Serotypic and Genotypic Characterization of Human Serotype 10 Rotaviruses from Asymptomatic Neonates

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Human rotaviruses were isolated from asymptomatic neonates at various hospitals and clinics in the city of Bangalore, India, and were found to be subgroup I specific and possess long RNA patterns (M. Sukumaran, K. Gowda, P. P. Maiya, T. P. Srinivas, M. S. Kumar, S. Aijaz, R. R. Reddy, L. Padilla, H. B. Greenberg, and C. D. Rao, Arch. Virol. 126:239–251, 1992). Three of these strains were adapted to tissue culture and found by serotype analysis and neutralization assays to be of serotype 10, a serotype commonly found in cattle but infrequently found in humans and not previously identified in neonates. By RNA-RNA hybridization, a high level of relatedness to a serotype 10 bovine rotavirus strain and a low-to-medium level of relatedness to a human rotavirus strain were observed. Since this human isolate shares a genogroup with bovine rotavirus, it is likely that it originated by interspecies transmission. A human rotavirus strain isolated from asymptomatic neonates and similar to bovine rotavirus might represent a good vaccine candidate.

Rotaviruses, the major etiological agents of severe gastroenteritis, have been isolated from the young of many species, including humans and wild and domestic animals (17). Members of the genus *Rotavirus* have a genome that consists of 11 double-stranded RNA segments surrounded by a double-layered capsid. Rotaviruses have been classified serologically into seven distinct groups (A to G) on the basis of group-specific antigens detected by immunofluorescence, enzyme-linked immunofluorescent assay (ELISA), or immunoelectron microscopy. Group A rotaviruses, the most common group, are found in almost all species and are divided into at least 12 distinct G serotypes on the basis of the antigenicity of VP7, the major outer capsid glycoprotein (5, 7, 15, 37). In addition, the serological characteristics of VP4, called P type, are currently being defined. At least four human P types and two bovine P types are currently known (7). The P serotype of most rotavirus strains has not yet been determined, however. In addition to serological classification schemes, electrophoretic classification can be done on the basis of the migration of the 10th and 11th genes of the double-stranded RNA on polyacrylamide gels, and these patterns are called long and short electropherotypes. Several types of associations are commonly seen in group A human rotaviruses with respect to subgroup specificity, serotype, and electropherotype (16). For human rotavirus strains, short and long RNA patterns are associated with subgroups I and II, respectively, with a few notable exceptions (1, 3, 4, 11, 12, 18, 24, 25, 31, 34, 37). Animal rotaviruses usually have subgroup I specificity and belong to a variety of serotypes, including 3, 4, 5, 6, 7, 8, 10, and 11. Virtually all human serotype 2 strains have a short electropherotype and subgroup I specificity. Animal strains almost always have a long RNA electropherotype (14). Human rotavirus isolates

with subgroup I specificity and long RNA patterns have been postulated to have a high likelihood of animal origin (14).

Rotaviruses are further classified into genogroups on the basis of gene homology. Members of a genogroup have a high degree of genetic relatedness to each other but have significantly less genetic homology with members of other genogroups (23, 29). To date, human rotaviruses have been classified into three distinct genogroups: (i) Wa-like, (ii) DS-1-like, and (iii) AU-1-like (23, 27, 29). Rotaviruses from different species have been shown, in most cases, to belong to species-specific genogroups (21).

Neonatal human rotavirus infections differ from those of older children in some important clinical and epidemiological features. Neonatal infections are asymptomatic in most cases and occur throughout the year, and rotaviruses infecting neonates often have electropherotypes distinct from those of the rotaviruses infecting older children in the same community at the same time (36). Each of the four major human rotavirus serotypes (serotypes 1 to 4) has been isolated from asymptomatic neonates (13, 15, 36). The putative lack of virulence of isolates from neonates is, therefore, not related to a specific serotype.

Recently, human rotavirus strains were isolated from asymptomatic neonates in hospitals and clinics in the city of Bangalore, India (35). All of the fecal specimens testing positive for group A rotavirus antigens showed subgroup I specificity, a long RNA pattern, and an undetermined serotype that did not appear to correspond to human serotypes 1 through 4 (35). In addition, these asymptomatic strains appeared to have related but not identical electropherotypes. In the present study, fecal specimens containing rotavirus, taken from asymptomatically infected newborn infants in Bangalore, were adapted to tissue culture, and their serotypes were determined. Further investigation of these strains by genotyping by RNA-RNA hybridization led to some insights into the possible origin of these viruses.

