Murine Rotavirus Genes Encoding Outer Capsid Proteins VP4 and VP7 Are Not Major Determinants of Host Range Restriction and Virulence

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Simian rotavirus (RRV) and murine rotavirus (EDIM-RW) differ dramatically in the oral inoculum required to cause diarrheal disease in neonatal mouse pups and in their ability to spread and cause disease in uninoculated littermates. A genetic approach was used to explore the molecular basis of these differences. Reassortant viruses were produced in vivo by coinfecting infant mice with RRV and EDIM-RW. Reassortant viruses were isolated by plaque purification of progeny virus obtained from mouse pup intestines on MA104 cells. The plaque-purified reassortants were evaluated for 50% diarrhea dose (DD₅₀) and for the ability to spread and cause diarrhea in uninoculated littermates. The parental RRV strain had a DD₅₀ of 10⁵ PFU per animal, while the EDIM-RW parental strain had a DD₅₀ of less than 1 PFU per animal. RRV never spreads from inoculated to uninoculated littermates and causes disease. Twenty-three reassortants were tested. Of great interest were the reassortants D1/5 and C3/2, which derived genes 4 and 7 (encoding VP4 and VP7) from RRV. These viruses had a DD₅₀ similar or identical to that of EDIM-RW and spread efficiently from inoculated mouse pups to uninoculated pups. We conclude that the major outer capsid proteins VP4 and VP7 are not primarily responsible for virulence or host range restriction in the mouse model using a homologous murine rotavirus.

Rotaviruses are one of the leading causes of viral gastroenteritis in humans and animals worldwide (5, 12, 27). They are also an excellent model system with which to study mucosal immunity and the molecular pathogenesis of a mucosal pathogen (1, 2, 10, 38, 40).

Rotaviruses are members of the *Reoviridae* family and consist of three concentric capsids surrounding an 11-segmented, double-stranded RNA genome which has a characteristic, strain-specific migration pattern on polyacrylamide gels (12). In vitro translation studies have demonstrated that most but not all gene segments are monocistronic and code for a single protein (12). Viral protein 1 (VP1), VP2, VP3, and VP6 are structural proteins of the inner capsids, VP4 and VP7 are the outer capsid structural proteins, and the products of genes 5, 7, 8, 10, and 11 are the nonstructural proteins NS53, NS34, NS35, NS28, and NS26 (12).

Rotaviruses typically exhibit marked species specificity. In nature, rotaviruses from one species (homologous host) have only rarely been isolated from another species (heterologous host) (17, 35). The inability of rotaviruses to spread efficiently from one species to another and to persist in the new host is called host range restriction (HRR). Under experimental conditions, studies in animals and humans have shown that cross-species infections with rotaviruses can occur and occasionally result in diarrheal disease (23, 28, 39). Such symptomatic heterologous infections, however, require large inocula, and heterologous rotaviruses do not usually appear to spread efficiently in the heterologous host.

As much as a 10⁵-fold difference has been found between

the abilities of a homologous virus and a heterologous virus to cause disease (23). The difference in 50% diarrhea dose (DD₅₀; the ability to cause diarrhea in 50% of inoculated animals) between rotavirus strains is described as a difference in virulence (37). Rotavirus infection in a heterologous host can usually be described as semipermissive, since symptoms are typically dependent on exposure to a large inoculum and spread among susceptible individuals does not occur or occurs very inefficiently.

The natural attenuation of heterologous rotavirus strains in humans has been exploited to create a number of vaccine candidates. This strategy has been termed Jennerian because it uses animal rotavirus strains to immunize humans (28). Other strategies have made use of the ability of rotaviruses to undergo gene reassortment at high frequency during mixed infection to improve on the Jennerian approach by reassorting the outer capsid protein most strongly involved with serotype-specific protective immunity (VP7) onto a heterologous virus background (9, 13). These Jennerian or modified Jennerian approaches are based on the fact that host range barriers greatly, but not absolutely, attenuate virulence and restrict spread of heterologous rotaviruses in humans (14). Of note, none of the Jennerian vaccine candidates studied to date have consistently induced high levels of protection, indicating that protection from rotavirus illness may be quite complex (28, 29, 45).

