed by the first letter of the name of the virus with the least-represented genes and the number of the gene segment. For example, RRV/E4 is a reassortant with gene 4 from EHP and all other genes from RRV.

Infectivity of the parental and reassortant strains in neuraminidase-treated cells in vitro. To determine how efficiently these viruses infected MA-104 and CaCo-2 cells with or without sialic acid residues on the surface, a viral infectivity assay in which the titers of the strains were determined in parallel in cells treated and untreated with neuraminidase was performed. Figure 1A shows that the infectivity of RRV was reduced between 75 and 90% by treatment with neuraminidase, depending on the enzyme origin, while the infectivity of EHP was unaffected. Similarly, the infectivity of SA-11 was reduced 80% by treatment with either of the neuraminidases used (Fig. 1B), while the infectivity of DS-1 was, unexpectedly, slightly increased (approximately 1.5-fold in relation to the control). When we examined the infectivity titers of the monoreassortants between EHP and RRV or SA-11 and DS-1, the difference in neuraminidase-dependent infectivity between strains cosegregated with the parental origin of gene 4. Reassortants EHP/R4 and DS-1/S4 exhibited phenotypes comparable to those of RRV and SA-11, respectively; that is, in the absence of sialic acid on the target cell, their infectivity was reduced between 50 and 80% compared with the controls. Despite the minor differences observed between strains and between enzymes, it is clear that reassortants containing gene 4 from RRV or SA-11 show a reduced infectivity in neuraminidase-treated MA-104 cells, like the RRV or SA-11 parental strains and unlike EHP and DS-1. On the other hand, the infectivity patterns of reassortants RRV/E4 and SA-11/D4 were similar to those of EHP and DS-1, respectively, despite the fact that these reassortants derived only gene 4 from EHP or DS-1.
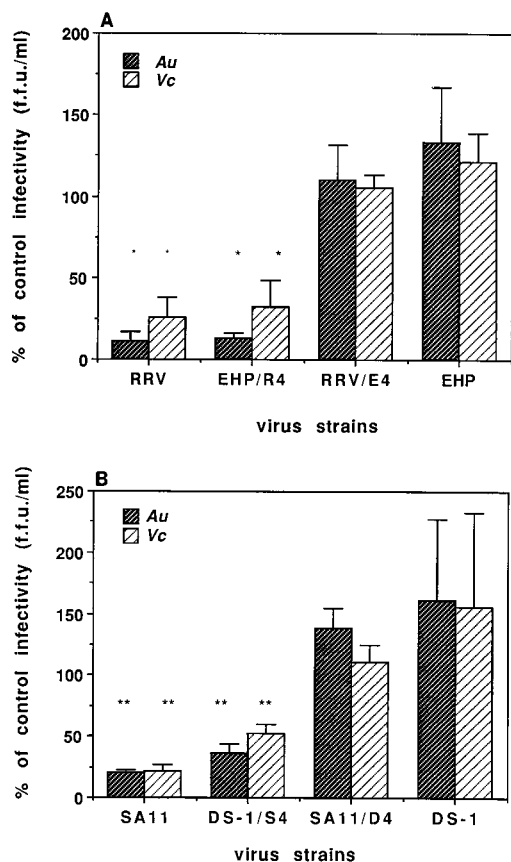
FIG. 1. Effects of neuraminidase (*Au*, *A. ureafaciens*; *Vc*, *V. cholerae*) treatment of MA-104 cells on infectivity of parental and reassortant rotaviruses. (A) RRV and EHP parental strains and corresponding VP4 monoreassortants; (B) SA-11 and DS-1 parental strains and corresponding monoreassortants. Titers of the parental and reassortant viruses were determined in parallel by serial dilutions in cells untreated or treated with the indicated neuraminidase as described in Materials and Methods. Results are expressed as percentage of FFU per milliliter obtained in cells treated only with PBS. Results represent the means of at least two experiments with standard deviations as indicated. *, significant differences ($P < 0.001$) were found with either RRV/E4 or EHP; **, significant differences ($P < 0.04$) were found with either SA-11/D4 or DS-1 except between the pair DS-1/S4 and SA-11/D4 with *V. cholerae* neuraminidase ($P = 0.1$).
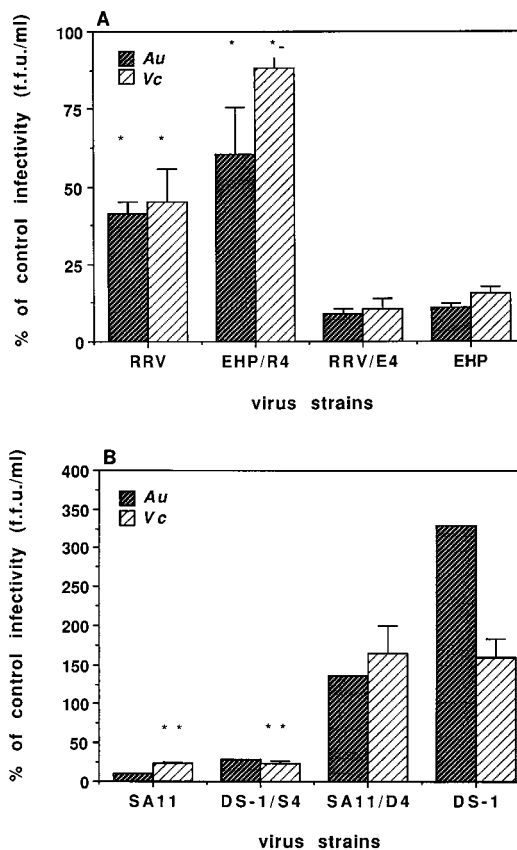

