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Sequence Analysis Demonstrates that VP6, NSP1 and NSP4 Genes of Indian Neonatal Rotavirus Strain 116E are of Human Origin

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Abstract. We have sequenced the genes encoding the inner capsid protein VP6 and the nonstructural proteins NSP1 and NSP4 of the Indian neonatal serotype P8[11]G9 human/bovine reassortant candidate vaccine rotavirus strain 116E. These three genes share a high degree of sequence and deduced amino acid homology with human prototype strain Wa. Our results confirm and extend those of previous RNA-RNA hybridization studies which suggested that these genes are of human origin, and will facilitate examination of the host immune response to 116E induced by natural infection and vaccination.

Key words: rotavirus, neonate, 116E, nucleotide sequence, vaccine

Introduction

Group A rotaviruses are the leading cause of severe gastroenteritis in infants and young children worldwide, and the development of an effective vaccine is expected to significantly reduce this disease burden (1). Rotaviruses are unenveloped, double-stranded RNA viruses of the family Reoviridae that possess 11 genome segments enclosed within a triple-layered protein shell (2). Each gene segment encodes a discrete protein, including six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and five nonstructural proteins (NPS1 to NSP5). The outer capsid is composed of the surface glycoprotein VP7 (encoded by segment 7, 8 or 9, depending on the strain) and the spike protein VP4 (encoded by segment 4), which is the viral attachment protein. Both VP7 and VP4 independently induce neutralizing antibodies that are used to define rotavirus G (for glycoprotein) and P (for protease-sensitive) serotypes, respectively, and have been implicated in the development of a protective immune response (3).

The current rotavirus vaccines most advanced in their development are live, attenuated reassortant strains containing VP7 genes of the most common rotavirus G serotypes and, in one case, the most common P serotype, on a background of rhesus monkey (RRV) or bovine (WC3) rotavirus genes (''modified Jennerian approach''), and are targeted at infants (4). An alternative vaccination approach is based on studies of rotavirus infection of neonates which is asymptomatic and protects the infant from severe diarrhea on subsequent infection (5,6). These observations raised the possibility of using this natural phenomenon as a vaccine strategy through the administration of neonatal strains to newborns (7,8). One such neonatal strain is 116E, which we isolated from a newborn without diarrhea as part of a longitudinal study of rotavirus infections in New Delhi, India (6). Sequence analysis demonstrated that the VP7 protein of 116E is closely related to that of the human serotype G9 strains, while the VP4 protein is closely related to that of the serotype P8[11]G10 bovine strain B223 (9,10). Plaque-reduction neutralization studies subsequently showed that strain 116E did belong to serotype G9 and was neutralized weakly by polyclonal sera to B223, suggesting that 116E is P serotype 8 (9). Strain 116E may be a particularly

appropriate vaccine candidate for use in India, since serotype G9 is a common community strain circulating in some regions there (11). RNA-RNA hybridization analysis demonstrated 116E to be a reassortant between strains from human (Wa) and bovine (KK3-like, P8[11]G10) rotaviruses, possessing at least seven genes from the former and at least one gene, gene 4, from the latter (9). However, the origin of the 10 remaining segments could not be determined conclusively, and this was considered important if 116E was to be further evaluated as a candidate rotavirus vaccine.

In this study, we have analyzed the genes encoding VP6, NSP1 and NSP4 of 116E. These genes have been implicated in animal models in either protective immunity to rotavirus (VP6) (12), or rotavirus virulence (NSP1 and NSP4) (13,14). We wished to confirm the likely species of origin of these genes and to detect any sequence changes that may aid further understanding of the host immune response to this strain, and its altered virulence characteristics, that may be of relevance to future vaccine studies. Our results demonstrate that the VP6, NSP1 and NSP4 genes of 116E are highly homologous to strain Wa, confirming their human origin.

