Genetic Reassortment between Two Human Rotaviruses Having Different Serotype and Subgroup Specificities

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SUMMARY

Two reassortant viruses were selected from a mixed infection of MA104 cells with human rotavirus strains Wa (serotype 1-subgroup II) and HN126 (serotype 2-subgroup I). Antigenic characterization and genotype analysis by polyacrylamide gel electrophoresis revealed that they were reassortants with novel antigenic compositions, i.e. serotype 1–subgroup I (C116) and serotype 2–subgroup II (C15). Furthermore one of them, C15, was considered to have a mosaic antigenicity defined by two serotype-specific antigens, namely the serotype 1-specific VP3 antigen and the serotype 2-specific VP7 antigen. Although this reassortant was shown to be a serotype 2 virus on the basis of its preferential reactivity in neutralization reactions with serotype 2 antiserum, unexpectedly the antiserum prepared against C15 equally neutralized both serotype 1 and serotype 2 strains.

INTRODUCTION

Rotavirus particles contain a genome of 11 segments of double-stranded RNA which can be separated into distinct bands by gel electrophoresis. The gel electrophoretic pattern of the segments (electropherotype) has been used for the characterization and identification of rotavirus isolates, since the genomic RNA of individual isolates always gives a reproducible migration pattern (Espejo et al., 1977, 1979; Lourenco et al., 1981). Using this technique, many investigators have shown the great variability in RNA electropherotype among field isolates of human rotavirus (HRV) (Espejo et al., 1980a, b; Rodger et al., 1981). It seems important at this time not only to understand rotavirus genetics but also in view of the epidemiology of rotavirus infections to elucidate the mechanism causing this genomic variability. In this regard, recent studies using animal and human rotaviruses have demonstrated that reassortment of genome segments readily occurs in cell cultures in vitro (Matsuno et al., 1980; Greenberg et al., 1981; Chenon et al., 1984). Evidence for reassortment in vivo was shown recently in mice which had been orally infected with two different simian rotavirus strains (Gombold & Ramig, 1986). These results, together with the detection in clinical specimens of HRV strains with electropherotypes containing extra RNA segments as compared with the usual 11 segments (Lourenco et al., 1981; Spencer et al., 1983; Rodriguez et al., 1983; Nicolas et al., 1984), seem to indicate that the genomic variability of rotaviruses in nature is due to reassortment of viral genes.

The antigens of group A human rotavirus are presently classified into four main categories, group-specific, subgroup-specific, serotype-specific and strain-specific antigens, although an additional antigen(s) shared by certain strains, irrespective of serotype or subgroup specificities, has also been suggested (Taniguchi et al., 1985; K. Taniguchi et al., unpublished results). The group-specific (or common) antigenicity is considered to be mainly associated with VP6 (42K protein), a major component of the inner capsid of the virus, which is encoded by RNA segment 6 (Greenberg et al., 1983a; Taniguchi et al., 1984). Regarding subgroup and serotype specificities, two different subgroups (I, II) and four serotypes (1 to 4)
have so far been reported. Gene coding assignments (Smith et al., 1980; McCrae & McCorquodale, 1982), analysis of reassortant viruses (Kalica et al., 1981a; Greenberg et al., 1983b) and studies with monoclonal antibodies (Greenberg et al., 1983a, c; Taniguchi et al., 1984) have shown that subgroup specificity is also associated with VP6, whereas serotype specificity is defined by VP7 (34K to 38K glycoprotein) on the outer capsid and encoded by RNA segment 8 or 9. Recent studies with monoclonal antibodies, however, revealed that in addition to VP6 and VP7 mentioned above, VP2 (90K protein) and VP3 (82K protein probably encoded by RNA segment 4) carry subgroup-specific and serotype-specific antigens, respectively (Greenberg et al., 1983c; Taniguchi et al., 1985, 1986). The association of the remaining antigenic specificities including strain specificity with viral polypeptides has not yet been fully investigated.

Regarding the relationship between RNA electropherotype and antigenic specificity, it has been reported that serotype 2 strains are distinguishable from strains of other serotypes on the basis of the 'short' or 'long' migration patterns of RNA segments 10 and 11 (the so-called short or long electropherotypes) (Kalica et al., 1978, 1981b; Flores et al., 1982). One of the curious facts from an epidemiological point of view regarding rotavirus infection in humans is that all HRVs isolated to date are either strains having the antigenic specificity of serotype 1,3 or 4-subgroup II (long electropherotype) or strains having the specificity of serotype 2–subgroup I (short electropherotype). Neither strains of serotype 1,3 or 4–subgroup I nor strains of serotype 2–subgroup II have been detected. In the present study, we first tried to obtain, by in vitro reassortment experiments, a human rotavirus having an antigenic composition undetected among field isolates, and then we further analysed the relationship between genome segments and both serotype and subgroup antigens.