FIG. 2. Effects of neuraminidase (*Au*, *A. ureafaciens*; *Vc*, *V. cholerae*) treatment of CaCo-2 cells on infectivities of parental and reassortant rotaviruses. (A) RRV and EHP parental strains and corresponding VP4 monoreassortants; (B) SA-11 and DS-1 parental strains and corresponding monoreassortants. The procedure was as explained in the legend to Fig. 1. Results represent the means of two experiments with standard deviations as indicated except that in panel B, only one experiment was done with *A. ureafaciens* neuraminidase. *, significant differences ($P < 0.01$) were found with either RRV/E4 or EHP; however, a significant difference ($P = 0.002$) was also found between RRV and EHP/R4 when *V. cholerae* neuraminidase was used. **, significant differences ($P < 0.002$) were found with either SA-11/D4 or DS-1.

Hence, both susceptibility and resistance to neuraminidase treatment of MA-104 cells mapped to gene 4.

We next tested the infectivity phenotypes of the same group of parental and reassortant viruses on CaCo-2 cells untreated or treated with neuraminidase (Fig. 2). The infectivities of SA-11 and DS-1 and of reassortants DS-1/S4 and SA-11/D4 paralleled those found in MA-104 cells (Fig. 2B). However, to our surprise, RRV and EHP showed a phenotype in neuraminidase-treated CaCo-2 cells different from that observed in MA-104 cells (Fig. 2A). EHP showed a high susceptibility (up to 90% reduction in infectivity) to neuraminidase treatment of CaCo-2 cells, while RRV showed only moderate susceptibility (approximately 50% reduction). The phenotypes of reassortants EHP/R4 and RRV/E4 resembled those of RRV and EHP, respectively, indicating that despite the altered phenotype of the parental strains in comparison with MA-104 cells, susceptibility of the infection to neuraminidase treatment of CaCo-2 cells still cosegregated with the parental origin of gene 4.

To further explore the role of VP4 in the susceptibility to infection in neuraminidase-treated cells and to evaluate a pos-

sible role for VP7 in this process, the infectivities of monoreassortants for VP7 from parental strains RRV and EHP were also evaluated in MA-104 cells. Reassortant RRV/E7 is susceptible to neuraminidase treatment of MA-104 cells to the same degree as RRV, while the infectivity of reassortant EHP/R7 after neuraminidase treatment is unaffected (Fig. 3). Thus, VP7 does not appear to play a primary role in determining the susceptibility or resistance of neuraminidase-treated MA-104 cells to these viral strains.