The genetic basis of attenuation and HRR of the Jennerian human vaccine candidates has not been thoroughly investigated. Previous studies investigating the genetic basis of virulence of rotavirus have implicated gene 4, the gene encoding VP4. Using a heterologous murine model system and bovine × simian viral reassortants, Offit et al. linked

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gene 4 to differences in DD_{50} (37). In these studies, both the simian and the bovine rotaviruses were highly attenuated in mice in comparison with murine strains. However, the simian virus was approximately 50-fold more virulent than the bovine strain, and this virulent phenotype mapped to VP4 (37). Gorziglia et al. (19) compared the nucleic acid sequences of genome segment 4 of several purportedly virulent and avirulent human rotavirus strains and found consistent sequence similarities in the attenuated and virulent strains, respectively. This observation led these investigators to suggest that gene segment 4 might be important in determining virulence of human rotaviruses (19). The putative virulent and attenuated human strains studied by Gorziglia et al. were not directly tested in an animal model or in a volunteer trial to determine whether these phenotypes were a genetic property of the viruses in question or due to host factors.

The proposal that the outer capsid structural protein VP4 is important in virulence appears logical since VP4 has been found to have several other biologically important properties that might be linked to virulence. It is the viral hemagglutinin, is involved in neutralization, contains the trypsin activation site, and is important in protease-enhanced plaque formation (12, 22, 26, 30). It has also been linked to restricted growth of human and murine rotaviruses in cell culture and has been implicated in viral attachment to intestinal cells (2). In contrast to the genetic and molecular studies of virulence, there has been very limited examination of the basis of rotavirus HRR, and no specific gene or genes have been associated with this phenotype.

In this study, we further investigate genetic determinants of rotavirus virulence and HRR in the homologous murine system. We used an *in vivo* reassortment strategy to create a library of reassortant rotaviruses derived from the coinfection of mouse enterocytes with a virulent homologous murine rotavirus (EDIM-RW) and an attenuated heterologous simian strain (RRV). These two strains exhibited a greater than 10^5 -fold difference in DD_{50} . Our preliminary observations indicate that in this specific genetic cross, neither outer coat protein, VP4 or VP7, appears to be a primary determinant of DD_{50} or HRR.

MATERIALS AND METHODS

Cells and viruses. MA104 cells were grown as monolayers in medium 199 as previously described (47). Seed stocks of rotavirus strains with known titers were used for cell and mouse inoculations. The following rotavirus strains were used: RRV, a rotavirus of simian origin (serotype 3) originally obtained from N. J. Schmidt (24), and a tissue culture-adapted virulent murine virus, EDIM-RW, originally isolated by E. Kraft and obtained from M. Collins, Microbiological Associates, Bethesda, Md., in 1980 (49). This murine strain was adapted to cell culture (49) and is called EDIM-RW to differentiate it from other similar or identical EDIM isolates also derived from the original E. Kraft murine strain. The precise serotype of the EDIM rotavirus remains controversial, with some investigators classifying it as a type 3 virus (36) and others classifying it as not type 1, 2, 3, 4, 5, or 6 (23). The EDIM virus is not neutralized by a broadly reactive high-titer type 3 monoclonal antibody (MAb) (23), but the sequence of EDIM gene 9 (encoding VP7) is related to sequences of other type 3 strains (36).

Mice. Untimed pregnant (15 to 17 days of gestation) rotavirus-seronegative CD-1 (Swiss-Webster) female mice

were obtained from Charles River Breeding Laboratories, Portage, Mich., and shipped air freight in filtered crates. Mice were housed individually in microisolator cages (Lab Products, Maywood, N.J.) which were located in a HEPA-filtered positive-pressure Bioclean tent (Hazleton Systems, Inc., Aberdeen, Md.). Seronegative status was confirmed by bleeding and testing selected dams on arrival by immunoprecipitation and/or enzyme-linked immunosorbent assay (ELISA).

