Genetic Relatedness among Human Rotavirus Genes Coding for VP7, a Major Neutralization Protein, and Its Application to Serotype Identification

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Antigenic characterization of human rotavirus by plaque reduction neutralization assay has revealed four distinct serotypes. The outer capsid protein VP7, coded for by gene 8 or 9, is a major neutralization protein; however, studies of rotaviruses derived from genetic reassortment between two strains have confirmed that another outer capsid protein, VP3, is in some cases equally important in neutralization. In this study, the genetic relatedness of the genes coding for VP7 of human rotaviruses belonging to serotypes 1 through 4 was examined by hybridization of their denatured double-stranded genomic RNAs to labeled single-stranded mRNA probes derived from human-rotavirus reassortants containing only the VP7 gene of their human rotavirus parent. A high degree of homology was demonstrated between the VP7 gene of (i) strain D and other serotype 1 human rotaviruses, (ii) strain DS-1 and other serotype 2 human rotaviruses, (iii) strain P and other serotype 3 human rotaviruses, and (iv) strain ST3 and other serotype 4 human rotaviruses. Hybrid bands could not be demonstrated between the VP7 gene of D, DS-1, P, or ST3 and the corresponding gene of human rotaviruses belonging to a different serotype. RNA specimens extracted from the stools of 15 Venezuelan children hospitalized with rotavirus diarrhea were hybridized to each of the reassortant probes representing the four human serotypes. All five viruses with short RNA patterns showed homology with the DS-1 strain VP7 gene; two of these were previously adapted to tissue culture and shown to be serotype 2 strains by tissue culture neutralization. Of the remaining 10 viruses with long RNA patterns, 2 hybridized only to the D strain VP7 gene, 6 hybridized only to the P strain VP7 gene, and 2 hybridized only to the ST3 strain VP7 gene. Hybridization using single human rotavirus gene substitution reassortants as probes may provide an alternative method for identifying the VP7 serotype of field isolates that would circumvent the need for tissue culture adaptation.

Human rotaviruses are the major etiologic agents of gastroenteritis in infants and young children in most parts of the world (18, 20). They belong to the family Reoviridae, have a genome consisting of 11 segments of double-stranded RNA (dsRNA), and are composed of four distinct serotypes (3, 12, 22, 30, 33, 34, 35). A comparison of the electrophoretic mobilities of the genomic dsRNAs (i.e., electrophero-
type) of numerous rotavirus strains has provided evidence for the genetic diversity of human rotaviruses (4, 28). However, characterization by electropherotype does not indicate the serotype of human rotaviruses, except for serotype 2 strains which, thus far, always exhibit a pattern of slow migration of RNA segments 10 and 11 (2, 15, 16, 21, 34). In addition, RNA-RNA hybridization studies, using single-stranded RNA (ssRNA) transcripts as probes, have revealed two major human rotavirus families; one includes strains related to the prototype strain Wa (serotype 1) family, and the other includes strains related to the prototype strain DS-1 (serotype 2) family (6, 7). However, this technique is not useful for serotype analysis, since serotypes 1, 3, and 4 all belong to the Wa family.

Four distinct serotypes of human rotaviruses have been identified by several techniques, including neutralization, enzyme-linked immunoassay (ELISA), and solid-phase immune electron microscopy (3, 8, 15, 30-35). Studies correlating the phenotype and genotype of cultivatable reassort-ant rotaviruses have indicated that the VP7 outer capsid protein, a major neutralization protein, is coded for by either gene 8 or 9 of the human rotaviruses studied to date (W, Wa, and D [serotype 1]; DS-1 [serotype 2]; P [serotype 3]; ST3 [serotype 4]) (10, 17, 23, 24). The VP7 gene sequences of only three human rotaviruses, Wa (serotype 1), Hu-5 (serotype 2), and S2 (serotype 2), have been reported (9, 11).

Thus, comparisons among the VP7 gene sequences of human rotaviruses belonging to the same serotype have been limited to serotype 2, and comparisons among the VP7 gene sequences of human rotaviruses representing each of the four serotypes have not been possible.