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TABLE 1. Serotype analysis of cultivated strains from asymptomatic neonates by ELISA with serotype-specific monoclonal antibodies

Strain	A_{405} (10^{-3}) with monoclonal antibody (serotype specificity):						
	5E8 (1)	1C10 (2)	159 (3)	ST3 (4)	5B8 (5)	IC3 (6)	B223/N7 (10)
I321	71	49	115	35	56	52	400
I422	102	53	165	32	63	44	642

The strains of rotavirus used in this study included Wa (human serotype 1), DS-1 (human serotype 2), S2 (human serotype 2), AU-1 (human serotype 3), FRV-1 (feline serotype 3), K9 (canine serotype 3), RRV (simian serotype 3), ST3 (human serotype 4), OSU (porcine serotype 5), NCDV (bovine serotype 6), 69M (human serotype 8), WI61 (human serotype 9), KK-3 (bovine serotype 10), B223 (bovine serotype 10), and L26 (human serotype 12). Strains representing avian serotype 7 and porcine serotype 11 rotaviruses were not available for study. Serotype-specific VP7-derived monoclonal antibodies used include 5E8 (serotype 1), 1C10 (serotype 2), 159 (serotype 3), ST2G7 (serotype 4), 5B8 (serotype 5), IC3 (serotype 6), and B223/N7 (serotype 10) and have been described elsewhere (30, 41).

Three representative isolates (I195, I321, and I422) of the Indian rotaviruses obtained from asymptomatic newborns in Bangalore were adapted to tissue culture as previously described (39). After two passages in primary AGMK cells followed by two further passages in MA104 cells, the three strains were plaque purified and further characterized. The three cultivatable strains had the same electropherotypes as the parental strains before cell culture adaptation (data not shown). The strains were then retested in a monoclonal antibody-based subgrouping and serotyping ELISA (32). Strains I321 and I422 were further tested in a neutralization assay with high-titer VP7-specific neutralizing monoclonal antibodies to serotypes 1, 2, 3, 4, 5, 6, and 10 (5E8, 1C10, 159, ST2G7, 5B8, IC3, and B223/N7, respectively) to determine their potential serotypes (20, 32, 41). Strain I321 was then used to produce two hyperimmune guinea pig serum samples to test for neutralization activity against prototypic viruses representing serotypes 1 to 6, 8 to 10, and 12 as well as the homologous viruses I321 and I422 by previously described methods (15, 32).

Genogrouping by RNA-RNA hybridization was carried out by previously described methods with hybridization conditions allowing for up to an 18% mismatch in nucleotide sequence (8–10, 21–23, 25, 27). Hybrids were separated by polyacrylamide gel electrophoresis after hybridization in solution, and homologous bands were identified as those segments that comigrated with the corresponding genomic RNA segments, while hybrids with lesser homology were

seen as aberrantly migrating bands with lesser intensity. The number of hybrids formed and their intensities were considered to be indicative of the relative and overall gene homology, since the gene segments involved in hybrid formation are difficult to identify exactly (21).

Serotyping and subgrouping results of the tissue culture-adapted isolates from neonates confirmed results reported previously for the original fecal specimens (35). All three isolates were subgroup I specific. Furthermore, neither I321 nor I422 reacted with monoclonal antibodies specific for serotypes 1 through 6 in an ELISA, but they did react with serotype 10-specific monoclonal antibody (Table 1).

To further investigate the serotypic characteristics of the neonatal Indian rotavirus strains, hyperimmune sera against strain I321 were raised in two guinea pigs. These antisera did not neutralize viruses representing serotypes 1, 2, 3, 5, 6, 8, 9, or 12 at high titers but did neutralize the prototypic serotype 10 virus (B223), the I422 isolate, and the homologous virus I321 (Table 2). One of the two antisera (Table 2, antiserum 1) did neutralize the ST3 serotype 4 strain at a significant titer. When monoclonal antibodies specific for rotavirus serotypes 1 to 6, 9, and 10 were tested against the I321 and I422 strains in a neutralization assay, only the serotype 10-specific monoclonal antibody had activity (Table 3). These data indicate that both the I321 and I422 strains are of serotype 10, a serotype commonly found in cattle (33) but previously identified in only one immunosuppressed child and never previously detected in healthy children or neonates (2).