Materials and Methods

Virus strain 116E was propagated in MA104 cells as previously described (9). Genomic double-stranded RNA was extracted from 116E cell lysate with phenol-chloroform and precipitated with ethanol, followed by a glass powder procedure (15). Extracted RNA was used for reverse transcription-polymerase chain reaction (RT-PCR) amplification of the VP6, NSP1 and NSP4 genes. The oligonucleotide primers used for RT-PCR amplification were based on published sequences of strain Wa (VP6 (16), NSP1 (17), and NSP4 (18). The primers were complementary to regions at or near the 5′ and 3′ ends of the respective genes: for VP6, nucleotides 1–20 (5′) and 1336–56 (3′); NSP1, nucleotides 1–20 (5′) and 1537–52 (3′); and NSP4, nucleotides 1–19 (5′) and 721–38 (3′). For nucleic acid sequencing, the ethidium bromide-stained PCR products were extracted and purified from a 1.2% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, Maine), using the QIAquick Gel Extraction Kit (Qiagen Inc., Chatsworth, Calif.). DNA sequencing was performed by the dideoxynucleotide chain termination method, using the PRISM TM Ready Reaction DyeDeoxy TM Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The primers for PCR amplification were then used for cycle sequencing, using the obtained PCR product as the template. Based on the sequences obtained, further primers were then designed to enable at least the whole open reading frame (ORF) to be sequenced. The cycle sequencing products were purified on Centrisep columns (Princeton Separations, Adelphia, NJ) and sequenced using a model 377 automated DNA sequencer (Applied Biosystems, Inc.). A partial sequence of the 116E NSP4 gene has been reported previously (N.A. Cunliffe et al., in press).

Overlaps in sequence between the different products were analyzed with the Sequencher program (Gene Codes Corporation, Inc., Ann Arbor, Mich.). Comparison to other rotavirus sequences was performed using the University of Wisconsin Genetics Computer Group programs (19).

The VP6, NSP1 and NSP4 gene sequences have been assigned GenBank Accession numbers U78558, U85998 and U85999, respectively.

Results

The entire gene 6 was sequenced, excluding the 5′ and 3′ terminal primer sequences used to generate the PCR products. The fragment (data not shown) was 1315 nucleotides in length (nucleotides 21–1335) and contained a single long ORF extending from nucleotide 24 to 1214, giving a deduced protein (VP6) of 397 amino acids. The VP6 nucleotide and amino acid sequences of 116E were compared with those of various other human and animal rotavirus strains (Table 1) and showed greatest homology to strain E210, an unusual P[4]G2 reassortant rotavirus with a short RNA migration pattern (electropherotype) but subgroup II VP6 specificity, (20). sharing 91.6% nucleotide identity and 98.5% amino acid identity. Comparable homology was found with the VP6 genes of the subgroup II strains Wa (P1A[8]G1) and RV3 (P2A[6]G3). Alignment of the amino acid sequence with Wa identified a few scattered substitutions throughout the Wa VP6 protein (data not shown).

The NSP1 gene fragment sequenced (data not shown) was 1480 nucleotides in length (nucleotides

Table 1. Nucleotide (nt) and amino acid (aa) percent identities between VP6, NSP1 and NSP4 genes of 116E and other rotaviruses

VP6[a]				NSP1[b]				NSP4[c]			
Strain	Species	nt	aa	Strain	Species	nt	aa	Strain	Species	nt	aa
Wa	Human	91.5	98.0	Wa	Human	89.4	87.9	Wa	Human	95.7	95.4
E210	Human	91.6	98.5	DS-1	Human	75.4	69.3	S2	Human	84.2	83.4
RV3	Human	89.8	98.0	AU-1	Human	64.6	55.4	M37	Human	94.5	93.1
1076	Human	79.5	92.7	Hochi	Human	88.9	85.8	RV4	Human	95.1	94.3
I321	Human	80.0	91.7	69M	Human	75.0	67.5	RV5	Human	83.1	82.3
RF	Bovine	80.3	91.7	M37	Human	82.8	79.8	A28	Human	83.1	84.6
UK	Bovine	80.8	92.4	I321	Human	88.0	86.4	UK	Bovine	83.7	84.0
Gottfried	Porcine	89.3	97.2	A44	Bovine	64.6	56.2	YM	Porcine	92.7	94.9
YM	Porcine	83.0	93.5	B223	Bovine	65.4	56.6	SA11	Simian	82.1	81.7
SA11	Simian	79.0	92.2	OSU	Porcine	83.6	82.9	RRV	Monkey	82.4	82.9

[a]The following VP6 sequences were obtained from published reports and the GenBank database: strain Wa (16; accession no. K02086); E210 (20; U36240); RV3 (38; U04741); 1076 (39; D00325); I321 (S. Aijaz, Unpublished; X94618); RF (40; K02254); UK (41; X53667); Gottfried (39; D00326); YM (42; X69487); and SA11 (43; M27824). Includes nucleotides 21–1335.