In the present study, the relatedness of the genes coding for VP7 of tissue culture-adapted human rotavirus strains of serotype 1, 2, 3, or 4 was analyzed by hybridization to 32P-labeled ssRNA transcripts prepared from human-bovine rotavirus reassortants which possessed only a single human rotavirus gene, that which codes for VP7. In some cases, the genomic dsRNAs of each of the reassortants were hybridized to a probe derived from the human rotavirus being tested. These reassortants contained only the VP7 gene of their human rotavirus parent (D, DS-1, P, or ST3), and the remaining 10 genes were derived from their bovine rotavirus strain UK parent (23, 24). In addition, RNAs extracted from the stools of 15 children who were hospitalized with rotavirus diarrhea in Venezuela were hybridized to these reassortant rotavirus probes to determine if this technique could discriminate among the VP7 sequences present in the reassortant viruses.
could be used to identify the VP7-mediated serotype of rotaviruses present in clinical stool specimens.

**MATERIALS AND METHODS**

**Viruses.** The following human rotaviruses, which had been adapted to tissue culture and serotyped by neutralization in tissue culture, were studied: D, Wa, and Mo (serotype 1); M37 (intertype, VP3 serotype 4 and VP7 serotype 1); DS-1, HN144, HN90II, and 1076 (serotype 2); P, M, and McN (serotype 3); and ST3, VA70, and Hosokawa (serotype 4) (8, 14, 34, 35). Bovine rotavirus strain UK and the following reassortant rotaviruses derived from earlier in vitro experiments were also studied: D × UK (47-1-1), DS-1 × UK (66-1-1), P × UK (22-1-1), and ST3 × UK (52-1-1) (23, 24).

Each of these reassortants derived only the gene segment coding for VP7 from its human rotavirus parent (gene 9 of D or ST3, gene 8 of DS-1, and gene 8 or 9 of P) and the remaining 10 genes from its bovine rotavirus parent. In addition, rotavirus-positive stool specimens from 15 children admitted between December 1981 and November 1982 to the Hospital de Ninos “J. M. De Los Rios,” Caracas, Venezuela, were used as a source of human rotavirus RNAs (7).

**RNA preparation.** Genomic dsRNAs of tissue culture-adapted rotavirus were extracted from infected lyses of MA104 cells by using phenol-chloroform as described previously (23). However, for strains 1076 and McN, which tend to grow poorly, tissue culture lyses were first centrifuged in a Beckman Ti45 rotor at 35,000 rpm for 90 min; the pellets were then fluorocarbon extracted, and the virus in the aqueous phase was sedimented through a 30% sucrose cushion by centrifugation for 2 h in a Beckman SW27 rotor at 25,000 rpm. The pellets were then fractionated in a 40 to 55% CsCl gradient by overnight centrifugation at 35,000 rpm in a Beckman SW40 rotor. Fractions with densities of 1.38 to 1.36 g/ml were pooled and pelleted out of CsCl, and then the genomic dsRNAs were extracted with phenol-chloroform. Approximately 1 g from each of 15 stool specimens from Venezuelan children was fluorocarbon extracted and processed for RNA in a manner similar to that used for the poorly growing tissue-culture-adapted rotaviruses. The dsRNAs were transcribed from viral cores of selected rotaviruses by using previously described methods, except that newly synthesized transcripts were precipitated with 2 M lithium chloride (5). Labeled transcripts were stored at −70°C until used.

**Hybridization.** To assess the genetic relatedness of human rotavirus VP7 genes, denatured genomic dsRNAs of different human rotaviruses were hybridized to 32P-labeled ssRNA probes. These probes were derived from a human-bovine rotavirus reassortant bearing a VP7 with serotype 1, 2, 3, or 4 specificity. In some instances, dsRNAs from each of the four reassortant rotaviruses were hybridized to probes derived from different human rotaviruses. The dsRNAs were diluted in 1 mM EDTA, heated at 100°C for 2 min, and then quenched on ice for 2 min. Approximately 10,000 cpm of the 32P-labeled RNA probe in hybridization buffer (100 mM NaCl, 50 mM Tris [pH 8], 0.1% sodium dodecyl sulfate) was added to the denatured dsRNAs, and hybridization was allowed to take place at 65°C for 14 h. Hybridized RNAs were precipitated with 2 or 3 volumes of ethanol, and after being kept overnight at −20°C, the RNAs were pelleted (12,000 × g) and taken up in 20 μl of sample buffer and electrophoresed in 10% polyacrylamide gels at 20 mA for 14 h. After the gels were stained with ethidium bromide and examined under UV light, they were dried and autoradiographed on Kodak X-Omat AR film for about 24 to 48 h.

