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Attenuation of a Human Rotavirus Vaccine Candidate Did Not Correlate with Mutations in the NSP4 Protein Gene

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The NSP4 protein of a simian rotavirus was reported to induce diarrhea following inoculation of mice. If NSP4 is responsible for rotavirus diarrhea in humans, attenuation of a human rotavirus may be reflected in concomitant mutations in the NSP4 gene. After 33 passages in cultured monkey kidney cells, a virulent human rotavirus (strain 89-12) was found to be attenuated in adults, children, and infants. Nucleotide sequence analysis of the NSP4 protein gene revealed only one base pair change between the virulent (unpassaged) and attenuated 89-12 viruses, which resulted from a substitution of alanine for threonine at amino acid 45 of the encoded NSP4 protein. Because both threonine and alanine have been found at position 45 of NSP4 in symptomatic and asymptomatic human rotaviruses, neither amino acid in this position could be established as a marker of virulence. Therefore, attenuation of rotavirus strain 89-12 appears to be unrelated to mutations in the NSP4 gene.

Rotavirus infection is a cause of severe diarrheal disease in numerous mammalian species, including humans. In commercially important domestic animals and in humans, rotavirus infection appears to be primarily restricted to the mature enterocytes on the tips of the intestinal villi, where extensive destruction has been reported (7, 9). It has been suggested that this destruction results in malabsorption of nutrients, electrolyte imbalance, and diarrhea. The villus stunting that occurs in large mammals following destruction of the mature enterocytes is very limited in mice following infection with murine rotaviruses even though neonatal mice can experience severe rotavirus diarrhea for up to 15 days of life (14, 20). For these reasons, Osborne et al. (13) suggested that rotavirus diarrhea in mice was due to vascular damage rather than enterocyte destruction.

Recent studies with mice suggested a third mechanism for rotavirus diarrhea due, at least in part, to enterotoxin-like properties of viral proteins. Shaw et al. (15) reported that inoculation of neonatal mice with large quantities of purified rhesus rotavirus inactivated by UV psoralen treatment produced moderate to severe diarrhea in most animals. These results indicated that mere attachment or uptake of rotavirus particles was sufficient to induce diarrhea in neonatal mice. Very recently, Ball et al. (1) reported that baculovirus-expressed preparations of the NSP4 protein from the simian rotavirus strain SA11 induced diarrhea when they were administered to neonatal and sometimes older mice. Furthermore, an SA11 peptide composed of amino acids 114 to 135 of NSP4 also induced diarrhea in the young animals. The suggested mechanism was potentiation of chloride secretion by a calcium-dependent signaling pathway. It was further suggested that NSP4 produced during viral replication was released into the lumen of the gut, where it interacted with a cellular receptor, thus triggering a signal transduction pathway.

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The significance of the results found with the SA11 NSP4 protein in the mouse model will depend on their applicability to rotavirus disease in larger mammals and humans. Very recently, Zhang et al. (21) reported that tissue culture attenuation of a virulent porcine rotavirus correlated with amino acid changes in the NSP4 protein, particularly those between amino acids 131 and 140. This result is consistent with the possibility that the NSP4 protein behaves as an enterotoxin in piglets. It was, therefore, of interest to determine whether this protein can also be associated with rotavirus diarrhea in humans.

The 89-12 strain of human rotavirus was obtained from a fecal specimen of a child with diarrheal disease in Cincinnati, Ohio, in December 1988 (3). The electropherotype of this G1[P8] strain was found to be indistinguishable from almost all (>95%) other rotavirus isolates obtained from approximately 100 children in Cincinnati with rotavirus disease during that season. Therefore, these virulent isolates were presumed to belong to the same strain as 89-12. Because subjects infected with 89-12-like rotaviruses developed neutralizing antibody to all major rotavirus serotypes (19) and were protected from subsequent rotavirus infection (2), the 89-12 strain was developed as a vaccine candidate. Attenuation of the virus was performed by multiple cell culture passages. To determine whether attenuation of the 89-12 strain was achieved, the passaged virus was administered first to adults and then to children with rotavirus antibody and finally to naive infants, the target population of the vaccine. In all three groups of subjects, administration of 10^5 PFU of the 89-12 vaccine preparation did not produce a significantly increased incidence of gastrointestinal symptoms relative to that found in placebo recipients (4). Therefore, multiple passages of this virus in cell culture attenuated its pathogenesis properties in humans, including its ability to induce diarrhea.