[b]The following NSP1 sequences were obtained from published reports: strain Wa (17; accession no. L18943); DS-1 (17; L18945); AU-1 (31; D45244); Hochi (25; Z12106); 69M (25; Z32552); M37 (44; U11491); I321 (26; U08418); A44 (30; U23726); B223 (25; Z12105); and OSU (25; Z12107). Includes nucleotides 21–1500.

[c]The following NSP4 sequences were obtained from published reports and the GenBank database: strain Wa (18; accession no. K02032); S2 (E.A. Palombo et al., unpublished; U59104); M37 (E.A. Palombo et al, unpublished; U59109); RV4 (E.A. Palombo et al, unpublished; U59108); RV5 (E.A. Palombo et al., unpublished; U59103); A28 (45; D01145); UK (46; L03384); YM (42; X69485); SA11 (47; K01138); and RRV (B. Tang et al, unpublished; L41247). Includes nucleotides 20–694.

21–1500) and contained a single long ORF extending from nucleotide 31 to 1488, giving a deduced protein of 486 amino acids, the same length as Wa. Comparison of the NSP1 nucleotide and amino acid sequences of 116E with those of other human and animal strains showed greatest homology to Wa (89.4% nucleotide identity and 87.9% amino acid identity), and slightly less homology to Hochi (P1A[8]G4), another prototype subgroup II strain and I321 (P8[11]G10), a subgroup I neonatal strain isolated in India (21). In contrast, low homology was found with the bovine P8[11]G10 strain B223 (65.4% nucleotide identity and 56.6% amino acid identity) (Table 1). Alignment of the amino acid sequence with Wa identified scattered substitutions throughout the Wa protein, and B223 had multiple substitutions. (Fig. 1). The conserved zinc binding motif extends from position 37 to 81 and includes the highly conserved cysteine-rich motif (C-X2-C-X8-C-X2-C-X3-H-X-C-X2-C-X5-C) that extends from position 42 to 72. Histidine is conserved at position 61 and proline at position 78. A second postulated zinc-finger motif, extending from amino acids 314 to 327 (H-X2-C-X6-C-X2-C), was fully conserved in strain B223, but only partially conserved in 116E. The first cysteine residue within this region (amino acid 317) was replaced by leucine in strain 116E.

The NSP4 gene fragment sequenced (data not shown) was 675 nucleotides in length (nucleotides 20 to 694) and contained a single long ORF extending from nucleotides 42 to 566, giving a protein of 175 amino acids. The NSP4 nucleotide and amino acid sequence of 116E was compared with those of other strains and demonstrated greatest homology to Wa (95.7% nucleotide identity and 95.4% amino acid identity), and less homology to S2 (84.2% nucleotide identity and 83.4% amino acid identity) (Table 1). Alignment of the amino acid sequence with Wa identified a few scattered substitutions throughout the Wa protein, while comparison with S2 showed numerous amino acid differences, which include the carboxy end of the proposed ''toxic peptide'' region (amino acids 114–135). Common structural features of the NSP4 protein, such as N-linked glycosylation sites at amino acids 9–11 and 18–20, and the sequence of the amino terminal region of the toxic peptide were conserved (data not shown).

Discussion

We have sequenced the VP6, NSP1 and NSP4 genes of Indian neonatal strain 116E, which is currently being developed as a live, attenuated vaccine