**TABLE 1. Relationships among human rotavirus genes coding for VP7 as determined by hybridization of genomic dsRNAs to reassortant rotavirus probes under stringent conditions**

<table>
<thead>
<tr>
<th>Strain (serotype)a from which dsRNAs derived</th>
<th>VP7 hybrid band detected with indicated probe (serotype)b</th>
<th>Serotype by hybridization assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>D × UK (1 VP7)</td>
<td>DS-1 × UK (2 VP7)</td>
<td>P × UK (3 VP7)</td>
</tr>
<tr>
<td>D (1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wa (1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mo (1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M37 (1f)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DS-1 (2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1076 (2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HN144 (2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HN90II (2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P (3)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M (3)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>McN (3)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ST3 (4)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VA70 (4)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hosokawa (4)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Homology between VP7 genes was seen only when the rotaviruses belonged to the same serotype.
b Determined by neutralization assay.

**RESULTS**

Cross hybridization between tissue culture-adapted human rotavirus strains (serotypes 1 through 4) and human-bovine reassortant rotaviruses. Genomic dsRNAs of human rotavirus strains containing a VP7 with serotype 1, 2, 3, or 4 specificity were hybridized to 32P-labeled ssRNA probes derived from bovine rotavirus UK strain (serotype 6) and reassortants D × UK (47-1-1), DS-1 × UK (66-1-1), P × UK (22-1-1), and ST3 × UK (52-1-1) (Table 1). These reassortants contain only gene 8 or 9, which codes for VP7, from their human rotavirus parent, and by neutralization they exhibit serotype 1, 2, 3, or 4 specificity, respectively. The remaining 10 genes are derived from their bovine rotavirus parent, UK (23, 24). When hybridized to the D × UK (47-1-1) probe, serotype 1 human rotavirus strains (D, Wa, Mo, and M37) formed an intense hybrid band with a migration pattern very similar, if not identical, to gene 9 of the homologous hybridization (D × UK dsRNA hybridized to D × UK probe) (Fig. 1). This hybrid band was absent when these strains were hybridized to UK and the reassortants bearing the VP7 gene of serotype 2, 3, or 4, indicating the specificity of the reaction between the VP7 genes of serotype 1 human strains and the corresponding gene present in the serotype 1 reassortant. A faint band with a migration pattern similar to gene 6 was seen when the serotype 1 human rotaviruses were hybridized to the UK probe (Fig. 1) as well as to each of the reassortant probes, indicating partial homology between a human rotavirus gene and a bovine rotavirus gene other than VP7 (Fig. 1).
with the reassortant probe containing a VP7 gene of the same serotype. In most cases, the VP7-specific hybrid bands exhibited a migration pattern similar, if not identical, to gene 8 or 9 of the homologous hybridization (as shown for serotype 3 human rotaviruses in Fig. 2). The exceptions were strains 1076 (serotype 2) and VA70 (serotype 4). In both these instances, the VP7-specific hybrid band migrated between genes 6 and 7 of the homologous hybridization. Additional hybrid bands seen in the hybridizations between the different human strains and the reassortants sharing the same VP7 serotype appeared to represent partial homology between human rotavirus and bovine rotavirus genes other than the VP7 gene, since corresponding hybrids were seen in the hybridizations involving human rotaviruses and the UK probe.

Cross hybridization between viral RNAs derived from rotavirus-positive stools of children hospitalized with diarrhea and labeled reassortant rotavirus probes (serotypes 1 through 4). The genomic dsRNAs derived from the stools of 15 children hospitalized in Venezuela for rotavirus diarrhea were hybridized to the VP7 serotype-specific probes derived from the reassortant rotaviruses D × UK (47-1-1), DS-1 × UK (66-1-1), P × UK (22-1-1), and ST3 × UK (52-1-1) as described above. In each case, the genomic dsRNAs of a given stool specimen formed a hybrid with the VP7 gene of only one of the four reassortant rotavirus probes. Additional bands were seen in several specimens, but these could be attributed to partial homology with the UK genes present in the reassortant probes, since corresponding hybrids were seen following hybridization with each of the four reassortant probes (Fig. 3). All five RNA specimens with short RNA patterns hybridized to the VP7 gene of DS-1 (serotype 2). Rotaviruses from two of these five specimens were adapted previously to tissue culture and identified as serotype 2 by neutralization assay (35). Of the 10 specimens with long RNA patterns, 2 hybridized only to the VP7 gene of D (serotype 1), 6 hybridized only to the VP7 gene of P (serotype 3), and 2 hybridized only to the VP7 gene of ST3 (serotype 4) (Table 2). The stool specimens from 14 of the 15 children were also tested by ELISA with VP7 serotype-specific monoclonal antibodies directed at human rotavirus serotype 1, 2, 3, or 4 (31a). Ten specimens were successfully serotyped by using this method, and the serotype was identical to that predicted by the hybridization assay. Two specimens (HN152 and HN159) that could not be serotyped by ELISA were subgroup I viruses with a short RNA pattern, and therefore they were most likely serotype 2, as predicted by the hybridization assay. The two remaining specimens (HN116 and HN171) that could not be serotyped by ELISA were both subgroup II viruses with a long RNA pattern. One of the specimens (HN144) was not tested in the serotyping ELISA but had been previously typed by neutralization assay (35).