Generation of reassortants. In our hands, the cell culture-adapted murine virus (EDIM-RW) grew poorly in MA104 cells, and it was difficult to carry out mixed infection studies using a high multiplicity of infection of EDIM-RW. We therefore decided to attempt to isolate reassortants from mixed infections carried out *in vivo* in the suckling mouse gut. Four-day-old CD-1 mouse pups were orally inoculated with 10^3 PFU in 100 μ l of EDIM-RW murine virus at time 0 and subsequently inoculated with 10^7 PFU in 100 μ l of RRV simian virus at 0, 8, 16, or 24 h postinoculation with EDIM-RW. All pups were euthanatized by CO_2 inhalation 24 h after inoculation with RRV, and the intestines were removed from duodenum to rectum. Intestines were frozen, thawed, minced, and homogenized by sonication and vortexing. Intestinal homogenate was aliquoted and stored at $-70^\circ C$. Reassortants were selected from plaque titrations of trypsin-activated (5 μ g/ml) intestinal homogenates on MA104 cell monolayers as previously described (16). RRV plaqued more efficiently than EDIM-RW or EDIM-RW-like reassortants in tissue culture. To prevent RRV overgrowth, MAb 57-8 was added to the viral inoculum prior to plaque titration (32). This MAb has VP7-specific neutralizing activity against serotype 3, 4, and 6 rotaviruses. At a 1/200 concentration, it was effective in inhibiting growth of RRV but not EDIM-RW.

Reassortant progeny isolation and gene assignment. After a 6-day incubation at $37^\circ C$ and 5% CO_2 , titrations of intestinal homogenate were overlaid with neutral red (5%), and viral plaques were picked. Viruses extracted from these plaques were isolated, expanded, and genotyped by polyacrylamide gel electrophoresis as described previously (23, 26). Briefly, viral RNA was extracted from tissue culture cell supernatant with phenol-chloroform and electrophoresed for 24 h at 40 mA on 41-cm 12% polyacrylamide gels at $4^\circ C$. The migration of silver-stained reassortant RNA bands was visually compared with that of parental strains, and gene assignments were made. Because bands in the 7-8-9 region comigrate, definitive gene assignment of gene 8 encoding NS34 was made by using a ^{32}P -labeled probe for RRV gene 8 under stringency conditions in which the probe annealed only to gene 8 derived from RRV. The full-length RRV gene 8 probe expressed by pBluescript KS (3) was labeled by using random primers (Prime-it; Stratagene). Washes were carried out with $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 30 min followed by two washes with $0.1\times$ SSC at $65^\circ C$ for 30 min each time. Reassortant viral strains of interest were selected and plaque purified one additional time, and seed pools were grown, titered, regentyped, and assayed in mice.

Viral inoculation of suckling mice: phenotypic characterization. The mouse dams were allowed to deliver, and pups were inoculated via 24-gauge stainless steel feeding needles (Popper & Sons) with 100 μ l of reassortant or parental virus strains at 4 to 7 days of age. Litters, consisting of 8 to 12 pups, were divided in half. One half was inoculated with the test virus, and the other half was left uninoculated within the same cage to observe whether diarrheal disease spread

naturally within the litter. Pup abdomens were gently palpated to check for diarrhea as previously described (23) and scored positive if evidence of unformed or liquid stool (including perineal staining) was observed. Spread was deemed to have occurred when any uninoculated pup developed diarrhea. Reassortant viruses were initially screened in one litter for ability to cause diarrhea and/or spread by inoculating pups with 10^3 to 10^5 PFU of virus, a substantially higher dose than that which causes diarrhea greater than 90% of the time with the murine parental type (DD_{50} of $<10^1$ PFU) but at least 1 to 2 logs less than the DD_{50} of the simian parent, RRV. Reassortant viruses causing any signs of disease or spread at the screening dose were then investigated more fully by serial titration in suckling mice, noting DD_{50} and observing the ability to spread to uninoculated littermates at each dilution.

Quantitation of virus shedding. The amount of virus shed in the stool following inoculation with the two parental strains and the reassortants D1/5 and A11/1 was determined by using a double-antibody capture ELISA to detect antigen in a 10% mouse stool suspension. The goat capture antibody was raised to human type 1 virus, and the detection guinea pig antibody was raised to RRV. The assay appeared to be 5- to 10-fold more sensitive for RRV than for EDIM-RW.

Statistical analyses. The abilities of reassortants to cause diarrhea in inoculated mouse pups (virulence) and to cause disease in uninoculated littermates (HRR) were scored as phenotypically resembling that of either the murine parent (EDIM-RW-like) or the simian parent (RRV-like) or as being intermediate. Single gene and gene pair association with virulence or spread was tested by using the chi-square statistic from contingency table analysis (Stateview) of each of the genes except for RRV gene 4. RRV gene 4 could not be evaluated because it was present in every virus except the murine parent.