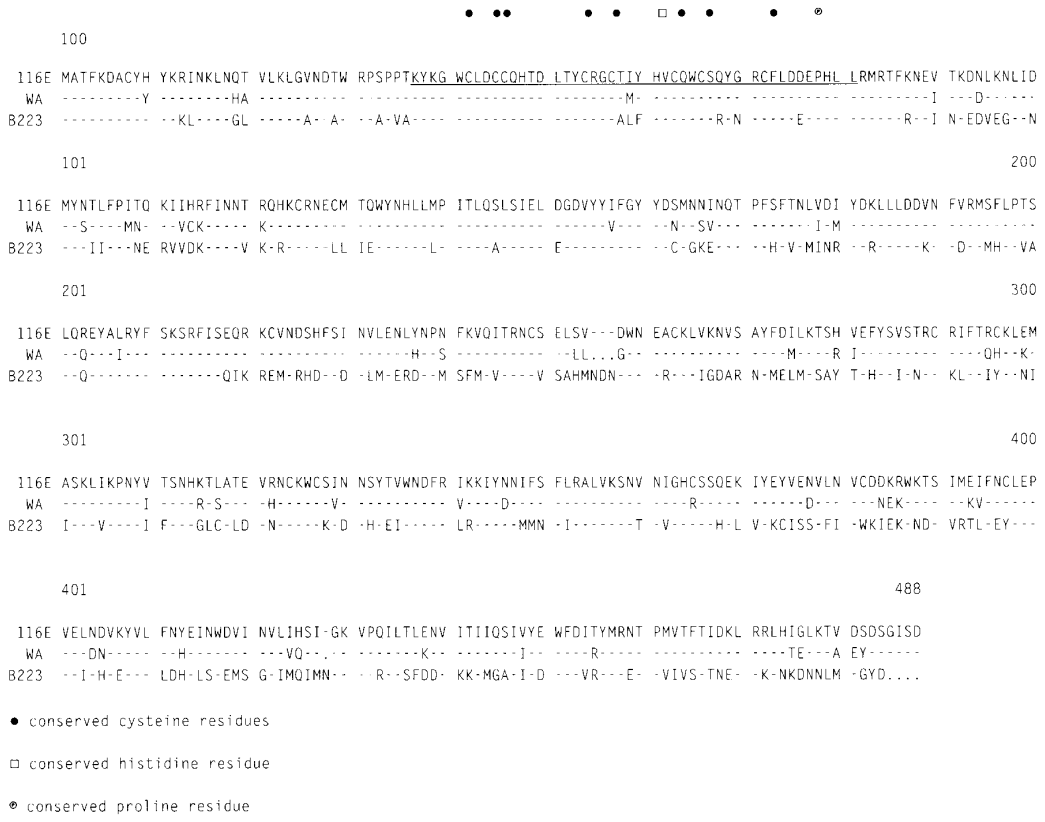

Fig. 1. Comparison of deduced amino acid sequences of the NSP1 protein of strains 116E, Wa and B223. Sequence alignments were prepared by the GCG program PILEUP, using a progressive alignment method (19). The sequence of strain 116E (top line) was used as the reference and the amino acids that differ from 116E are shown for Wa and B223, using the single letter amino acid code. The first zinc finger region (amino acids 37–81) is underlined. Conserved cysteine, histidine and proline residues within this zinc finger are identified.

candidate strain. Comparative analyses of the VP6, NSP1 and NSP4 genes of 116E to the cognate genes of other rotaviruses showed that they all share very high homology with Wa. These results confirm and extend findings of previous studies (9), which showed that 116E was genetically related to human Wa genogroup members by RNA-RNA hybridization, subgroup (SGII), serotype (G9) and RNA gel migration pattern (long electropherotype). In contrast, the VP4 gene of 116E was related to bovine strain B223 (10). It remains to be confirmed whether the genes encoding VP1 to VP3, NSP2, NSP3 and NSP5 are also related to Wa genogroup strains. One limitation of this study was that the sequences were derived from templates made by RT-PCR so that we cannot exclude the possibility that the minor variation between the 116E genes and those of strain Wa could be due to incorporation errors during PCR.

Each gene analyzed in this study has been recently implicated either in aspects of rotavirus virulence or in protection from infection. VP6 is the inner capsid protein, is highly immunogenic and defines group and subgroup specificity (3). Antibodies directed at VP6 are non-neutralizing *in vitro*, and VP6 was not previously thought to be an important target of the protective immune response. However, in a recent ‘‘backpack tumor’’ murine model, non-neutralizing IgA monoclonal antibody to VP6 was shown to prevent primary rotavirus infection, and resolve chronic rotavirus infections; it was proposed that secretory IgA may determine *in vivo* intracellular viral inactivation and may be a host defense mechanism against rotavirus infection (12). Furthermore, cytotoxic and T-helper lymphocyte responses to VP6 documented in mice could also play a role in host defense (22,23). A protective immune response

directed at VP6, if operative in rotavirus infections of humans, could explain the heterotypic immunity observed in some studies of natural rotavirus infections and vaccine trials (24). VP6 of strain 116E is virtually identical to that of Wa.