Binding of parental and reassortant rotavirus strains to cultured cells treated with neuraminidase. Previous studies (16, 21, 28, 35) demonstrated that the decreased infectivity produced by neuraminidase treatment was caused by decreased viral binding to cells. To ensure that the effect of neuraminidase on each of our reassortants was due to changes in viral binding, an assay to measure the amount of infectious virus bound to treated and untreated MA-104 cells was performed (1). We found that the relative amounts of parental strains RRV and EHP and reassortant strains EHP/R4 and RRV/E4 bound to target cells cosegregated with the genetic origin of VP4 (Fig. 4A). After neuraminidase treatment, the binding of reassortant EHP/R4 was reduced approximately
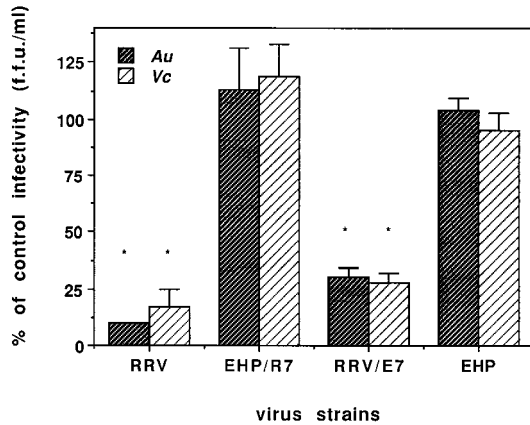
FIG. 3. Effects of neuraminidase (*Au*, *A. ureafaciens*; *Vc*, *V. cholerae*) treatment of MA-104 cells on the infectivities of RRV and EHP parental strains and corresponding VP7 monoreassortants. The procedure was as explained in the legend to Fig. 1. Results represent the means of two experiments with the standard deviations as indicated. *, significant differences ($P < 0.002$) were found with either EHP/R7 or EHP.

90% in relation to the controls. On the other hand, the amount of infectious reassortant RRV/E4 bound to cells was unaffected by treatment of the cells with neuraminidase.

Strains SA-11 and DS-1 and the corresponding monoreassortants also bound to treated MA-104 (Fig. 4B) or CaCo-2 (Fig. 5) cells in a manner that paralleled their infectivity. Again, with these viruses, the capacity of the parental and reassortant strains to bind to cells treated with neuraminidase cosegregated with the origin of VP4.

Infectivity of the parental and reassortant strains in cells treated with neuraminidase in vivo. To study the infectivity of virus in vivo in the presence or absence of sialic acid, we took advantage of an intestinal loop model in infant mice in which neuraminidase and virus are inoculated directly into the lumen of a closed intestinal segment. Although there is no published evidence that treatment of intestinal loops with neuraminidase will effectively remove sialic acid residues from enterocytes, we reasoned that such an effect would be indicated by a reduction in the infectivity of neuraminidase-sensitive but not -insensitive viruses in the treated loops. For the in vivo experiments, a reassortant containing only gene 4 from RRV in an EW background (EW/R4) was used (5). Reassortants derived from EHP × RRV could not be used in the loop model because the cell culture-adapted EHP strain is highly attenuated in mice (7) and shows very few infectious foci in the loop model. In addition, the infectivity of heterologous (nonmurine) viruses in this system is also severely restricted, and loops inoculated with RRV show very few infected enterocytes, even when concentrated preparations of virus are used (21a). The infectivity of EW in MA-104 cells is augmented by treatment of the cells with either of the neuraminidases used (Fig. 6A), and a similar effect is observed in CaCo-2 cells (data not shown). Meanwhile, the same treatment reduces the infectivity of reassortant EW/R4 in MA-104 cells more than 80% (Fig. 6A). EW/R4 also showed a phenotype similar to that of RRV in treated CaCo-2 cells (data not shown). We next determined if the decreased infectivity observed with EW/R4 when sialic acid was removed from the cell surface was due to a defect in binding (Fig. 6B). As was the case with other reassortants, binding as well as infection cosegregated with the origin of gene 4.

We also examined the behavior of EW and EW/R4 in vivo. The result from a typical in vivo experiment is shown in Fig. 7.
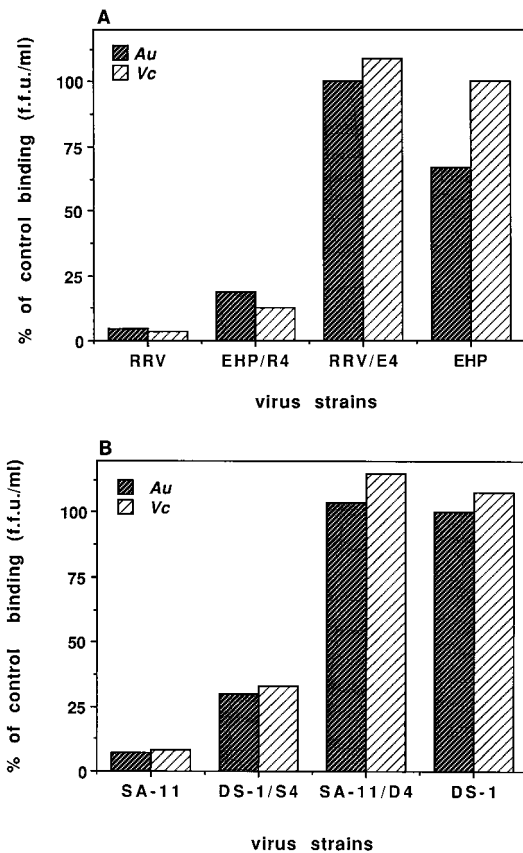