METHODS

**Viruses.** Two cultivable HRV strains, HN126 (serotype 2–subgroup I) and Wa (serotype 1–subgroup II), kindly provided by R. G. Wyatt, NIH, Bethesda, Md., U.S.A. (Wyatt et al., 1983), were passaged several times in MA104 cells in the presence of trypsin before use (Urasawa et al., 1984).

**Preparation of serotype-specific antisera.** Antisera against serotype 1 and serotype 2 HRVs were prepared in weaning rabbits by two intravenous injections (with a 2 week interval) of purified Wa (serotype 1) and S2 (serotype 2) HRV strains, respectively (Urasawa et al., 1982), and employed for serotyping viruses. The neutralizing titres of both antisera against the homologous strains were 1:16384. The serotype 1 and 2 antisera were then made specific by incubating them at 4 °C for 48 h with concentrated $2 and Wa virus, respectively, followed by low-speed centrifugation. The cross-absorbed antisera were incorporated in an agar overlay to select reassortment clones efficiently (the neutralizing titres of these antisera after absorption were both 1:4096 for the homologous strains and <1:8 for the heterologous strains used for absorption). Rabbit antisera against the reassortment virus C15 were similarly prepared in weanling rabbits and used without absorption with any HRV strain.

**Mixed infections.** MA104 cell monolayers formed in plastic 24-well microtitration plates (Falcon) were mixedly infected with Wa and HN126 strains pretreated with acetylated trypsin (10 μg/ml, at 37 °C for 30 min) at a m.o.i. of 2 to 4 p.f.u./cell. After absorption for 1 h at 37 °C the cell monolayers were washed twice and to each well was added 1 ml Eagle’s MEM containing 1 μg/ml acetylated trypsin. After an incubation period of 48 h the virus yield from the dually infected culture was similarly treated with acetylated trypsin, diluted ten- to 100-fold, and plated on CV-1 cell monolayers in 5 cm Petri dishes. Then plaques were allowed to develop under a purified agar overlay medium (Urasawa et al., 1982) containing acetylated trypsin (3 μg/ml) and a 1:100 dilution of either serotype 1- or serotype 2-specific antiserum. Four to 5 days later, discrete plaques were picked after staining with neutral red, and were propagated once in CV-1 cell monolayers in 24-well plates.

**Subgrouping and serotyping.** Subgroup determination of reassortant viruses was carried out by an enzyme-linked immunosorbent assay (ELISA) using three monoclonal antibodies, subgroup I-specific S2-37 (directed at VP6), subgroup II-specific YO-5 (directed at VP6), and subgroup II-specific YO-60 (directed at VP2) (Taniguchi et al., 1984, 1986).

Serotype determinations of reassortant viruses were performed in a plaque neutralization test using serotype-specific rabbit antisera. The neutralizing titre of each serum was measured by the 60% plaque reduction method (Urasawa et al., 1984).

**Extraction of viral RNA and gel electrophoresis.** Genomic RNA was extracted from concentrated virus samples with phenol and precipitated with ethanol. Polyacrylamide gel electrophoresis (PAGE) of genomic RNA was performed on 10% (w/v) polyacrylamide slab gels as previously described (Taniguchi et al., 1982).
RESULTS

Screening of reassortants

MA104 cells were mixedly infected with the parental strains Wa (serotype 1–subgroup II) and HN126 (serotype 2–subgroup I). Serotype-specific antisera were incorporated in an agar overlay medium to select reassortant virus clones with the desired antigenic properties from the mixedly infected tissue culture fluids.

One-hundred and twenty-three plaques which developed under the selective pressure of serotype 2 antiserum were picked, propagated once in cell culture and examined for their subgroup specificity by ELISA. One progeny isolate (no. 116) of the 123 reacted with monoclonal antibody $2-37$ and was found to be in subgroup I, while the remaining isolates were all determined as subgroup II based on their reactivity with monoclonal antibody YO-5. In contrast, only one progeny isolate (no. 15) out of 152 plaques that developed in the presence of serotype 1 antiserum was determined as subgroup II and the remaining 151 were of subgroup I according to the same criteria. Since the plaque progenies, no. 116 and no. 15 (presumptive reassortant viruses), were possibly composed of a heterogeneous population of viral particles in terms of their genome composition, they were further cloned by three plaque-to-plaque passages.