RESULTS

Viral reassortment. Initial attempts to isolate reassortants from mixed infections of RRV and EDIM-RW carried out in vitro appeared to be hampered by the poor growth and low titer of EDIM-RW. No reassortant plaques were recovered from these matings despite the use of MAb 57-8 to suppress the growth of the RRV parent. To circumvent this problem, we attempted to isolate reassortants from mixed infections carried out in vivo.

The in vivo matings yielded a total of 23 different reassortants from approximately 450 plaques picked from the intestinal homogenates of eight different mouse pups inoculated with EDIM-RW and RRV parental virus strains (Tables 1 and 2). Reassortants were isolated from mice sacrificed at each time point (0, 8, 16, and 24 h, with eight, nine, one, and five reassortants isolated, respectively). We did not isolate enough reassortants to determine whether the frequency of reassortment varied with time of inoculation. There appeared to be a tendency to isolate reassortants with more EDIM-RW genes at the later sampling time points (8, 16, and 24 h) (Table 3), but more isolates would have to be studied to draw definitive conclusions on this issue. We conclude that reassortment can occur in vivo between homologous and heterologous rotaviruses and that reassortment can occur when as much as 24 h separates the time of parental strain inoculation.

Viral genotypes. Reassortant genotypes were assigned by comparing the electrophoretic mobilities of reassortant gene segments with parental virus gene segment migration. All

TABLE 1. Genotypes of reassortants (EDIM-RW $\times$ RRV) with phenotypes resembling that of RRV

Virus	Gene segment ^a :											DD_{50}	Spread
	1	2	3	4	5	6	7	8	9	10	11		
RRV	R	R	R	R	R	R	R	R	R	R	R	$\geq 10^5$	No
EDIM-RW	e	e	e	e	e	e	e	e	e	e	e	≤ 1	Yes
B7/2	R	R	e	R	R	R	R	R	R	R	R	$>10^3$	No
D11/2	R	R	R	R	e	R	R	R	R	R	R	$>10^4$	No
A15/1	R	R	R	R	R	R	e	R	R	R	R	$>10^5$	No
A9/1	R	R	R	R	R	R	e	R	e	R	R	$>10^3$	No
E10/1	R	R	R	R	R	R	e	e	R	R	R	$>10^5$	No
D10/2	R	R	R	R	e	R	e	R	R	R	e	$>10^4$	No
A4/1	R	R	R	R	R	R	e	R	e	R	e	$>10^3$	No
F1/1	R	R	e	R	e	R	e	R	R	R	R	$>10^3$	No
B2/1	R	e	e	R	R	R	e	R	R	R	e	$>10^5$	No
B4/1	R	R	e	R	R	R	e	e	e	R	R	$>10^4$	No
B6/1	R	e	e	R	R	R	e	R	R	R	e	$>10^3$	No
D2/3	R	R	e	R	R	e	e	e	R	R	R	$>10^3$	No
C2/2	R	R	R	R	e	e	e	R	e	R	e	$>10^4$	No
E4/1	R	R	e	R	e	R	e	e	R	R	R	$>10^5$	No
E14/2	e	e	e	R	R	e	e	R	e	e	R	$>10^4$	No
H5/1	e	e	e	R	R	e	e	e	R	e	R	$>10^3$	No
E9/1	e	e	e	R	R	e	e	e	e	e	R	$>10^3$	No
D4/3	e	e	e	R	R	e	e	e	e	e	e	$>10^5$	No

^a R, RRV gene segment; e, EDIM-RW gene segment. Genes have been converted into functional equivalents based on the RRV gene-protein assignment.

genes could be unambiguously assigned except for gene segments 7, 8, and 9 which, although clearly separable between parental strains, tended to migrate too closely together in some reassortants to be reliably distinguished. In these cases, a ^{32}P -labeled probe for RRV gene segment 8 (encoding NS34) was used (Fig. 1) to clearly define the parental origin of gene 8. By using this strategy, gene origin assignments for the 7-8-9 region could be unambiguously made. Reassortants containing each gene, except EDIM-RW gene 4, from either parent were isolated. Presumably, reassortants containing EDIM-RW gene 4 were not isolated because viruses containing this gene plaqued very poorly. This supposition is consistent with prior studies indicating that gene 4 is associated with cultivability and plaque formation (26, 43).