The purpose of this study was to compare the nucleotide sequences of the NSP4 protein gene for unpassaged 89-12 and 89-12-like viruses with that of the 89-12 strain both during and after attenuation by passage in cell culture. The results were then evaluated to determine whether the attenuation process affected the deduced amino acid sequence of the 89-12 NSP4

protein and, if so, whether such changes could account for the observed attenuation of the virus.

The unpassaged 89-12 strain obtained from the stool of a child with gastrointestinal illness in Cincinnati in December 1988 was passaged 26 times in primary African green monkey kidney (AGMK) cells, as previously described (18), and 7 additional times in an AGMK cell line, the last passage of which became the vaccine preparation. Multiple 1-ml aliquots of this preparation were prepared and stored at −70°C. The titer of this preparation was 5×10^5 PFU/ml. Stool specimens containing the unpassaged 89-12 isolate and 89-12-like rotaviruses, collected during the 1988 to 1989 rotavirus season in Cincinnati, were stored as 10% suspensions, also at −70°C.

The methods used to administer the vaccine and monitor for safety and immunogenicity have been described elsewhere (4). In brief, one dose of 10^5 PFU was first administered to adults and children to determine its safety. Infants aged 6 to 26 weeks were then administered two doses of 10^5 PFU (21 subjects) or a placebo (tissue culture medium; 21 subjects) separated by 6 to 10 weeks. Subjects were monitored for gastrointestinal illnesses during the 7 days following each vaccination. Blood specimens were collected before and after each vaccination to measure the immunogenicity of the vaccine. Stool specimens were collected just prior to vaccination (day 1) and on days 4, 7, 14, and 21 following each vaccination to monitor for viral shedding and intestinal rotavirus immunoglobulin A responses. Shedding of rotavirus was determined initially by a sensitive enzyme-linked immunosorbent assay (18). The relative quantities of rotavirus antigen present in the positive specimens were further determined by this enzyme-linked immunosorbent assay by a standard curve procedure. The standard was a lysate from cells infected with culture-adapted strain 89-12 which was arbitrarily assigned a value of 10,000 rotavirus antigen U/ml. The limit of detection by this assay was 30 U/ml.

For nucleotide sequence determinations, viral RNA was extracted from stool specimens according to a modification of the procedure developed by Ushijima et al. (16). A 10% suspension of stool (250 μl) was diluted twofold with Earle's balanced salt solution, and an equal volume of 6.8 M guanidine thiocyanate in 50 mM Tris (pH 7.5) was added to disrupt the virus while the sample was being incubated on ice for 5 min. Samples were centrifuged (10,000 × *g*, 2 min) to remove debris, and 10 μl of Rnaid beads (Rnaid kit; Bio 101, Inc., Vista, Calif.) was added. The beads were kept in suspension for 10 min at room temperature by occasional mixing and then pelleted (650 × *g*, 2 min) and washed three times with 400 μl of the kit wash buffer. RNA was eluted by adding 30 μl of water and heating at 65°C for 10 min. The beads were removed by centrifugation (10,000 × *g*, 2 min), and the supernatant was stored at −20°C until it was used in the reverse transcription (RT) procedure.