FIG. 4. Effects of neuraminidase (*Au*, *A. ureafaciens*; *Vc*, *V. cholerae*) treatment of MA-104 cells on binding of parental and reassortant rotaviruses. (A) RRV and EHP parental strains and corresponding VP4 monoreassortants; (B) SA-11 and DS-1 and corresponding monoreassortants. MA-104 cells were treated with 100 mU of *A. ureafaciens* or *V. cholerae* neuraminidase per ml or, as a control, PBS. Concentrated preparations of parental and reassortant rotaviruses were added to the treated and untreated cells. After incubation at 4°C for 1 h, cells were washed three times and freeze-thawed three times, and the amount of infectious virus bound to cells was measured by titration on new MA-104 cell monolayers, using an FFU assay (1). The results are expressed as the percentage of virus bound to untreated cells.

The infectivity of EW was not affected by the treatment of the loop with neuraminidase (Table 2 and Fig. 7). However, the infectivity of reassortant EW/R4 was reduced more than 80% by treatment of the enterocytes in the loop with neuraminidase (Table 2 and Fig. 7). The reciprocal reassortant, RRV/EW4, was not available for analysis (5) but presumably would not be infectious in the loop system because the parental RRV strain was not infectious.

DISCUSSION

Most of the animal rotaviruses isolated to date require the presence of sialic acid on the cell surface to bind and infect cells in vitro, while most human rotaviruses and some animals isolates do not (16, 21, 24, 28, 32, 35, 39). Using monoreassortant rotaviruses derived from three separate sets of parental strains with different requirements for sialic acid, we found that gene 4 appears to encode all or most of the cell attachment activity in each case. This genetic linkage accounts for both sialic acid-dependent and -independent binding, indicating that rotaviruses containing VP4s of either phenotype use VP4 for the first step in cell attachment in vitro. In addition, we
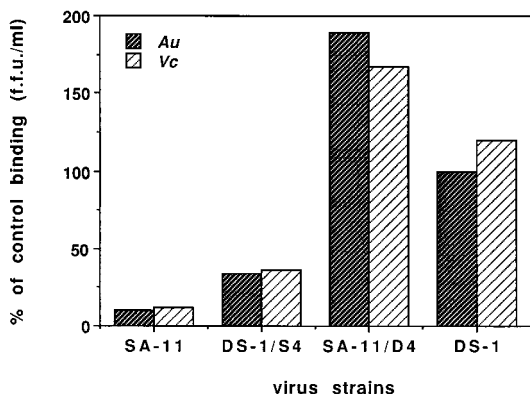
FIG. 5. Effects of neuraminidase (*Au*, *A. ureafaciens*; *Vc*, *V. cholerae*) treatment of CaCo-2 cells on binding of SA-11 and DS-1 parental strains and the corresponding monoreassortants. The procedure was as explained in the legend to Fig. 4.

obtained data which suggest that the role of VP4 in neuraminidase-dependent attachment seen in vitro is highly likely to be operative in vivo as well.

Méndez et al. (24) isolated RRV mutant strains whose infectivity was no longer dependent on the presence of sialic acid on the target cell surface. The mutation site was mapped to gene 4 by genetic analysis. Since these mutants were still able to bind sialic acid, the authors proposed the existence of a second, sialic acid-independent binding site on VP4, most likely VP5*. Our results with reassortant rotaviruses RRV/E4 and SA-11/D4 demonstrated that the sialic acid-independent binding site, present in most humans and some animals isolates, is also located in VP4. Whether these two sites map to the same location on the VP4 molecule cannot be determined from the present data. The construction of chimeric VP4 molecules may allow us to address this question.