Of the 23 different reassortants, 18 contained EDIM-RW gene 9, encoding murine VP7. This finding is not unexpected since reassortants were isolated after exposure to a neutralizing MAb directed at RRV VP7. However, five reassortants with VP7 encoded by RRV were isolated. These viruses did

TABLE 2. Genotypes of reassortants (EDIM-RW $\times$ RRV) with phenotypes resembling that of EDIM-RW

Virus	Gene segment ^a :											DD_{50}	Spread
	1	2	3	4	5	6	7	8	9	10	11		
RRV	R	R	R	R	R	R	R	R	R	R	R	$\geq 10^5$	No
EDIM-RW	e	e	e	e	e	e	e	e	e	e	e	≤ 1	Yes
D6/2	e	e	e	R	e	e	e	e	e	e	e	≤ 1	Yes
C7/4	e	e	e	R	e	e	e	e	e	e	R	≤ 1	Yes
D1/5	R	e	e	R	e	R	R	e	e	R	e	≤ 1	Yes
C3/2	R	e	e	R	e	R	R	e	e	R	e	≤ 1	Yes
A11/1	R	R	R	R	e	R	R	R	e	R	R	≤ 10	No

^a R, RRV gene segment; e, EDIM-RW gene segment. Genes have been converted to functional equivalents based on the RRV gene-protein assignments.

TABLE 3. Frequencies of EDIM-RW gene selection in reassortants isolated from mice coinfecting with RRV and EDIM-RW at time 0 versus 8, 16, and 24 h^a

0 h		8 h		16 and 24 h	
Reassortant designation	No. of genes derived from EDIM-RW ^b	Reassortant designation	No. of genes derived from EDIM-RW ^c	Reassortant designation	No. of genes derived from EDIM-RW ^c
B7/2	1	D10/2	1	E10/1	2
A15/1	1	D11/2	3	F1/1	3
A9/1	2	D2/3	4	E4/1	4
A4/1	3	C2/2	5	E14/2	6
B2/1	3	D4/3	9	E9/1	8
B4/1	4	D6/2	10	H5/1	7
B6/1	4	C7/4	9		
A11/1	2	D1/5	6		
		C3/2	6		

^a All mice were inoculated with EDIM-RW at time 0.^b Average of 2.5 EDIM-RW genes per reassortant at time 0.^c Average of 5.5 EDIM-RW genes per reassortant obtained at times 8, 16, and 24 h ($P = 0.003$).

not appear to be MAb 57-8-selected escape variants since they remained fully susceptible to neutralization and hemagglutination inhibition by MAb 57-8 and several other RRV VP7-specific MAbs with specificities similar to that of MAb 57-8 (data not shown). Rather, these isolates would appear to represent breakthrough plaques not fully inhibited by initial exposure to MAb 57-8.

Viral phenotypes. Parental strains exhibited at least a

10^5 -fold difference in the amount of infectious virus required to induce diarrhea in infant mice. The infectivity titer of the RRV inoculum (10^8 PFU/ml) was determined by plaque assay in cell culture. The infectivity titer of the EDIM-RW virus was determined by plaque assay (10^3 PFU/ml) and titration in suckling mice (10^8 mouse infectious units per ml). For purposes of comparison, the infectivity titer of EDIM-RW in vivo was used. RRV causes diarrhea in infant mice (but never spreads) up to a dilution of 10^3 -fold, in contrast to EDIM-RW, which causes diarrhea and spreads efficiently up to a 10^8 -fold dilution in neonatal mice.

Virulence and spread phenotypes (host range) were determined by assessing DD_{50} and the ability to spread and cause diarrheal disease in uninoculated littermates. By using these criteria, reassortant progeny viruses tested in suckling mice were found to closely resemble either one or the other parental strain, with only one reassortant identified with an intermediate phenotype (A11/1; Table 2).

Eighteen of the reassortants exhibited the heterologous RRV parental phenotype, with DD_{50} s of $>10^3$ PFU and with no evidence of disease or spreading to uninoculated littermates (Table 1). This RRV-like phenotype was observed in some isolates which contained very few genes from the RRV parent. For example, reassortant D4/3 contains only genes 4 and 5 from RRV yet has a DD_{50} of $>10^5$ and was not able to spread and cause disease in uninoculated littermates (Table 1). Similarly, reassortants H5/1 and E9/1 were highly attenuated in mice yet derived only three and four genes, respectively, from RRV (Table 1).

Four separately isolated reassortants representing three genotypes that resembled the virulent murine parent were identified (Table 2). An additional isolate (A11/1) had an intermediate phenotype since it had a DD_{50} lower than that of RRV but higher than that of EDIM-RW and did not spread to uninoculated littermates. Of this group of virulent reassortants, two independently isolated but identical reassortants were particularly interesting.