Antigenic characterization of reassortant clones

The results of antigenic characterization of the two viral clones, C116 and C15, which were obtained after three plaque purifications are shown in Table 1. The antigenic characteristics of the parental strains are also shown as controls. Viral clone C116 reacted in ELISA with monoclonal antibody $S2-37$ but not with YO-5 or YO-60. This indicated that the specificities of the major subgroup antigen on VP6 and possibly that of the minor subgroup antigen on VP2 (because subgroup I and II antigens on VP2 are considered to be mutually exclusive) are both subgroup I-specific. In contrast, viral clone C15 reacted with monoclonal antibodies YO-5 and YO-60 but not with S2-37, confirming that the subgroup antigens on both VP6 and VP2 are subgroup II-specific. The serotypes of the two viral clones were examined in a plaque neutralization test using polyclonal antisera (Table 1). C116 and C15 were determined as serotype 1 and serotype 2, respectively, as had been expected from the specificity of the antisera initially used for selecting each viral clone. These results indicated that HRVs having unique antigenic compositions (i.e. serotype 1–subgroup I and serotype 2–subgroup II) not yet encountered in clinical specimens had been obtained under in vitro conditions, and that the HRVs thus obtained were indeed reassortants derived from the parental viruses, Wa and HN126.

Table 1. Antigenic characterization of two reassortant clones

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>In ELISA with monoclonal antibody</th>
<th>Subgroup</th>
<th>In neutralization test with monoclonal antibody</th>
<th>Serotype 1(Wa) antiserum</th>
<th>Serotype 2(S2) antiserum*</th>
<th>Ascribed subgroup</th>
<th>Ascribed serotype</th>
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</tr>
<tr>
<td>C116</td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>8192‡</td>
<td>&lt;512</td>
<td>I</td>
<td>1</td>
</tr>
<tr>
<td>C15</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>512</td>
<td>4096</td>
<td>II</td>
<td>2</td>
</tr>
<tr>
<td>Wa</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>16384</td>
<td>512</td>
<td>II</td>
<td>1</td>
</tr>
<tr>
<td>HN126</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>512</td>
<td>8192</td>
<td>I</td>
<td>2</td>
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* The neutralizing titre of this antiserum against the homologous S2 strain was 1:16384.
† Either S2-37, YO-5 or YO-60 monoclonal antibody adhering to the internal walls of microplate wells was reacted in turn with the following reagents: virus sample to be tested, rabbit anti-HRV serum, alkaline phosphatase-conjugated goat antibody to rabbit IgG and p-nitrophenylphosphate. When the sum of $A_{400} \times 1000$ in two wells exceeded 300, the reaction was considered positive (Taniguchi et al., 1984).
‡ 60% plaque reduction titre.
S. URAKAWA, T. URAKAWA AND K. TANIGUCHI

Fig. 1. Comparison of the genome RNAs from the parental strains Wa and HN126. Three pairs of co-migrating RNA segments of different parental origin are indicated by arrows.

Genotype analysis of reassortant viruses

The RNA gel electrophoretic patterns of the parental viruses and the reassortant viruses are shown in Fig. 1 and 2. The 11 RNA segments derived from each of the two parental viruses were clearly distinguishable by PAGE except for three segment pairs, i.e. the 3rd segment of HN126 and the same segment of Wa, the 9th segment of HN126 and 8th segment of Wa, and the 11th segment of HN126 and 10th segment of Wa, which co-migrated electrophoretically (Fig. 1). Co-electrophoresis of the RNAs from each of the reassortants with the RNAs of the parental viruses enabled us to identify the parental origin of the reassorted RNA segments except for the three co-migrating pairs of RNA segments mentioned above (Fig. 2, Table 2).