For RT, the viral double-stranded RNA was melted in 5% dimethyl sulfoxide at 97°C for 5 min. Usually, 2 μl of concentrated RNA was added to 17 μl of H_2O and 1 μl of dimethyl sulfoxide. The reverse transcriptase from GIBCO BRL (Gaithersburg, Md.) was added to reagents in the RT-PCR kit from Perkin-Elmer (Foster City, Calif.) for cDNA synthesis. Random primers, oligonucleotides, RNase inhibitor, salts, and buffers were used as described in the instructions for the kit. Incubation was at 42°C for 1 h. The samples were heated at 94°C for 5 min and placed on ice. Either the entire 20-μl sample or 5 μl was used in the subsequent PCR.

We used oligomers for PCR which were based upon the sequence of gene 10 of the human rotavirus strains Wa (12), SA11 (5), and DS1 (unpublished results). The 5′ oligomer was 5′-GAGAGAGCGCGTGCGGAAAGATG-3′ (primer A), and the 3′ oligomer was 5′-CCGTTCCTTCCATTAACGTCC-3′ (primer B). In order to obtain the sequence of only the coding region, additional oligomers which ended at either the initiation codon or the termination codon of gene 10 were designed. This permitted detection of a mismatch in the coding region of previously unsequenced wild-type viruses. The 5′-end-internal oligomer, which ended at the ATG initiation codon, was primer A described above, and the 3′-end-internal oligomer, which ended at the termination signal, was 5′-CGG CAGCTCAACCTCTC-3′. Other internal primers were used to make additional fragments for sequencing. Primer A was used with 5′-GTTCAATTTCACGAGTAG-3′, and primer B was used with 5′-GACGCCAGCTAGAAATGATTG-3′. The PCR primers generated DNA fragments which contained overlapping sequences that were used for confirmation. The PCR procedure was carried out with the kit from GIBCO BRL, and it was performed in 35 cycles with a three-step temperature program consisting of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s followed by a final incubation at 72°C for 7 min.

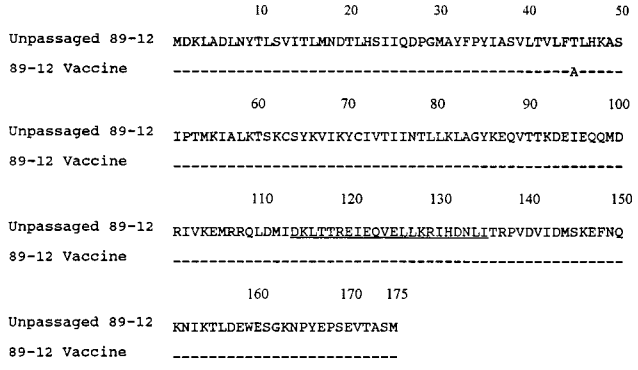

FIG. 1. Predicted amino acid sequences of the NSP4 protein of unpassaged 89-12 and the attenuated 89-12 vaccine preparation. Identical amino acids are denoted by dashes. Amino acids 114 to 135 are underlined.

Before sequencing, PCR-amplified DNA fragments were purified by electrophoresis (4 h, 200 V) in 1.5% agarose gels. DNA bands located with UV light were cut from the gels, and the DNA was extracted by the Qiaquick procedure (Qiagen, Chatsworth, Calif.). Purified DNA was then sequenced on an Applied Biosystems, Inc., automated DNA sequencer, model 373A, by using dye-labelled terminator chemistry (Perkin-Elmer, Branchburg, N.J.). DNA sequence was analyzed with the Intelligence software from Intelligenetics, Inc. (Mountain View, Calif.).