A series of genotyping studies was initiated to determine the relatedness of the genome of I321 to those of various viruses of the three human genotypes and animal genotypes, including the bovine genogroup. By using the criteria of Nakagomi and Nakagomi (21), in which a minimum level of gene homology is indicated by the presence of two or fewer hybrid bands, a low level of homology is indicated by the presence of three or four hybrid bands, a medium level is indicated by the presence of five to seven hybrid bands, and a high level is indicated by the presence of eight or more hybrid bands, it was observed that I321 hybridized with high homology to the bovine serotype 10 strain KK-3 (Fig. 1). This strain hybridized with a medium level of homology to the bovine serotype 6 strain NCDV. Both NCDV and KK-3 have subgroup I specificity and a long RNA pattern. These two serotypes of bovine rotavirus were shown in previous studies to belong to a single genogroup distinct from the human genogroups (19). The relatedness of I321 to other human viruses or animal viruses representing other genogroups was substantially less than its relatedness to the two bovine strains (Fig. 1). Thus, on the basis of overall nucleic acid sequence homology and VP7-specific serotype, the Indian strains isolated from asymptomatic individuals seem to be most closely related to bovine rotaviruses of serotype

TABLE 2. Neutralization titers against various rotaviruses as determined by focus reduction assay

Antiserum[a]	Neutralization titer of rotavirus (serotype)[b]:										
	Wa (1)	S2 (2)	RRV (3)	ST3 (4)	OSU (5)	NCDV (6)	69M (8)	WI61 (9)	B223 (10)	L26 (12)	I321 and I422
1	<200	<200	800	3,200	<200	800	<200	800	6,400	<512	<12,800
2	<200	<200	<200	800	<200	200	<200	200	12,800	ND[c]	<12,800

[a] Hyperimmune guinea pig antiserum to strain I321.
[b] Neutralization titer is expressed as the reciprocal of the serum dilution resulting in a 50% reduction in the number of plaques.
[c] ND, not determined.

TABLE 3. Monoclonal antibody neutralization titers against two rotavirus strains

Strain	Neutralization titer of monoclonal antibody (serotype specificity)[a]:							
	5E8 (1)	1C10 (2)	159 (3)	ST2G7 (4)	5B8 (5)	IC3 (6)	WI61 (9)	B223/N7 (10)
I321	<200	<200	<200	<200	<200	<200	<200	<512,000
I422	400	<200	<200	<200	<200	<200	<200	<512,000

[a] Neutralization titer is expressed as the reciprocal of the serum dilution resulting in a 50% reduction in the number of plaques.

10 specificity. A low- to medium-level hybridization of I321 to the Wa strain indicated that I321 might have arisen through reassortment between a bovine strain and a human strain belonging to the Wa genogroup. In the reciprocal hybridizations between Wa and I321 (Fig. 1), three bands are clearly seen. One band migrated between genes 4 and 5 and the other two migrated as a doublet at the approximate position of the gene 7-gene 8-gene 9 complex.

Reassortment of viruses with segmented genomes has been viewed as a potential mechanism for rapid viral evolution (6). Evidence for natural reassortment of genes within the human rotavirus genogroups was shown in studies in which viruses in the Wa and DS-1 genogroups reassorted segments to form progeny with shared genotypic characteristics (40). Molecular evidence of rare interspecies transmission of rotavirus from animals to humans is strong (28), and such strains transmitted between species usually have sub-

