Molecular Basis of Rotavirus Virulence: Role of Gene Segment 4

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Bovine rotavirus NCDV and simian rotavirus SA-11 exhibited markedly different patterns of gastrointestinal tract disease when inoculated orally into newborn mice. A genetic approach was used to define the molecular basis of these differences. The SA-11 strain of rotavirus was more virulent than the NCDV strain when inoculated orally into newborn mice; the dose of SA-11 required to cause diarrhea in 50% of infant mice was 50-fold less than that required for NCDV. Nineteen reassortant viruses were derived by coinfection of MA-104 cells in vitro with the SA-11 and NCDV strains. The parental origin of reassortant virus double-stranded RNA segments was determined by gene segment migration differences in polyacrylamide gels and hybridization with radioactively labeled parental viral transcripts. The neutralization antigen phenotype of reassortant viruses was determined by plaque reduction neutralization. We found that the dose of SA-11 and NCDV rotavirus required to induce gastroenteritis in newborn mice was determined by gene segment 4. The results suggest that rotavirus virulence may be manipulated by modification or reassortment of gene segment 4.

Rotaviruses are the single most important cause of gastroenteritis requiring hospitalization of infants and young children in developed countries (2, 9). In developing countries, where malnutrition and poor access to medical care are constant problems, rotavirus-induced gastroenteritis is a significant cause of mortality in children less than 2 years of age (1, 19). The worldwide impact of these viruses has excited interest in disease prevention by vaccine (10, 17, 18). However, little is known about the precise molecular mechanisms determining the relative capacity of rotaviruses to produce illness in an infected host. This information may be relevant to the development of a successful vaccine.

Rotaviruses selectively infect the mature villus enterocytes of the small intestine and exhibit a predilection for the young of many animal species. One approach to understanding some of the molecular aspects of pathogenesis involves identification of the rotaviral gene or genes which determine different patterns of gastrointestinal tract disease exhibited by different strains. We have developed a murine model system for oral induction of infection and gastroenteritis with a tissue culture-adapted primate rotavirus (simian strain SA-11) (13). We determined that bovine rotavirus NCDV was less virulent for the mouse than was the SA-11 strain as indicated by the dose required to induce diarrhea. Using a genetic approach that took advantage of the high frequency of reassortment that occurs in vitro during mixed infection with two rotavirus strains, we found that genomic RNA segment 4 determined the difference between these two strains in the dose required to induce diarrhea in newborn mice.

MATERIALS AND METHODS

Animals. Conventionally bred pregnant CD-1 mice obtained from Charles River Breeding Laboratories, Inc.

(Wilmington, Mass.) were housed in isolation units. Dams were bled on arrival by retroorbital capillary plexus puncture, and sera were tested for rotavirus-specific antibodies by radioimmunoassay as previously described (14); litters whose dams were seronegative were used in these studies.

Cells and virus. MA-104 cells were grown in antibiotic-free BHK cell medium (11) supplemented with 10% newborn bovine serum.

A seed stock of SA-11 virus was obtained from H. H. Malherbe (University of Texas, San Antonio). The bovine rotavirus strain NCDV, adapted to growth in tissue culture at the University of Nebraska, was provided by Robert Yolken (Baltimore, Md.). Plaque-purified stocks of the SA-11 and NCDV strains for use in these studies were prepared in MA-104 cells.

Reassortant viruses were derived by coinfection of MA-104 cells with the SA-11 and NCDV strains of rotavirus each at a multiplicity of infection of 5.0. After 48 h of incubation at 37°C, cultures infected with SA-11 and NCDV rotaviruses were harvested by freezing and thawing. Progeny virus was titrated by plaque assay as previously described (15). Individual progeny plaques were picked, passaged twice in MA-104 cells, and characterized as described below.

Genotypic analysis of reassortant viruses by SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detection of RNA bands by silver nitrate staining were performed as previously described (15). Reassortant virus gene segment assignments were determined by a comparison of gene segment migration in SDS-PAGE with parental strains.

Genotypic analysis of reassortant viruses by RNA-RNA hybridization. The construction of ³²P-labeled, single-stranded RNA probes prepared from parental viruses and the application of these probes in hybridization studies with reassortant virus genome RNA segments were performed as previously described (4, 8).