It is of interest that the resistance to neuraminidase of reassortants RRV/E4 and SA11/D4, the sensitivity to neuraminidase of reassortant DS-1/S4 in MA-104 cells (Fig. 1), and the sensitivity of reassortant EHP/R4 in CaCo-2 cells (Fig. 2A) are not as pronounced as those observed with the parental strains from which the gene 4's were derived. It has previously been reported that the antigenicity of VP4 (10), as well as other specific phenotypes associated with this protein (9), can be affected by the recipient genetic background in rotavirus reassortants. In light of this evidence, a possible explanation for the somewhat modified phenotype shown by the monoreassortants in certain circumstances is that VP7, VP6, or both proteins in the recipient virus modify the function of VP4 as an attachment protein. Alternatively, VP7 may play a minor but direct role in the interaction of the rotavirus virion with the cells. The results obtained with the monoreassortants for VP7 (Fig. 3) support the first hypothesis, but additional genetic data are needed to fully clarify this point. However, it is clear from our genetic studies that VP4 is the primary determinant of cell binding in vitro.

To our surprise, the infectivity of EHP in CaCo-2 cells was highly dependent on the presence of sialic acid on the cell surface, while the infectivity of this strain in MA-104 cells was completely independent of sialic acid residues. At present, we do not have an explanation for this discrepancy, but this result indicates that the distinction between neuraminidase-sensitive and -insensitive strains may be influenced by the cell type used to carry out the assays as well as the sequence of VP4. In any case, the results obtained with reassortant RRV/E4 demon-

strate that the resistance or sensitivity of EHP infection to neuraminidase treatment cosegregates with the genetic origin of gene 4 irrespective of the specific phenotype of the parental virus.

We have tried to expand our observations on viral attachment to infection in vivo. Because of difficulties in isolating ideal reassortants, our in vivo studies are not as extensive as the in vitro analysis. However, our results with the loop model strongly suggest that the mechanism operating for rotavirus binding in vitro is operative in vivo as well; that is, VP4 acts as the rotavirus attachment protein for enterocytes. The result obtained with reassortant EW/R4 directly suggests that this is the case with RRV, and from the results obtained in vitro, it is logical to suppose that VP4 is also the attachment protein for the homologous neuraminidase-insensitive strain EW.

In agreement with previous studies (8, 38, 39), our results suggest that sialic acid is a constituent of the receptor for RRV (but not of the receptor for EW) in the intestines of newborn mice. Superti and Donelli (35) reported that gangliosides, specifically GM1, could interact with SA-11 virus and that such interactions were neuraminidase sensitive. Rolsma et al. (28) purified two gangliosides (GMX and GM1) from the small intestines of young piglets, demonstrated that these gangliosides were capable of binding OSU rotavirus in vitro, and showed that the binding activity was also neuraminidase sen-
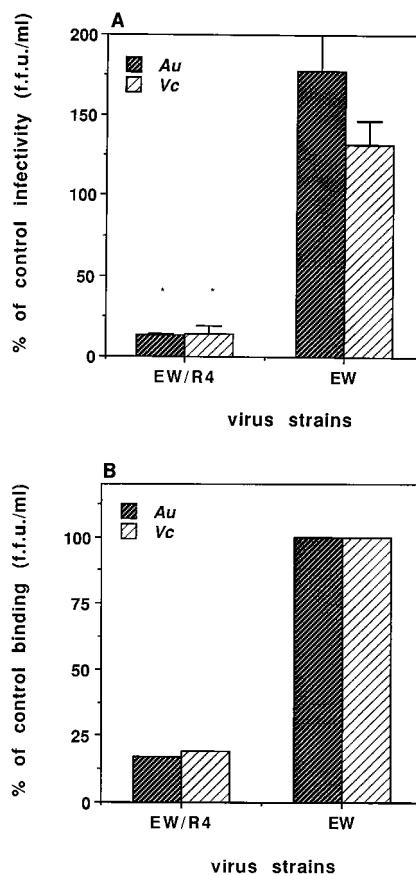