The nucleotide sequence of the NSP4 protein gene of the unpassaged 89-12 strain isolated from the stool of an ill child was determined directly from its RT-PCR product. This sequence was compared to the NSP4 gene sequences of the same strain after only two passages in cell culture and after 33 passages (i.e., the attenuated vaccine preparation). In every case, the sequence obtained was a consensus of the viral genotypes within the sample. That is, the viruses were not plaque purified, nor were the NSP4 genes cloned prior to sequence analysis. Following this analysis, no changes in the NSP4 gene were detected after two passages in cell culture and only one change from A to G at nucleotide 135 of the open reading frame was found in the attenuated vaccine preparation. This change resulted in a predicted amino acid change from threonine to alanine at position 45 of NSP4 (Fig. 1). In addition to the unpassaged 89-12 strain, the NSP4 gene sequences of three isolates of unpassaged 89-12-like rotaviruses obtained during

TABLE 1. A comparison of amino acids at position 45 of NSP4 from published and GenBank sequences compared to those found for virulent and attenuated 89-12

Rotavirus strain	G type	Source of virus	Amino acid at position 45	Source or reference
Unpassaged 89-12	G1	Ill child	Threonine	This study
89-12 after 33 passages	G1	Ill child	Alanine	This study
Wa	G1	Ill child	Threonine	12
YM	G11	Piglet	Alanine	10
SA11	G3	Monkey	Alanine	5
RRV	G3	Monkey	Threonine	L41247[a]
UK	G6	Calf	Threonine	17
RV3	G3	Asymptomatic neonate	Threonine	U42628[a]
1076	G2	Asymptomatic neonate	Threonine	U59105[a]
M37	G1	Asymptomatic neonate	Threonine	U59109[a]
ST3	G4	Asymptomatic neonate	Threonine	U59110[a]
RV5	G2	Ill child	Threonine	U59103[a]
S2	G2	Ill child	Threonine	U59104[a]
E201	G2	Ill child	Threonine	U59106[a]
E210	G2	Ill child	Threonine	U59107[a]
RV4	G1	Ill child	Alanine	U59108[a]

[a] GenBank accession number.

the same 1988 to 1989 rotavirus season were also determined. All three were from young children with gastroenteritis, and all had electropherotypes that were indistinguishable from those of 89-12. When analyzed, all three had the same NSP4 gene sequence as the unpassaged 89-12 strain.

To determine whether the single amino acid change at position 45 selected during multiple passages of 89-12 could account for the observed attenuation of this strain, the amino acid found in this position was compared to those of a variety of symptomatic and asymptomatic strains. From published sequences and those found in GenBank (a total of 14), only threonine (11 cases) or alanine (3 cases) was present at amino acid position 45 (Table 1). Of particular interest is that asymptomatic neonatal strains RV3 (G3), 1076 (G2), M37 (G1), and ST3 (G4) all contained threonine at position 45, the same amino acid as that found in unpassaged virulent 89-12 and 89-12-like strains. Furthermore, a cloned NSP4 gene of the culture-adapted G1 Wa strain also contained threonine at this position. However, the culture-adapted G1 RV4 strain, obtained from a symptomatic child, contained alanine at position 45. Moreover, the NSP4 gene of simian strain SA11, which was found to induce diarrhea in mice and formed the basis for the suggestion that NSP4 may be the cause of rotavirus-induced diarrhea (1), also contained alanine at position 45. These results are inconsistent with the possibility that a single amino acid change from threonine to alanine at position 45 of NSP4 accounts for attenuation of strain 89-12 in humans.

It is possible that attenuation of 89-12 during passage in cell culture resulted in mutations in genes other than that encoding NSP4 that restricted virus replication in humans. If this was the case, a concomitant reduction in the production of NSP4 would be expected, possibly to levels insufficient to induce diarrhea. To determine whether rotavirus replication was restricted in vaccinees administered the attenuated 89-12 preparation, the quantity of rotavirus antigen shedding was measured at different time points after vaccination in previously uninfected infants. By day 4 after 89-12 vaccination, 60% of the 20 previously uninfected infants aged 6 to 22 weeks were shedding detectable amounts of rotavirus (Fig. 2). Shedding peaked on day 7 and gradually declined. However, even by day 21, nearly one-half of the subjects were still shedding detectable amounts of rotavirus.