Inoculation of animals. Five-day-old CD-1 mice were orally inoculated with 100 µl of a virus preparation. Serial

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TABLE 1. Gene segment assignments and in vivo virulence characteristics of a series of SA-11 × NCDV reassortant rotaviruses

Parent/ reassortant designation	Gene segment assignments ^a											DD ₅₀ ^b
	1	2	3	4	5	6	7	8	9	10	11	
NCDV	N	N	N	N	N	N	N	N	N	N	N	6.7
SA-11	S	S	S	S	S	S	S	S	S	S	S	5.0
S-4	N	N	N	S	N	N	N	N	N	N	N	5.2
N-4	S	S	S	N	S	S	S	NA	S	S	S	7.0
S-2,4	N	S	N	S	N	N	N	NA	N	N	N	5.0
S-2,4,11	N	S	N	S	N	N	N	N	N	N	S	4.8
S-4,7,8,9,10	N	N	N	S	N	N	S	S	S	S	N	5.3
S-4,6,7,9,11	N	N	N	S	N	S	S	NA	S	N	S	5.3
S-2,4,7,9	N	S	N	S	N	N	S	NA	S	N	N	5.3
N-5,7,11,	S	S	S	S	N	S	N	NA	S	S	N	5.0
N-1,3,6	N	S	N	S	S	N	S	NA	S	S	S	5.5
N-5	S	S	S	S	N	S	S	NA	S	S	S	5.3
N-7	S	S	S	S	S	S	N	NA	S	S	S	5.3
N-10	S	S	S	S	S	S	S	NA	S	N	S	5.2
N-9	S	S	S	S	S	S	S	NA	N	S	S	5.0
S-1	S	N	N	N	N	N	N	NA	N	N	N	7.0
S-2,11	N	S	N	N	N	N	N	NA	N	N	S	6.7
S-3,10	N	N	S	N	N	N	N	NA	N	S	N	6.4
S-7,10	N	N	N	N	N	N	S	N	N	S	N	6.7
S-7	N	N	N	N	N	N	S	NA	N	N	N	6.6
S-10	N	N	N	N	N	N	N	NA	N	S	N	7.0

^a Reassortant gene segment assignments were determined by comparison of gene segment migration in SDS-PAGE with the SA-11 and NCDV parent strains (N, NCDV; S, SA-11). We were able to distinguish SA-11 and NCDV gene segments 1, 2, 3, 4, 5, 6, 7, 10, and 11 but were unable to distinguish NCDV gene segments 8 and 9 from SA-11 gene segment 8. To determine the origin of gene segment 8, we performed RNA-RNA hybridization analysis with reassortant viruses S-4, S-7,10, S-2,4,11 and S-4,7,8,9,10. The parental origin of gene segment 8 for reassortant viruses not tested by RNA-RNA hybridization was not assigned (NA).

^b Five-day-old CD-1 mice were orally inoculated with 100 μ l of a parent or reassortant virus tissue culture fluid preparation. Serial fivefold dilutions of parent and reassortant viruses were prepared in phosphate-buffered saline, and 8 to 12 mice were inoculated at each dilution. Mice were inspected daily for the presence of diarrhea by gentle palpation of their abdomens. The DD₅₀ was defined as that parent or reassortant virus titer at which 50% of inoculated animals developed diarrhea. Titers are expressed as log₁₀ PFU/ml.

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Statistical analysis. The Student's *t* test for unpaired values was used to determine the significance of the differences in

FIG. 1. SDS-PAGE of the SA-11 and NCDV parent and S-4 reassortant rotavirus strains as detected by staining with silver nitrate. Lanes: A, NCDV; A + B, NCDV plus S-4; B, S-4; B + C, S-4 plus SA-11; C, SA-11. RNA gene segments are identified by number in order of increasing mobility. N, NCDV; S, SA-11.

DD₅₀ among reassortant viruses containing SA-11 or NCDV gene segment 4.