The results obtained from the genotype analysis of the reassortants could explain the antigenic character of the reassortants described above. Segment 6 of C116 coding for the major subgrouping protein VP6 was derived from the HN126 parent, which corresponded with the observation that the virus belonged to subgroup I. While RNA segment 9 of Wa has been proved to code for the major serotype-specific protein VP7 (Richardson et al., 1984; Gunn et al., 1985), accumulated results (Kalica et al., 1981a, 1983; Greenberg et al., 1983b, c; Hoshino et al., 1985b) strongly suggest that RNA segment 4 of Wa codes for the other serotype-specific protein VP3. The reassortant that contained segments 4 and 9 from the Wa parent in fact showed the specificity of serotype 1, confirming the previously known gene coding assignments for serotype-specific neutralizing proteins.

In contrast, the reassortant C15 which received segment 6 coding for the subgrouping protein VP6 from the Wa parent was shown to be subgroup II as expected. Regarding serotype, this reassortant, while receiving segment 4 possibly coding for the minor neutralizing protein VP3 from the Wa parent, did not contain segment 9 of Wa, which codes for the major serotype-specific antigen VP7. Consequently, the VP7 of this reassortant was considered to be encoded by the genome segment (either the 8th or the 9th segment) derived from the HN126 parent.
Genetic reassortment between two HRVs

(a) Wa + C116 + HN126 + C15 + Wa
   C116 HN126 C15 C15

(b) Wa + C116 + HN126 + C15 + Wa
   C116 HN126 C15 C15

Fig. 2. PAGE profiles of virion RNAs from the parental strains Wa and HN126, and reassortants C116 and C15. (a) RNAs from reassortants co-electrophoresed with Wa or HN126 as indicated. (b) Schematic drawing of the same PAGE profiles. The derivations of particular RNA segments in the reassortants are shown by arrows.
Table 2. Parental origin of gene segments in reassortants as determined by PAGE

<table>
<thead>
<tr>
<th>Reassortant virus</th>
<th>RNA segment</th>
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<tbody>
<tr>
<td>C116</td>
<td>Wa₁ (Wa₅ or HN126₉) Wa₄ Wa₅ HN126₆ Wa₇ (Wa₉ or HN126₁₀) Wa₉ (Wa₁₀ or HN126₁₁) Wa₁₁</td>
</tr>
<tr>
<td>C15</td>
<td>Wa₁ Wa₂ (Wa₃ or HN126₃) Wa₄ Wa₅ Wa₆ Wa₇ HN126₈ (Wa₈ or HN126₀) Wa₈ (Wa₁₀ or HN126₁₁) Wa₁₁</td>
</tr>
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</table>

* Waₓ indicates segment no. x was derived from the Wa parent and similarly for HN126. Parentheses indicate that the origin of the segment remains to be determined.

Table 3. Neutralization of parental rotavirus strains, serotype 1 Wa and serotype 2 HN126 by antiserum against reassortant virus, C15

<table>
<thead>
<tr>
<th>Virus tested</th>
<th>Neutralization titre of antiserum against C15</th>
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<tbody>
<tr>
<td>Wa</td>
<td>4096</td>
</tr>
<tr>
<td>HN126</td>
<td>4096</td>
</tr>
<tr>
<td>C15</td>
<td>8192</td>
</tr>
</tbody>
</table>

Thus, reassortant C15 is thought to be a serotypic mosaic strain in terms of its genomic constitution although it was determined as phenotypically serotype 2 (Table 1) possibly because of the presence of the major neutralizing protein VP7 of HN126 origin.

**Immunogenicity of reassortant C15**

The finding that reassortant C15 was an antigenic mosaic strain in terms of serotype specificity raised the interesting question as to whether antiserum against C15 would neutralize one or both of the parental strains. Hyperimmune antiserum was prepared in rabbits by multiple injections of the reassortant virus, and its neutralizing activity was examined against the parental strains. The antiserum against the reassortant virus strongly neutralized both the parental strains as well as reassortant C15 (Table 3).