It is unknown how these levels of virus shedding compare to that occurring in infants with rotavirus diarrhea because the time of maximum shedding after infection and the duration of shedding after natural infection have not been reported. In an attempt to obtain comparative data, we measured the quantities of rotavirus antigen in stools of subjects collected during the peak of illness following natural infection with 89-12 and 89-12-like viruses. The range of shedding was from 44,000 to 863,000 U/g of rotavirus antigen, and the geometric mean titer (GMT) was 179,000 U/g, significantly ($P = 0.02$, Student's t test) more than that found in vaccinees on day 7 after administration of the 89-12 vaccine. However, 6 of the 20 (30%) previously uninfected vaccinees shed larger amounts of rotavirus antigen on day 7 after vaccination with 89-12 than the GMT of rotavirus antigen found in specimens from ill subjects.

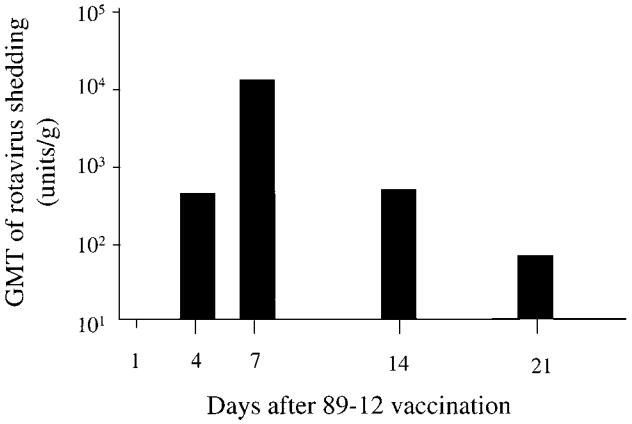

FIG. 2. Shedding of rotavirus antigen in 6- to 22-week-old infants following administration of 10^5 PFU of the 89-12 vaccine. Values represent the GMTs of rotavirus antigen found in stools of rotavirus naive subjects (one vaccinee had experienced a natural rotavirus infection before vaccination based on the presence of rotavirus immunoglobulin A) who shed detectable amounts of virus on any day following vaccination. Rotavirus antigen was found in 0 of 20 subjects (0%) on day 1, 12 of 20 subjects (60%) on day 4 (range, 71 to 553,000 U/g), 17 of 20 subjects (85%) on day 7 (range, 117 to 979,000 U/g), 15 of 20 subjects (75%) on day 14 (range, 120 to 8,000 U/g), and 9 of 20 subjects (45%) on day 21 (range, 40 to 800 U/g).

Therefore, based on the duration of shedding and quantity of rotavirus shed, extensive 89-12 replication occurred in the vaccinated infants, which required the production of large amounts of NSP4, presumably sufficient to induce diarrhea if the NSP4 protein of 89-12 were responsible for this activity.

The mutations that caused attenuation of 89-12 may have occurred in any one or several of the 11 rotavirus gene segments. Initial studies using a mouse model suggested that virulence of rotavirus was associated with the VP4 protein encoded by segment 4 (11). Later studies with mice, however, provided evidence that neither VP4 nor the other outer capsid protein, VP7, was primarily responsible for virulence in a mouse model with homologous murine rotaviruses (6). In that study, the protein most significantly associated with virulence was NSP1, encoded by segment 5 ($P < 0.008$). A more recent study conducted with human (DS-1) and porcine (SB-1A) reassortants linked virulence in piglets to the presence of four rotavirus proteins (VP3, VP4, VP7, and NSP4), all of which were absolutely and exclusively required (8). Thus, it is unclear whether specific rotavirus proteins are responsible for virulence or whether virulence is merely a combined effect resulting from the interactive properties of some or all of the 11 rotavirus proteins. From this uncertainty it follows that attenuation of 89-12 may have resulted from mutational events in genes coding for any one or several of these proteins. The results reported here, however, indicate that mutations in the NSP4 protein gene were not involved in this attenuation process. Therefore, attenuation of 89-12 appeared to be associated with genes other than that encoding NSP4.

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