RESULTS

Gene segment assignments and neutralization phenotypes of reassortant rotaviruses. Nineteen reassortant rotaviruses were isolated, and gene segment assignments were determined by comparison of segment migration in SDS-PAGE with the SA-11 and NCDV parent strains (Table 1). Reassortant viruses were designated S (SA-11) or N (NCDV) based upon the least represented parental gene segments. Gene segments from the least represented parent virus were listed by number following the letter designation. For example, S-4 contained SA-11 gene segment 4 and NCDV gene segments 1, 2, 3, 5, 6, 7, 8, 9, 10, and 11. An example of the comparison of reassortant gene segments with parental gene segments by SDS-PAGE with the S-4 reassortant virus is shown in Fig. 1. All reassortant viruses were analyzed by comigration with parent strains in SDS-PAGE. We were able to distinguish all parental gene segments except for NCDV gene segments 8 and 9 and SA-11 gene segment 8 which comigrated in SDS-PAGE under our conditions of electrophoresis.

The parental virus assignments for gene segment 8 were determined for reassortment viruses S-4, S-7,10, S-4,7,8,9,10, and S-2,4,11 by RNA-RNA hybridization analysis (data not shown). S-4, S-7,10, and S-2,4,11 reassortant rotaviruses contained gene segment 8 from the NCDV parent; S-4,7,8,9,10 contained gene segment 8 from the SA-11 parent.

Pathogenic properties of the SA-11 and NCDV parent and reassortant rotavirus strains. The DD₅₀ of SA-11 virus was 50-fold less than that required for NCDV (Table 1). This difference between the SA-11 and NCDV strains was determined by gene segment 4 based on a comparison of the DD₅₀s among 19 reassortant viruses containing SA-11 or NCDV gene segment 4 ($P < 0.001$ by the Student's *t* test for unpaired values). The role of gene segment 4 was clearly illustrated by the S-4 and N-4 reassortant viruses. S-4 had the DD₅₀ phenotype characteristic of SA-11 virus despite containing only gene segment 4 from SA-11, and N-4 had the DD₅₀ phenotype of NCDV despite containing only gene segment 4 from NCDV. There were no obvious differences in the clinical disease induced by the SA-11, NCDV, or reassortant rotaviruses.

In vitro growth of the SA-11 and NCDV parent and reassortant rotavirus strains. In contrast to the differences in growth among the SA-11, NCDV, and S-4 strains in murine gastrointestinal tracts, these viruses grew well in vitro in fetal rhesus monkey MA-104 cells. At 48 h after inoculation of 10⁶ cells at 5.0 PFU per cell, virus yields were 50, 25, and 25 PFU per cell for the SA-11, NCDV, and S-4 strains, respectively.

DISCUSSION

The dose of simian rotavirus SA-11 required to cause diarrhea in orally inoculated infant mice was 50-fold less than that required to cause disease with bovine rotavirus NCDV. Using reassortant rotaviruses derived from cells coinfecting with these two strains, we found that gene segment 4 determined this difference in gastrointestinal tract virulence. Previous investigators have shown that fastidious human rotaviruses which do not undergo a productive infection in tissue culture could be rescued by genetic reassortment during mixed infection with a cultivable bovine rotavirus; genetic analysis of cultivable reassortants re-

vealed that protease-enhanced plaque formation and restriction of growth of the fastidious viruses in tissue culture was also a function of gene segment 4 (5, 8).

Biochemical studies of SA-11 virus indicated that gene segment 4 coded for an 88-kilodalton nonglycosylated outer capsid structural protein that could be modified in vitro by the proteolytic action of trypsin; cleavage of this outer capsid polypeptide was correlated with an enhancement of viral infectivity in cell culture (3). It is now clear that the trypsin-sensitive protein product of rotavirus gene segment 4 plays not only a role in the adaptation of rotaviruses to growth in tissue culture and the control of viral infectivity in vitro, but also a major role in gastrointestinal tract virulence. Determination of the degree to which our findings are predictive of the genetics of virulence in homologous host rotavirus infections awaits further study.

Rotaviruses and reoviruses are frequently compared since they are both members of the family *Reoviridae*. Because of the ready cultivability of reoviruses and the existence of several convenient systems for inducing reovirus disease in the mouse, many biological and biochemical correlates of reoviruses have been defined (7). Differences among reovirus strains in central nervous system and gastrointestinal tract pathogenicity were determined by the chymotrypsin-sensitive outer capsid protein product of gene segment M2 (6, 16). Virulence in both the reovirus and rotavirus systems appears to be determined by a protease-sensitive outer capsid protein.

Our results suggest that rotavirus virulence may be manipulated by reassortment of gene segment 4 between attenuated and virulent strains. These findings may be relevant to the development of a successful reassortant vaccine (12).

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