**DISCUSSION**

Reassortment of viral genome segments has been reported to occur during co-infection of cultured cells with two rotaviruses isolated from different animal species (Matsuno et al., 1980; Greenberg et al., 1981) and with two human rotaviruses (Chenon et al., 1984). In the latter study some of the reassorted viruses exhibited mixed RNA patterns containing extra genomic segments and these supplementary segments segregated after successive passages in cell cultures. Chenon et al. (1984) surmised that the mixed RNA patterns are an intermediate step in the establishment of stable reassorted viruses. Recently, conclusive evidence for genetic reassortment in vivo was presented by Gombold & Ramig (1986) in mice infected orally with a mixture of simian rotavirus SA 11 and rhesus rotavirus RRV. They reported that reassortment of genome segments was observed in 252 of 662 (38%) clones analysed and that a proportion of clones yielded mixed genotypes containing more than 11 segments. Previous reports (Espejo et al., 1980a; Rodger et al., 1981; Follett & Desselberger, 1983; Chiba et al., 1984) have described the variability in RNA electropherotype among field isolates of HRV prevailing at a given time in a given population. Under such circumstances as in large, densely populated cities where a number of HRV strains with different electropherotypes are circulating simultaneously, the occurrence of sequential or simultaneous infections in individuals by more than one HRV strain seems probable. Such conditions may lead to reassortment between different strains, producing many reassortant strains with unique electropherotypes. The results reported by several investigators indicating that 10% or more of HRV isolates examined were ones with electropherotypes containing extra RNA segments (Lourenco et al., 1981; Spencer et al., 1983;
Genetic reassortment between two HRVs

Rodriguez et al., 1983; Nicolas et al., 1984) are also compatible with this view. Epidemiological evidence that genetic reassortment of genomic RNAs accounts for an antigenic shift in rotaviruses is also presented by Street et al. (1982).

In the present study, we found that rotaviruses not encountered to date, having the antigenic specificities of serotype 1–subgroup I and serotype 2–subgroup II, occurred in an in vitro reassortment experiment. One of the reassortants, C15, having the latter specificity showed a long pattern of RNA segments 10 and 11 (long electropherotype), which is contrary to the previously accumulated data showing that all HRVs with short RNA electropherotypes belong to serotype 2. These results led us to assume that such strains having novel antigenic constitutions and electrophoretic patterns will be found in clinical specimens in the future. The genotype analysis of the reassortants in the present study showed that most of their genomic segments were derived from the Wa parent; the reassortant C116 possessed at least seven Wa segments, at least one HN126 segment and three segments of undetermined origin; in the reassortant C15 at least seven genes were from Wa, at least one from HN126 and the remaining three were of undetermined origin. The reason for, and the significance of, these results, however, remains to be determined, although the observed tendency of the parental Wa strain to grow better than the HN126 strain in the cell culture used might be an influence.

The gene coding assignment for the minor subgroup-specific VP2 protein seems worthy of mention. Initially, we surmised that in HRV, as in other animal rotaviruses (Mason et al., 1983; Dyall-Smith & Holmes, 1981; McCrae & Faulkner-Valle, 1981), RNA segment 2 codes for VP2. The results obtained in the present study, however, seem incompatible with this view. Although segment 2 of both reassortants was derived from the Wa parent (Fig. 2 and Table 2), the reactivity of these reassortants with the VP2-directed monoclonal antibody YO-60 was different (Table 1): C15 reacted with YO-60 whereas C116 did not. This result seems to indicate that the genome segment encoding VP2 is segment 3 rather than segment 2 as far as the two HRV strains used in this study are concerned. This point, however, could not be conclusively proven in the present study because the two segment 3s of the parental strains were indistinguishable by gel electrophoresis. Thus, the gene coding assignment for HRV VP2 requires further study.

The antigenic and genetic properties of reassortant C15 deserve special mention. Genotype analysis strongly suggested that it was a mosaic virus having genome segments encoding the two serotype-specific proteins: it possessed serotype 1-specific VP3 protein and serotype 2-specific VP7 protein. On the other hand, cross-neutralization tests revealed that while the reassortant was defined as serotype 2 by its reactivity with serotype 1 and serotype 2 antisera (Table 1), antiserum against the C15 virus strongly neutralized not only serotype 2 but also serotype 1 viruses. A similar asymmetric neutralization was observed with a field isolate of rotavirus, M37, having dual neutralization specificities: serotype 4 by its VP3 neutralization specificity and serotype 1 by its VP7 neutralization specificity (Hoshino et al., 1985a, b).

Although the genetic reassortments described in this study occurred under experimental conditions, the results obtained seem useful for understanding the mechanism generating antigenic diversity in rotavirus isolates. The results also suggest that the distinction between rotavirus serotypes is not so clear-cut as was suggested by initial studies (Beards et al., 1980; Urasawa et al., 1982; Wyatt et al., 1983). In addition, reassorted antigenic mosaic viruses seem worthy of further investigation as promising candidates for vaccine production, since they may be able to confer a broader immunity.

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REFERENCES


Genetic reassortment between two HRVs


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