FIG. 2. Hybridization of serotype 3 human rotavirus strains to serotype 3 human-bovine rotavirus reassortant P × UK (22-1-1) and to bovine rotavirus strain UK. Lane 1 shows the homologous hybridization between the genomic dsRNAs and the 32P-labeled mRNA probe derived from the reassortant P × UK (22-1-1). This reassortant contains gene 8 or 9 of P, which codes for VP7, but its remaining 10 genes are derived from UK (23). In lanes 2 through 4, serotype 3 human rotavirus strains P, M, and McN are hybridized to the serotype 3 reassortant probe, yielding a hybrid band which is similar if not identical in its migration pattern to gene 8 or 9 of the homologous hybridization. No corresponding bands are seen in the hybridization of these serotype 3 strains to the UK probe (lanes 5 through 7), indicating that these hybrids represent homology between the VP7 genes of P and other serotype 3 human rotavirus strains. Additional hybrid bands (with migration patterns between RNA segments 5 and 6) seen in the hybridization between these serotype 3 human rotavirus strains and the P × UK probe could be attributed to homology between the human rotavirus and bovine rotavirus genes, since corresponding hybrids were seen in the human rotavirus-UK control hybridizations.

FIG. 1. Hybridization of serotype 1 human rotavirus strains to serotype 1 human-bovine rotavirus reassortant D × UK (47-1-1) and to bovine rotavirus strain UK. Lane 1 shows the homologous hybridization between the genomic dsRNAs and the 32P-labeled mRNA probe derived from the reassortant D × UK (47-1-1). This reassortant contains gene 9 of D, which codes for VP7, but its remaining 10 genes are derived from UK (23). In lanes 2 through 5, serotype 1 human rotavirus strains D, Wa, Mo, and M37 are hybridized to the serotype 1 reassortant probe, yielding a hybrid band which is similar if not identical in its migration pattern to gene 9 of the homologous hybridization. No corresponding bands are seen in the hybridization of these serotype 1 strains to the UK probe (lanes 6 through 9), indicating that these hybrids represent homology between the VP7 genes of D and other serotype 1 human rotavirus strains.

DISCUSSION

Serotype identification of human rotaviruses is needed for any comprehensive study of the epidemiology of infection and diarrheal disease caused by these viruses. It is also needed for evaluating the efficacy of different rotavirus vaccine candidates, especially with regard to the question of heterotypic immunity (19). Identification of serotype has
been accomplished by various neutralization assays, including neutralization of immunofluorescent foci, plaque reduction neutralization, and tissue culture tube neutralization, each of which requires the adaptation of human rotaviruses to growth in tissue culture. Because human rotaviruses are fastidious in their requirements for growth in culture, adaptation of naturally occurring strains to tissue culture is often time-consuming and frequently unsuccessful. As a result, other approaches to serotype identification have been explored, such as solid-phase immune electron microscopy using absorbed hyperimmune rabbit sera to reference serotypes of human rotaviruses and ELISA using either hyperimmune sera or monoclonal antibodies (8, 31, 32). Serotype 1 or 3 tissue culture-adapted rotavirus strains were successfully identified by using the latter technique (31). More recently, an ELISA has been developed that allows typing of human strains belonging to serotypes 1 through 4. This assay has successfully typed tissue culture-adapted strains and has also allowed serotype identification of approximately 70% of rotavirus-positive stool specimens collected from infants and children with diarrheal illness (31a).