FIG. 6. Effects of neuraminidase (*Au*, *A. ureafaciens*; *Vc*, *V. cholerae*) treatment of MA-104 cells on the infectivities (A) and binding (B) of EW and reassortant EW/R4. (A) The procedure was as explained in the legend to Fig. 1. Results represent the means of two experiments with standard deviations as indicated. (B) The procedure was as explained in the legend to Fig. 4. *, significant differences ($P < 0.01$) were found with EW.
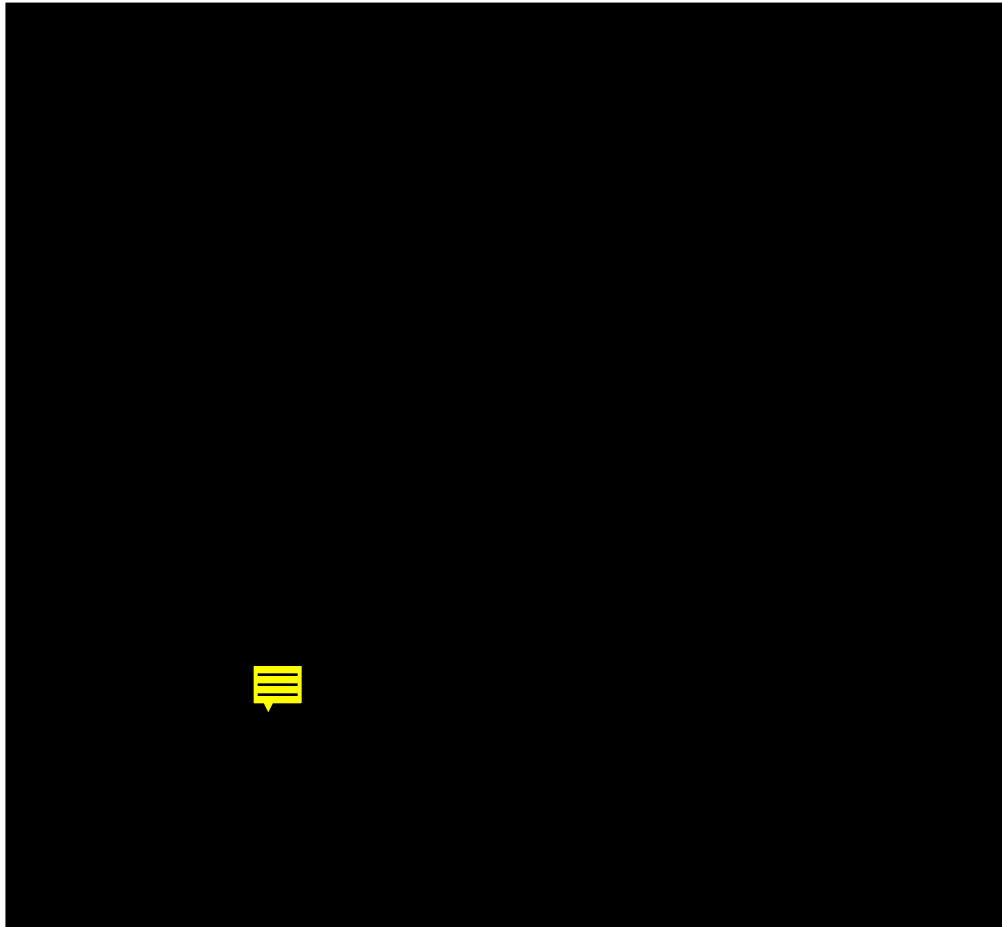
FIG. 7. Effects of neuraminidase treatment of closed mouse intestinal segments on the infectivities of EW and reassortant EW/R4. Treatment of closed intestinal segments of neonatal mice with *V. cholerae* neuraminidase for 30 min was followed by direct inoculation of the indicated viruses. At 12 h postinfection, the closed segments of intestine were fixed, embedded in paraffin, and processed for immunohistochemistry. The infectivity of reassortant EW/R4 was reduced by the prior treatment with neuraminidase, while that of EW was not affected. All panels were photographed at the same magnification. Bar = 20 μm.

sitive. On the other hand, asialoglycolipids (GA1 and GA2) isolated from MA-104 cells or adult mouse intestine have been shown to bind to rotavirus (34, 36). Equal levels of binding to GA1 were observed with both neuraminidase-sensitive strains (RRV) and neuraminidase-insensitive strains (Wa and DS-1) (36). GM1 and other sialic acid-containing gangliosides are the major gangliosides present in the small intestines of newborn mice. After 3 weeks of age, they are replaced by neutral asialoglycosphingolipids (31, 36). Given the temporal correlation between the presence of sialic acid-containing gangliosides, the observed age-dependent susceptibility of mice to infection with

simian rotaviruses (27), and the finding that sialic acid is a component of the receptor for RRV in the murine intestine, it is tempting to suggest that these sialoglycolipids actually play a role in the infection of mice with RRV. In addition, sialic acid glycoproteins may also play a role, since RRV is capable of binding in a viral overlay protein blot assay to glycoproteins isolated from brush border membranes prepared from enterocytes of suckling mice. This binding was abolished by neuraminidase treatment of the membranes and by competition with baculovirus-expressed VP4 (2). A similar speculation cannot be made with respect to the asialoglycosphingolipids and the murine strains because at least some murine rotaviruses are equally infectious in newborn and adult mice (7).

