Short communication

Multiple-gene rotavirus reassortants responsible for an outbreak of gastroenteritis in central and northern Australia

Enzo A. Palombo,* Helen C. Bugg, Paul J. Masendycz, Barbara S. Coulson, Graeme L. Barnes and Ruth F. Bishop

Department of Gastroenterology, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia

Two rotavirus strains, E210 and E212, implicated in epidemics of gastroenteritis in children in central and northern Australia during 1993–1994, exhibited the unusual combination of a ‘short’ RNA electrophoretic pattern and subgroup II specificity. The outer capsid protein VP7 was found by PCR typing and sequence analysis to be related to that of serotype G2 viruses. Both strains displayed a novel pattern of reactivity to G2-specific monoclonal antibodies that correlated with sequence variation in the antigenic regions of VP7. The VP4 serotype of E210 and E212 was determined as P1B in an enzyme immunoassay, consistent with other G2 viruses. Analysis of the VP6 gene indicated significant identity (98–99%) with other human subgroup II viruses. Northern hybridization analysis of E210 RNA using total genome probes derived from the prototype strains RV4 and RV5 indicated that E210 was derived from multiple gene reassortment between rotaviruses belonging to different genetic types.

Group A rotaviruses are the single major cause of severe gastroenteritis in infants and young children world-wide (Bishop, 1994) and the development of a safe and effective vaccine is a priority. Effective vaccine strategies require an understanding of the epidemiology of rotavirus infection and the degree of genetic and antigenic variation in strains causing disease in humans. The increasing reports of naturally occurring human rotaviruses derived from inter- and intraspecies genetic reassortment (Ward et al., 1990; Nakagomi & Nakagomi, 1991; Bishop, 1994; Kaga & Nakagomi, 1994; Palombo & Bishop, 1995) indicate that the incidence of such strains may be greater than previously appreciated and possibly provide a challenge to a rotavirus vaccine.

Rotavirus classification is based on group and subgroup (SG) determinants located on the inner capsid protein, VP6, while the outer capsid neutralization antigens VP4 and VP7 determine the P and G serotypes respectively (Estes & Cohen, 1989). The rotavirus genome consists of 11 segments of double-stranded RNA (Estes & Cohen, 1989) and the electrophoretic mobility of these determines the electropherotype of a strain. Epidemiological surveys of human Group A rotaviruses indicate particular associations between subgroups, G-types, P-types and electropherotypes (Gouvea & Brantly, 1995). Strains belonging to G serotypes 1, 2, 3 and 4 are the predominant types causing symptomatic infection in humans and, in general, G1, G3 and G4 strains are associated with VP4 serotype P1A (genotype P8), SGII specificity and a ‘long’ electropherotype, while G2 strains usually exhibit ‘short’ electrophoretic patterns and P1B (P4), SG1 specificity (Bishop, 1994; Gouvea & Brantly, 1995). The G2 and non-G2 strains also define the two major ‘genogroups’ of human rotaviruses, represented by the prototype strains DS1 and Wa respectively (Nakagomi & Nakagomi, 1993). A third ‘genogroup’ consists of the AU1-like viruses (Nakagomi & Nakagomi, 1993). The genomes of the prototypes do not exhibit significant cross-hybridization and are considered to be genetically distinct. To avoid confusion with the rotavirus ‘Group’ classification (Groups A–E), we have used the term ‘genotype’ rather than ‘genogroup’ in this paper when referring to the different genetic types.

Reassortment of gene segments is believed to be responsible for the rapid evolution of rotaviruses. Random reassortment between rotaviruses of different
we describe the molecular and antigenic characteristics munities in which they have been detected. In this report, these strains appear to have been derived from multiple wide-spread epidemics of severe diarrhoea in central and northern Australia between April and June 1994. Patients admitted to Royal Darwin Hospital in northern Australia with acute rotavirus gastroenteritis. Rotavirus-positive faecal samples, identified by latex agglutination, were collected within 48 h of hospitalization from more than 100 young children during 1993–1994. These strains appear to have been derived from multiple gene reassortment between wild-type viruses of the DS1 and Wa human genotypes.

Between December 1993 and August 1994, approximately 100 children were admitted to Alice Springs Hospital in central Australia with acute rotavirus gastroenteritis. Rotavirus-positive faecal samples, identified by latex agglutination, were collected within 48 h of admission to hospital, stored at −20 °C or −70 °C and transported to Melbourne where they were stored at −70 °C as 10% (w/v) homogenates.

Routine serotyping (G-typing) and subgroup determination by enzyme immunoassay (EIA) of all samples were carried out as previously described (Coulson et al., 1987) using G-type-specific neutralizing monoclonal antibodies (NMAbs) and subgroup-specific MAbs (Greenberg et al., 1983). Electropherotypes were determined by electrophoresis of viral RNA, isolated from faecal material by phenol–chloroform extraction, in 10% (w/v) polyacrylamide gels and staining with silver nitrate (Dyall-Smith & Holmes, 1984).