In the present study, the genetic relatedness among the genes coding for the major neutralization protein, VP7, of different human rotaviruses was examined by RNA-RNA hybridization under stringent conditions. Bovine-human rotavirus reassortants, which derived only the gene coding for VP7 from their human rotavirus parent (D, DS-1, P, or ST3, which represent human serotype 1, 2, 3, or 4, respectively) and the remaining genes from their bovine rotavirus parent (UK), were used as probes. Although there was some cross hybridization between portions of the bovine UK genome and the human rotavirus genome other than the VP7 gene, especially in the case of serotype 2 human rotavirus strains, these reassortant rotavirus probes provided a relatively easy means for detecting significant homology between the VP7 gene of a given human rotavirus strain and that of the corresponding gene present in one of the four serotype-specific reassortant probes. Hybrids that formed as a result of partial homology between bovine and human rotavirus genes other than the VP7 gene could be distinguished from hybrids that formed as a result of human rotavirus gene VP7 homology, because the former were observed during hybridization with each of the four reassortant probes (as well as with the UK probe), whereas the latter were observed during hybridization with only one of the four reassortant probes. With this approach, it was shown that human rotaviruses belonging to each of the four recognized serotypes exhibited VP7 homology only with the reassortant probe that contained a VP7 gene of the same serotype. Thus, there was a strict correlation between the serotype determined by neutralization in tissue culture and that identified by RNA-RNA hybridization under stringent conditions.

Genomic RNAs extracted directly from stool specimens of children with rotavirus diarrhea were also examined by the hybridization method, and in each instance, these RNAs formed a hybrid specifically with the VP7 gene of only one of the four reassortant probes, indicating that they probably belonged to that particular serotype. The serotypes of 11 of these 15 specimens were successfully determined by tissue culture neutralization assay (2 specimens) or serotyping
ELISA (10 specimens) or both, and the results were in agreement with the serotype determined by hybridization. Two of the four specimens that could not be typed by ELISA belonged to subgroup I and had short RNA electropherotype patterns, making it highly likely that these were serotype 2 strains (2, 15, 16, 21, 34), as determined by the hybridization assay. Thus, it appears that this hybridization assay provides a sensitive and specific method of serotyping human rotaviruses in tissue culture or in stool specimens. Each of 14 well-characterized tissue culture-adapted rotavirus strains (serotypes 1 to 4) isolated from symptomatic or asymptomatic neonates, infants, and children from different geographic areas (United States, Venezuela, Sweden, Italy, United Kingdom, Australia, and Japan) was correctly serotyped by hybridization (Table 1). In addition, the 11 stool specimens from infants and children with rotavirus diarrhea which had been serotyped by tissue culture neutralization or ELISA or both were correctly typed by hybridization.

Preliminary results suggest that serotype determination of rotavirus strains from stool specimens by the hybridization assay may be more sensitive than ELISA, in that 15 of 15 specimens were typed by hybridization and only 10 of 14 of these specimens were typed by ELISA. However, serotype determination by ELISA can be easily applied in the field setting, whereas the hybridization assay is limited to research laboratories, is much more labor intensive, and requires a larger amount of stool. For example, the ELISA requires approximately 0.5 to 1 ml of a 10% stool suspension, whereas we routinely extracted 1 g of stool to obtain sufficient RNA for multiple hybridizations. This is consistent with other studies in which 1 g of stool (positive for rotavirus by electron microscopy or ELISA) from neonates (mostly asymptomatic) or infants and young children hospitalized with diarrhea yielded sufficient RNA in approximately 90% of the cases (1, 6, 7, 27, 29).

In summary, RNA-RNA hybridization under stringent conditions, using single human rotavirus VP7 gene substitution reassortants representing human serotypes 1 through 4 as probes, may provide an alternative method of serotyping field isolates. It circumvents the need for tissue culture adaptation, hyperimmune sera, or specific monoclonal antibodies and is able to identify viruses belonging to each of the four recognized serotypes. In addition, this method should help identify new serotypes, since viruses exhibiting a new VP7 antigen should fail to hybridize to any of the four reassortant rotavirus probes.

It should be noted, however, that the single gene substitution reassortants used in this study do not permit characterization of the other outer capsid protein, VP3, which also plays a role in determining serotype specificity (13, 25, 26).

LITERATURE CITED