Reassortants D1/5 and C3/2 (Table 2) obtained nearly half of their genes from the heterologous simian parent yet were fully virulent and spread efficiently. In these two reassortants, all three major structural proteins (VP4, VP6, and VP7) are derived from RRV. Although by quantitative criteria the virulent reassortants could not be distinguished from the murine parent, subtle differences were noted in some of the reassortants. Diarrheal disease appeared identical in time of onset, duration, and type of stool produced

FIG. 1. Parental gene assignment for the 7-8-9 region of selected reassortants. (A) Silver-stained polyacrylamide gel; (B) 32 P-labeled hybridization of a Northern (RNA) blot shown in panel A with an RRV gene 8 (encoding NS34) probe. Lanes: 1, RRV; 2, EDIM-RW; 3, reassortant A11/1; 4, D1/5; 5, H5/1; 6, E14/2; 7, E4/1; 8, C2/2. Gene assignments are as indicated in Tables 1 and 2.

FIG. 2. Single gene association with virulence and HRR in reassortants derived from mice coinfecting with EDIM-RW and RRV. Gene 7, encoding NS35, is the murine gene equivalent of RRV gene 9.

between EDIM-RW and reassortants D6/2 and C7/4. On the other hand, D1/5, C3/2, and C7/4, although having mouse DD_{50} s and spread characteristics similar to those of EDIM-RW, produced a pasty, less liquid type of stool and appeared to require 1 to 2 days longer than did EDIM-RW, at the corresponding dose, to produce visible diarrheal symptoms. Spread was typically an all-or-none phenomenon in that if spread occurred, then all contact pups exhibited diarrhea within 1 to 3 days.

No single gene could be associated with virulence or HRR in all reassortants. Chi-square analysis for single gene association with virulence and HRR identified the murine gene 5 as being highly associated ($P < 0.008$) with virulence in the EDIM-RW $\times$ RRV library (Fig. 2). The murine gene 7, encoding NS35, also achieved a significant association with virulence in the EDIM-RW $\times$ RRV library ($P < 0.027$). The remaining genes in the EDIM-RW $\times$ RRV library did not achieve significant association with either virulence or HRR, having P values of greater than 0.05 (Fig. 2).

Multiple gene combinations were studied. Several murine gene pairs were found to be statistically important at $P < 0.05$ (5+2, 5+7, 5+9, 5+8, 5+10, and 5+1), but only one pair, 5+2, found significance in virulence at $P < 0.01$. This pair was found in all of the fully virulent reassortants that spread but not in any of the nonvirulent reassortants. The 5+2 pair was also missing in the intermediate reassortant A11/1, which showed a DD_{50} similar to that of EDIM-RW but did not shed antigen or spread as did EDIM-RW. The pair 5+7 was present as the only EDIM-RW genes in the intermediate reassortant A11/1. It was also found in all of the fully virulent reassortants. However, this pair was found once in a nonvirulent reassortant, C2/2, suggesting that although very important in virulence, this pair alone is not sufficient to fully confer spread characteristics and that expression is also modified by the presence or absence of other genes. A more meaningful analysis of multiple gene associations with virulence or HRR cannot be done with the present numbers of reassortants. We can conclude, however, that virulence and HRR are most likely a multiple-gene phenomenon in this reassortant rotavirus system.

Further analyses of reassortants D1/5 and A11/1 were undertaken to more fully compare their biological behavior with that of the two parental virus strains. Rotaviral antigen shedding was measured in intestines collected from mouse pups infected with RRV (10^7 PFU in 100 μ l), EDIM-RW (10^3 PFU in 100 μ l), D1/5 (10^6 PFU in 100 μ l), or A11/1 (10^7 PFU in 100 μ l) (Fig. 3). D1/5 was shed in a pattern similar to that of EDIM-RW, while RRV and A11/1 shedding was undetect-

FIG. 3. Rotavirus antigen shedding in infant mouse stool suspensions (10% by weight) as determined by ELISA. Four-day-old mouse pups were orally inoculated with 10^3 PFU of EDIM-RW ($\square$), 10^6 PFU of D1/5 ($\blacklozenge$), 10^7 PFU of A11/1 ($\triangle$), and 10^7 PFU of RRV ($\blacksquare$). Each time point represents the average of three pups. Results shown were obtained from two separate experiments.

able, consistent with failure of these viruses to spread to uninoculated littermates.