NSP1 is a protein of variable length that contains a zinc finger region typical of nucleic acid binding proteins and may be involved in viral replication (2). Reassortant studies with mice demonstrated that the NSP1 gene was associated with virulence phenotype (13). NSP1 is highly divergent, displaying extensive interspecies variation, predominantly in the carboxy-terminal half of the protein (25,26). The selective force driving this high level of sequence divergence is not known, although it has been suggested that selection for a NSP1 gene which alters replication potential could influence pathogenicity (27). In this regard, it has been speculated that the selection of a Wa-like NSP1 gene may allow transmission of strain I321-like rotaviruses (P8[11]G10) among Indian neonates (28). Strain I321 (also a proposed rotavirus vaccine candidate) (29), shares 90.6% nucleotide and 87.6% amino acid identity with strain Wa. We have shown that 116E NSP1 is also related to the NSP1 of Wa genogroup strains, supporting the Wa genogroup origin of NSP1 and consistent with the hypothesis that NSP1 may influence spread. These data also support results of previous studies correlating NSP1 sequence homology with genogroup designation (30,31).

In contrast to the highly divergent carboxy-terminal half of the NSP1 protein, the zinc-finger region near the amino terminus (amino acid 37–81) is known to be conserved across all strains examined (25). The highly conserved, cysteine-rich region within this zinc finger has been shown by deletion mapping to be essential for RNA-binding (32), although a recent study found that bovine rotavirus mutants whose gene 5 lack this region were not defective (33). The cysteine-rich region in 116E lies between amino acids 42 and 72. Further evidence that the NSP1 gene of 116E is not of bovine origin comes from the observation that a proposed second zinc-finger motif identified in NSP1 genes of bovine rotaviruses RF, UK and B223 is not fully conserved in 116E.

The transmembrane glycoprotein NSP4 is known to be involved in virus assembly (34), and has recently been shown to be capable of inducing diarrhea in young mice (14). More specifically, a synthetic peptide corresponding to amino acid residues 114–135, the toxic peptide, reproduced this effect. It is proposed that NSP4 acts as a viral enterotoxin and stimulates a secretory diarrhea through a Ca+ dependent signaling pathway in small intestinal epithelial cells (14). The same peptide has a membrane destabilizing activity which may play a role in removing the transient rotavirus envelope during virus assembly (34). We have previously shown by sequence analysis that the NSP4 genes of human rotaviruses can be classified into two main genetic groups (which differ in sequence that includes the carboxy-terminal end of the toxic peptide region) and that the NSP4 genes of several animal and animal/human reassortant strains also fall into one of these groups (N.A. Cunliffe et al., in press). This division of human rotavirus NSP4 genes into two genetic groups correlates with genogroup designation by RNA-RNA hybridization analyses. The NSP4 gene of 116E is highly homologous with Wa, in agreement with the results of a partial sequence we have reported previously (N.A. Cunliffe et al., in press). The NSP4 gene of 116E differs from that of Wa at two positions in the highly variable carboxy-end of the toxic peptide (amino acids 131 and 133); the significance of these substitutions in 116E NSP4 is unknown, although in the mouse model substitutions in this region of the toxic peptide may play a role in attenuation of virulence (35).

It is not yet known whether inclusion of human rotavirus genes associated with virulence or protection (other than VP7 and perhaps VP4) in vaccine strains will have an impact on vaccine efficacy. However, as more information becomes available about the roles that other structural and non-structural proteins play in virulence and protection, the potential role of other genes in the development of better vaccines should become more clearly defined (36,37). A more complete genetic characterization of rotavirus vaccine strains will facilitate studies of the host immune response to them. In the example studied here, 116E (P8[11]G9) was shown to have VP6, NSP1 and NSP4 genes closely related to those of the Wa genogroup, and conceivably may share all genes but VP4 with Wa-like viruses. In contrast, I321 (P8[11]G10), the other candidate vaccine isolated in India, may contain 4 to 6 genes from the Wa genogroup and the remainder from a bovine rotavirus parent (29). A more complete molecular understanding of the genomes of these viruses could lead to improved assays to measure the immune response

to their individual proteins and subsequently allow a better understanding of the protective immunity induced by 116E and I321 in response to natural infection and vaccination.

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