group I specificity and a long RNA pattern (1, 4, 11, 12, 23–26, 29, 31, 34, 36). Occasionally, interspecies transmission appears to be caused by viruses that are reassortants between animal and human strains rather than purely animal strains (27, 29). Human isolates AU-1 and Ro1845 were found to be genetically related to feline rotavirus and to canine and feline rotavirus isolates, respectively (27, 29). Interspecies transmission is suggested when an isolate shares a genogroup with a virus from a different animal species (27). The neonatal serotype 10 isolates are similar to bovine rotaviruses in serotype and overall genetic homology. It is clear, however, that these viruses are well adapted to humans. Isolation of a large number of these strains with similar electropherotypes over several years from various hospitals and clinics indicates that the serotype 10 rotaviruses may be quite common in asymptomatic neonates in India. It is interesting to hypothesize that the close associa-
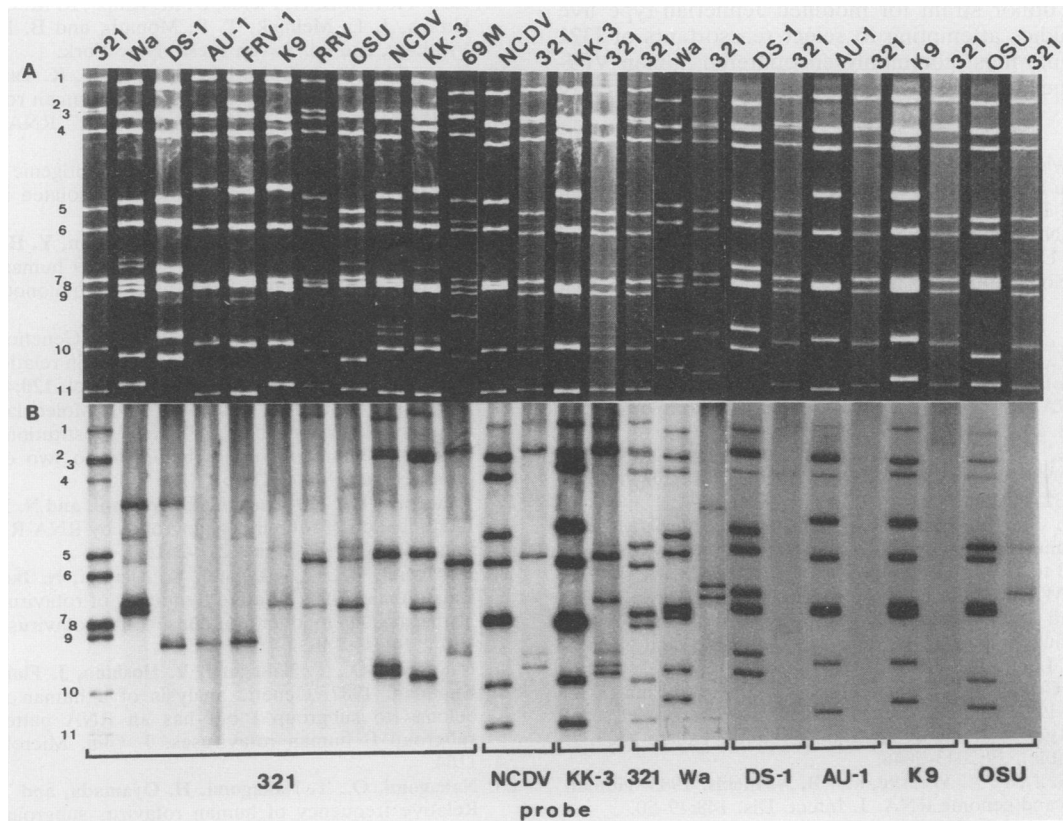

FIG. 1. Hybridization patterns obtained from ^{32}P-labeled single-stranded RNA probes hybridized to genomic RNA from various mammalian rotavirus strains. (A) Ethidium bromide-stained gels under UV illumination; (B) corresponding autoradiographs. The ^{32}P-labeled single-stranded RNA probe strains are indicated below the lanes, and the genomic RNA strains are indicated above the lanes. RNA segments are indicated on the left in each panel.

tion between humans and cattle in India facilitated the spread of a bovine virus into the human population. It seems reasonable to assume that the nonvirulent phenotype of this virus is due to its animal origin. The genetic basis for its ability to replicate well and persist in human remains to be determined. On the basis of the genogrouping and serotyping data, it does not appear that I321 VP4 or VP7 was derived from a human strain. The origin of the three primary bands that strongly cross-hybridize with Wa is not clear, but the best guess would indicate that they correspond to the genes encoding NS53, NS35, and NS34. The role that these nonstructural genes play in determining host range restriction remains to be determined. Whatever the genetic basis for the ability of the I321 virus to replicate efficiently in humans may be, this strain possesses several attributes that make it a logical choice for further studies as a potential vaccine strain. First, unlike the other current live vaccine candidates under study, it is a natural human isolate that grows well in humans or at least neonates. Second, it appears to be highly attenuated in the wild, and the attenuation is likely to be due to its animal origin. In this respect, it differs from the M37 neonatal isolate, that has already been studied as a vaccine candidate (38). M37 is entirely human in origin and did not perform well in very limited vaccine trials. However, its failure was due primarily to the very homotypic nature of the immune response generated after infection and to somewhat increased reactigenicity. The I321 isolate or a reassortant derived from this parent might well behave differently from M37. Third, I321 has been adapted to growth in cell culture. Presumably, this strain represents an excellent donor strain for modified Jennerian-type live vaccines. Studies attempting to select reassortants of I321 that contain the most commonly encountered human VP7-encoding genes and/or VP4-encoding genes are currently under way.

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