DISCUSSION

Role of the outer capsid structural proteins in virulence and HRR. Probably the most interesting findings of this study involve our clear demonstration of the absence of association of any of the three major rotavirus structural proteins, including the two outer capsid proteins, VP4 and VP7, with virulence or HRR. Genetic studies of reovirus have clearly linked the surface sigma 1 protein with virulence (42), and prior studies of rotavirus have also identified surface proteins as important factors in virulence (19, 37). Our findings, however, suggest no linkage between either VP4 or VP7 with virulence or HRR. The most striking examples of this phenomenon were the reassortants D1/5 and C3/2. These reassortants derived both surface proteins and the major inner capsid protein, VP6, from the attenuated simian parent. However, these two reassortants both have virulence characteristics similar, if not identical, to those of the murine parent EDIM-RW, and both spread readily to uninoculated littermates. It is highly unlikely that the virulent phenotype of these two reassortants is due to an extraneous mutation which occurred during selection in mice since they were isolated independently from separate matings and exhibit identical characteristics. The validity of our observation is further reinforced by other preliminary studies that we have carried out with reassortants isolated from the mating of the murine rotavirus EB with RRV (data not shown). In these studies, we also isolated fully virulent reassortants that contained gene 4 (and hence VP4) from the simian parent, RRV. Other recent studies from our laboratory also indicate that VP4 and VP7 are not major determinants of HRR. Asymptomatic rotavirus strains isolated from neonates in Bangalore, India, appear to have outer capsid proteins of bovine origin (11).

Our observations thus differ from previously published studies of mice implicating rotavirus VP4 in virulence. However, it must be remembered that the prior studies measured a relatively small differences in virulence (50-fold versus 10⁵-fold in the homologous system) between two

heterologous rotavirus strains, both of which replicated poorly in mice (37). Virulence with these heterologous strains was therefore not clearly associated with viral replication and may have been based on an early event such as attachment and entry, which indeed is likely associated with the product of gene 4, an outer capsid protein. Alternatively, the genetic basis of virulence in either system may have been highly dependent on the distinct parental virus pairs used for each study. As has been noted in previous work, recipient genetic context may effect phenotypic expression of donor genes (7, 8). However, whichever possibility proves correct, in this study we have attempted to determine the genetic basis for differences in virulence and HRR between a highly infectious and virulent murine virus and a relatively attenuated heterologous simian strain in the mouse model. In this homologous system, the major viral structural proteins do not appear to be critical determinants of virulence or HRR.

Prior studies have indicated that VP4 or VP7 or both might function as the viral attachment protein(s) or otherwise promote cell entry and hence influence virulence and/or HRR (2, 3, 15, 44). In this context, it should be noted that RRV, unlike many other heterologous strains such as human type 1 and 2 viruses, is quite capable of infecting mice and causing diarrhea, albeit only when given in larger doses than are homologous strains (4). Perhaps RRV VP4 and/or VP7 are capable of efficiently binding to and/or initiating infection in mice but other RRV gene products are not fully compatible with subsequent steps in the viral replicative cycle in mice such as assembly or release. If such a hypothesis were true, one might expect RRV to produce infection and disease in mice when administered at a high dose which infects many enterocytes on initial infection but to not be capable of multiple cycles of replication and amplification when administered at a low dose. One might expect human type 1 or 2 strains to be attenuated even when given at a high dose if they were blocked at the levels of binding or cell entry. In fact, it is the case that RRV and other serotype 3 strains can cause illness in mice when administered at a high dose but that mice appear to be highly resistant to disease when exposed to a type 1 or 2 strain (4). If reassortants were isolated from cells coinfecting with a human type 1 or 2 virus and a murine strain, the genetic basis of virulence might prove quite different for the current study. Since these human strains are substantially more attenuated in mice than is RRV and virtually never cause diarrhea, they might be restricted on the basis of binding or entry. If such were the case, murine reassortants with human serotype 1 or 2 VP4 and/or VP7 might demonstrate attenuation and/or HRR that mapped to VP4 or VP7. We are currently carrying out studies to test this hypothesis.