This analysis indicated that 90 specimens exhibited the unusual combination of a short electropherotype and SGII specificity; however, none were reactive with NMAbs specific for serotypes G1–4. Co-electrophoresis of RNA indicated the presence of two major electropherotypes, distinguished by characteristic mobility differences in segments 7, 8 and 9, designated E210 and E212. Strain E210 was also detected in 43 patients admitted to Royal Darwin Hospital in northern Australia between April and June 1994.

RNA from E210 and E212 was purified and used in the nested PCR G-typing method of Gouvea et al. (1990) with primers specific for G-types 1–4. Both E210 and E212 yielded cDNA products of 652 bp, as expected for serotype G2 viruses, indicating a VP7 gene that was G2-like. As E210 and E212 were untypeable in the standard EIA for G-typing, they were reacted with a panel of G2-specific NMAbs, namely RV5:1, RV5:3, RV5:4 and N3/B7 (Coulson et al., 1986, 1987, 1995), which included the NMAb (RV5:3) used in the original G-typing assay. Both E210 and E212 reacted only with NMAb RV5:1 (Table 1), albeit at a low level. In contrast, control G2 faecal extract and the standard G2 strain RV5 reacted to a significant level with all four and three of the four G2-specific NMAbs, respectively (Table 1).

Full-length VP7 cDNA (1062 bp) of E210 and E212 was generated by RT–PCR using VP7 gene end-primers (Gouvea et al., 1990) and cloned into plasmid pGEM-3Zf (Promega). The nucleotide sequences [GenBank accession numbers U36241 (E210 VP7) and U36242 (E212 VP7)] were determined using the fmol DNA sequencing system (Promega) and gene-specific primers. The VP7 genes of E210 and E212 differed by two nucleotides (99.8% identity), only one of which introduced a coding change (see below).

The deduced amino acid sequences of VP7 of both strains are shown in Fig. 1. The proteins were 326 amino acids in length and showed significant identity to the sequences of VP7 proteins from standard G2 viruses. The VP7 of E210 exhibited 99.7% identity to E212, with only a single amino acid difference (Gln → Glu at residue 104). E210 and E212 showed 94–97% and 94–96% identities to the VP7 of G2 viruses RV5, S2 and DS1, respectively, while these proteins were only 71–74% identical to the VP7 proteins of standard G1, G3 and G4 strains. Differences were observed in the antigenic regions A, C and F of VP7 which may explain the unusual pattern of reactivity of E210 and E212 with G2-specific NMAbs.

Recently, two monotypes (a and b) were defined within G2 rotaviruses based on very low levels of reactivity of NMAbs RV5:3 and RV5:4 with 1076 virus (G2b) compared with RV5, DS1 and S2 (G2a) strains.

### Table 1. Serotype G2 reactivity of G2 rotavirus-containing faecal extracts by serotyping EIA

<table>
<thead>
<tr>
<th>Virus</th>
<th>RV5:1</th>
<th>RV5:3</th>
<th>RV5:4</th>
<th>N3/B7</th>
</tr>
</thead>
<tbody>
<tr>
<td>E210</td>
<td>2.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>E212</td>
<td>2.1</td>
<td>1.2</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>G2 control†</td>
<td>4.8</td>
<td>24.4</td>
<td>3.6</td>
<td>19.9</td>
</tr>
<tr>
<td>RV5‡</td>
<td>6.7</td>
<td>32.0</td>
<td>Not done</td>
<td>28.0</td>
</tr>
</tbody>
</table>

* A faecal sample was considered positive if its A450 with a given NMAb (P) was at least twice the A450 of a pool of faecal extracts negative for rotavirus by EIA (Coulson et al., 1987) reacted with the same coating and detecting antibodies (N), i.e. a P/N value of ≥ 2.0. Values are the average of duplicate experiments.
† Control G2 rotavirus positive faecal extract, prepared by pooling five faecal extracts containing G2 rotaviruses of standard electropherotype, subgroup, P-serotype and G-serotype.
‡ NMAb P/N values from Coulson et al. (1987).
(Coulson et al., 1995). It is likely that E210 and E212 represent a third monotype (2c) as they were not detected by NMAbs RV5:3, RV5:4 and N3/B7 and were recognized by RV5:1 at a low level.

The VP7 gene of a 'normal' G2 strain (with respect to subgroup and RNA pattern) designated E201 isolated in Perth, Western Australia, prior to the central Australian outbreak showed significant homology to that of E210 and E212 (99.5–99.7% nucleotide sequence identity; unpublished data). Hence, it is possible that the VP7 genes of E210 and E212 may be derived from rotaviruses circulating in the Western Australian community. However, both strains also show significant homology to the VP7 gene (98–98.2% identity) of the unusual G2 strain M48 isolated in India in 1987/88 (Krishnan et al., 1994). Therefore, the origin of these genes remains uncertain.