Recently, evidence which suggests that binding of viruses to cells is often a multistep process whereby an initial loose binding is followed by a second, more stable binding step has been accumulating (18). Binding of animal rotaviruses to cells in culture might first occur via a rapid association to a sialic acid-containing cell receptor followed by a slower binding step through a sialic acid-independent receptor. The rapid association with sialic acid would facilitate a second, more slowly occurring step. Human and some animal rotaviruses, lacking the ability to bind to sialic acid, would rely only on the second binding step for infection, which could account, in part, for the difficulty in growing many of these strains in vitro. The ob-

TABLE 2. Effects of neuraminidase treatment of ligated murine intestinal segments on the infectivity of EW and reassortant EW/R4

Virus strain	Mean % of infected cells/villus[a] ± SE (no. of animals)		% of control infectivity
	PBS	Neuraminidase[b]	
EW	51.4 ± 6.0 (4)	51.0 ± 6.2 (4)	99.2
EW/R4	35.5 ± 5.3[c] (2)	6.2 ± 4.0[c] (2)	17.5

[a] A total of 15 villi per animal were counted.
[b] Neuraminidase from *V. cholerae*, 50 to 100 μl of a solution containing 250 mU/ml per loop, 30 min at room temperature.
[c] Significant difference (P = 0.0247).

served increase in infectivity of DS-1 and EW in MA-104 and CaCo-2 cells treated with neuraminidase could be explained by this model. We postulate that the removal of sialic acid from the cell surface would render the putative secondary receptor for these viruses more accessible, thereby facilitating the binding of the virion to cells. Since the reassortant SA-11/D4 also showed enhanced infectivity (although to a lesser degree) in the absence of sialic acid, it would follow that VP4 also plays a role in this second binding step. In support of this hypothesis is the observation that a monoclonal antibody-selected SA11 escape mutant which localized to VP8* lost hemagglutination activity and also grew more slowly than the parental strain, presumably as a result of a loss of primary binding to sialic acid (40).

A number of studies have indicated that VP4 is a determinant of viral virulence (6, 25). It is logical to hypothesize that virulence differences may be due to differences in the efficiency with which VP4 binds to specific target cells. However, no direct proof of this hypothesis is available. In fact, studies done in cell culture indicate that differences in binding efficiencies of rotavirus do not account for differences in cell tropism (1). Although VP4 appears to be primarily responsible for viral attachment, it obviously has other functions as well, such as playing a role in cell penetration. Future studies must focus on how the binding and penetration functions are linked and what changes in VP4 or other viral proteins are required for the viral replication cycle to progress from binding through cell entry.
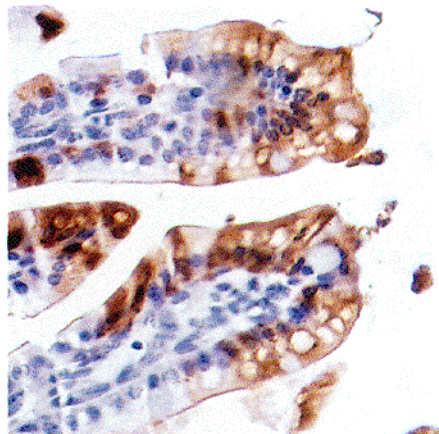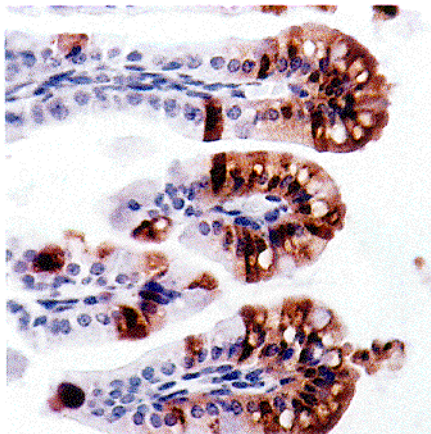
ACKNOWLEDGMENTS

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PBS　　　　　Neuraminidase

EW

EW/R4