Role of gene segment 5 in virulence. In our studies, only the murine gene 5 was very strongly associated with the virulence and spread phenotype. Of the five reassortants which exhibited the EDIM-RW phenotype (Table 2), all possessed EDIM-RW gene 5. However, this association was not absolute, as evidenced in reassortants D10/2, F1/1, C2/2, and E4/1, which contained the EDIM-RW gene segment 5 yet had the virulence phenotype of the simian parent, RRV. EDIM-RW gene 7 (encoding NS35) was the only other single murine gene found to be significantly associated with virulence. Gene 7 was found in all the virulent reassortant phenotypes but was also present in many nonvirulent reassortants. The data are not available to enable us to draw a conclusion as to the potential mechanisms by which gene 5 or 7 would affect virulence or HRR.

Little is known concerning the gene 5 product except that

it may be involved in replication and that it shows a surprising amount of sequence divergence between species (12). However, other rotaviral reassortant studies have strongly indicated an important role for it in conferring, possibly with other genes, a replication and growth advantage in neonatal mice (18). Gombold and Ramig (18), using two heterologous viruses in mice, conducted an *in vivo* reassortant study wherein they sampled mice at several time points postinfection. Although gene segment reassortment was strikingly random overall between the two heterologous viruses *in vivo*, they found a compellingly nonrandom occurrence of SA11 gene 5, and to a slightly lesser extent SA11 gene 3, in all mice and at all time points sampled. Another reassortant study implicating gene 5 as having an important role in conferring a growth advantage used naturally occurring genetically rearranged human viruses isolated from chronically infected, immunodeficient children crossed with a bovine virus in a cell culture experiment. This study found apparently nonrandom selection for single gene segments 2, 5, 6, 9, and 11 and a gene association with 5 and 9 and a stronger selection for the triple gene association between 5, 9, and 11. Selection was linked with the parental gene constellation and host cell type (20).

From our data, the murine gene pair 5+2 was the only one to have a statistically significant association with virulence, but the biological significance is speculative. Interestingly, Gombold and Ramig (18) found gene pair 5+3 to be strongly represented in their *in vivo* heterologous reassortant system. Both VP2 and VP3, the respective protein products of genes 2 and 3, are found in pre-core replicase intermediate complexes and, in association with VP1, NS53, NS35, NS34, and NS26, are required for the synthesis of viral double-stranded RNA in infected cells (16, 31, 41). How gene products involved in RNA replication might affect virulence or HRR remains unknown. In both studies, however, one involving a homologous and one involving a heterologous system, genes involved in the replication process appear important in enhancing growth *in vivo*.

Our findings, taken together with these other studies (18, 20), point strongly to gene 5 as having an important role in conferring a selective growth advantage *in vivo*. However, it is almost certain, at least in the context of the two parental strains that we have studied, that both virulence and HRR are multigenic phenotypes. Similar complex multigenic phenotypes have also been observed in the influenza virus and the reovirus systems (6, 33, 48). Further studies of additional reassortants isolated from the mating of EDIM-RW and RRV as well as studies using other heterologous and homologous strains will be needed to completely define the genetic basis of virulence and HRR.

Reassortment *in vivo*. Previously, rotavirus reassortment has been shown to occur *in vivo* (18) as well as *in vitro* (7, 8, 21, 37). The *in vivo* studies used two heterologous strains in a mouse model (18). In addition, several epidemiologic studies have provided indirect evidence that reassortment between two distinct homologous rotavirus strains can occur in humans (24). In the present study, we have demonstrated that reassortment can occur *in vivo* when a mouse is coinfecting with a murine rotavirus and a heterologous strain. Whether such reassortment can occur in the wild remains to be demonstrated. However, at least some of our reassortants appear to manifest host range phenotypes that would permit them to continue to spread among mice if they were produced in the wild. In the case of influenza virus, reassortment between animal and human viruses occasionally leads to the evolution of new and virulent human strains (34).

Finally, it is interesting to speculate as to the mechanism by which homologous viruses appear to be able to spread more efficiently than heterologous strains. Although D1/5 and A11/1 had similar DD₅₀s, only D1/5 spread to uninoculated littermates. In the antigen shedding study using these viruses, D1/5 shedding was similar to that of EDIM-RW while A11/1 shedding resembled that of RRV in that it was not detectable above background. This finding implies that the spread phenotype is associated with higher levels of virus excretion in the stool. The separation in the A11/1 virus of the HRR and virulence phenotypes implies that these two phenotypes are not entirely related. Further studies will be needed to determine the mechanisms which separate these two properties.

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