The VP4 serotype of E210 and E212 was determined by EIA using NMAbs specific for the common human P-serotypes P1A (NMAb F45:4), P1B (RV5:2) and P2 (ST3:3) (Coulson, 1993). E210 and E212 exhibited P1B
specificity as is usual for G2 viruses. In addition, gene 4 of E210 (encoding VP4) exhibited homology to RV5 as determined from hybridization analysis (see below). However, the VP4 genes of E210 and E212 have been recalcitrant to in vitro amplification so that these genes cannot be characterized at present.

VP6 gene cDNA (1356 bp) of E210 and E212 was generated by RT-PCR using gene 6 end-primers (Palombo & Bishop, 1994), cloned and sequenced (GenBank accession number U36240) and the deduced amino acid sequence of VP6 determined. Both strains carried identical VP6 genes and encoded proteins that were 397 amino acids in length. These exhibited significant identity (98–99%) with the VP6 of standard human SGII strains, RV3 (Palombo & Bishop, 1994) and Wa (Gorziglia et al., 1988), and less similarity (93% identity) to the human SGI strain 1076 (Gorziglia et al., 1988). The residues believed to be part of the SGI- and SGII-specific determinants on VP6 (aa 176, 305 and 306; Lopez et al., 1994) were conserved between all the SGII viruses.

The genetic and antigenic characterization of E210 and E212 suggested that these strains were the product of in vivo genetic reassortment. To investigate the origins of all gene segments, Northern hybridization analysis of E210 was carried out using whole genome probes derived from strains RV4 (G1P1A, SGII; Wa genotype) and RV5 (G2P1B, SGI; DS1 genotype) grown in MA104 cells. RV4 and RV5 probes were generated by labelling cDNA, derived by reverse transcription of gel-purified RNA segments, with digoxigenin (DIG)-11-dUTP (Boehringer Mannheim).

Northern hybridization was carried out at 50 °C using 10 ng/ml of probe, as previously described (Palombo & Bishop, 1995). RNA from virus or faecal sample was electrophoresed in a 10% (w/v) polyacrylamide gel, stained with ethidium bromide (middle panel), denatured and transferred to a nylon membrane (Boehringer Mannheim). Probe was detected using anti-DIG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and the chemiluminescent substrate CDP-Star (Boehringer Mannheim). Probe was removed prior to re-probing by incubation of the membrane at 70 °C in 50% formamide, 0.1 x SSC, 0.1% SDS for 60 min.

Fig. 2 shows the result of hybridization with RNA from RV5, RV4 and E210. Both probes reacted with all genes of the homologous virus but not significantly with the virus of the non-homologous genotype. Gene segments 2, 3, 4 (encoding VP4), 5 and 9 (encoding VP7) of E210 reacted with the RV5 probe, while the RV4 probe reacted with segments 6 (encoding VP6), 7 and 8. Genes 10 and 11 showed significant homology to both RV4 and RV5 probes while gene 1 did not react with either. In addition, E210 gene 1 did not exhibit homology to a probe derived from AU1 gene 1 suggesting that this gene originated from a non-human source or that strain E210 possesses a novel human gene 1. Nucleotide sequence analysis of this gene may help to determine its origins.

According to the hybridization conditions used (allowing hybridization with up to 20% sequence mismatch), genes 10 and 11 of the prototype strains RV4 and RV5 do not share significant sequence homology. However, our results suggest that genes 10 and 11 in strains currently circulating in Australia may have evolved to an extent that they show similarity to both prototypes, which were isolated in 1977 and 1981, respectively.

The increasing number of reports of rotavirus strains that are the result of interspecies or intergenotype reassortment indicates that reassortment is a mechanism by which rotaviruses rapidly evolve. However, strains exhibiting the unusual subgroup and electrophoretic pattern combination found in E210 and E212 are rare and to date only a single such isolate has been described in a large epidemiological survey in South Africa (Steele & Alexander, 1988), whereas strains E210 and E212 were responsible for major outbreaks and were recovered from the majority of hospitalized children during a 9 month period. These strains demonstrate that complex intergenotype reassortment events are possible in vivo.
and can result in strains with novel genetic and antigenic properties. Furthermore, such strains have the ability to spread widely and cause epidemics in young children. Further characterization of such viruses is important for the development of effective rotavirus vaccination strategies.

We thank Fran Morey and Rex Matters, Alice Springs Hospital, Kay Withnall and Andrew Loewe, Royal Darwin Hospital, and Roger Schnaagl, La Trobe University, for preparing and sending faecal specimens. This work was supported by grants from the Medical Research Council and the Public Health Research and Development Committee of the National Health and Medical Research Council of Australia and the Royal Children’s Hospital Research Foundation.

References


(Received 3 November 1995; Accepted 23 January 1996)