Cultivation and Characterization of Three Strains of Murine Rotavirus

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Three distinct strains of murine rotavirus were adapted to growth in cell culture. These strains are genetically related but not identical; they are serotypically heterogeneous. The cultivatable strains were substantially more infectious (~10⁶-fold) for suckling mice than heterologous simian rotaviruses were. Homologous murine rotavirus strains spread from inoculated to uninoculated litter mates and caused diarrhea, while heterologous rotaviruses did not spread and cause illness.

Acute rotavirus infection is an important cause of infantile diarrhea in humans and many other animals (5). A number of investigators, especially those interested in veterinary medicine, have studied rotavirus infection in large domestic animals, such as pigs, lambs, and calves (2, 4, 17, 26, 32). However, the first rotavirus to be studied was isolated from an infant mouse with diarrhea. Since its original description, the murine model of rotavirus-host interaction has proven to be extremely useful for studying a variety of aspects of rotavirus infection, including pathogenesis and immunity (1, 15, 16, 21, 24, 30). The small size of mice and the ease and low cost of study are obvious advantages of this homologous rotavirus infection model. Unfortunately, difficulties in cultivating murine rotavirus strains have limited the extent to which this model can be manipulated. Until now, only one strain of murine rotavirus was adapted to growth in cell culture, and the virulence of this cell culture-adapted strain in suckling mice has not been well characterized (27).

Recently, Offit and co-workers demonstrated that oral administration of a high-titer inoculum of simian rotavirus strain SA-11 caused diarrhea in suckling mice (19, 20). This heterologous model had several advantages over the homologous murine rotavirus model, including the fact that simian rotavirus strain SA-11 is well characterized and is easily cultivated (5). While a large inoculum (~10⁶ PFU per mouse) of simian rotavirus appears consistently to cause illness in susceptible suckling mice, the input heterologous rotavirus does not replicate efficiently (19). Because heterologous rotaviruses replicate poorly in newborn mice, this model is not well suited for studying the genetic basis of rotavirus host range or the mechanism of viral clearance following infection. In order to develop a model to study the molecular basis of rotaviral virulence, we adapted three strains of rotavirus to grow in tissue culture. Two of these strains were studied in some detail. The cultivatable murine strains infect newborn mice with substantially greater efficiency than heterologous rotavirus strains. In addition, they are shed in greater amounts in the stools and can spread from one pup to another, a characteristic not found during heterologous rotavirus infection.

MATERIALS AND METHODS

Viruses. The rhesus rotavirus was originally obtained from N. J. Schmidt (11). Rotavirus strain SA-11 was originally obtained from H. Malherbe (11). Strain EW of murine rotavirus was originally obtained from J. Wolfe (30). This strain had been passaged many times in suckling mice and was felt to be a descendant from the original EDIM isolate of E. Kraft (15). Murine rotavirus strain EB was isolated from the homogenized intestine of a suckling mouse with diarrhea at the National Institutes of Health mouse breeding colony in 1982. Murine rotavirus strain EHP was isolated from the homogenized intestines of newborn mice with diarrhea in Rio de Janeiro, Brazil, in 1981 by H. G. Pereira.

Cells. Murine rotaviruses were initially adapted to grow in primary African green monkey kidney (AGMK) cell roller tubes (M. A. Bioproducts, Walkersville, Md.). Rotavirus strains EB and EW were plaque purified on secondary AGMK cell monolayers by using standard methods (31). After plaque purification the murine rotavirus strains were passaged in continuous rhesus monkey kidney cell (MA104 cell) roller tubes 10 to 20 times. The simian rotaviruses were grown in MA104 cells as previously described (11). The cells were propagated in either Eagle minimal essential medium or medium 199 supplemented with penicillin, streptomycin, glutamine, and 5% fetal calf serum. The cells were washed free of fetal calf serum prior to infection. All viruses were propagated in either medium 199 or minimal essential medium supplemented with antibiotics and 0.5 µg of trypsin (type IX; Sigma Chemical Co., St. Louis, Mo.) per ml.

Neutralization assays. A standard 60% plaque reduction assay was used to study the simian rotavirus strains (11). The murine rotavirus strains were studied by using a modified tube neutralization assay. Serial fourfold dilutions of antisera were mixed with approximately 100 50% tissue culture infective doses (TCID₅₀) of either strain EW or strain EB. After incubation for 1 h, the virus-antiserum mixtures were inoculated into roller tubes. After an additional 1 h the tubes were washed once, fed with medium 199 supplemented with trypsin, and rolled for 1 week. The tubes were then frozen and thawed once and tested by using a standard rotavirus antigen detection enzyme-linked immunosorbent

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FIG. 1. Plaque titration of murine rotavirus strain EW on primary AGMK cells. Rotavirus strain EW was activated with trypsin (5 $\mu\text{g}/\text{ml}$) for 1 h. Subsequently, serial 10-fold dilutions were inoculated onto cell monolayers. Wells 1 and 5, 10^1 dilution; wells 2 and 6, 10^{-2} dilution; well 3, 10^{-3} dilution; well 4, 10^{-4} dilution. The wells were overlaid with agarose containing 0.5 μg of trypsin per ml. The plates were fixed and stained after 7 days of incubation at 37°C .

assay (ELISA). Neutralization was defined by the complete absence of rotavirus antigen in the ELISA.

ELISAs. A standard detection ELISA was used. In this assay we used preimmune and hyperimmune goat antisera to rotavirus (goat 930) as the capture antibodies and guinea pig hyperimmune antibody (National Institutes of Health reference reagent V710-511-558) as the detection antibody (14). The two hyperimmune antisera were raised against type 1 human rotaviruses; however, both cross-reacted with all group A rotaviruses. This assay was used to test tissue culture supernatants or suckling mouse intestinal cell homogenates for rotavirus antigen. Tissue culture fluid was tested undiluted. Suckling mouse intestines (small and large intestines) were finely minced with surgical scissors and suspended in 1 ml of medium 199; 25 μl of this material per well was tested in the ELISA. Specimens were tested in duplicate. Antigen tests were called positive when the optical densities in the wells coated with immune serum were 2.5 times or more greater than the optical densities in the wells coated with preimmune serum and the values were greater than 0.1.

Antiserum production. The production and characterization of hyperimmune antisera to strain RRV, strain SA-11, and strain Wa human rotavirus have been described previously (8, 11). The production and characterization of serotype 3-specific monoclonal antibody 159 have also been described previously (10). MA104 cell culture-grown pools of strain EW and EB rotaviruses were extracted with fluorocarbon and pelleted by centrifugation at $100,000 \times g$ for 1 h. The resulting pellets were taken up in small volumes of saline and used to immunize guinea pigs. Guinea pigs were injected with serial inoculations of virus mixed with Freund adjuvant as previously described (8).

RNA analysis. Viral RNA was extracted from MA104 tissue culture cells as described previously (12). RNA electropherotypes were studied by using 12% polyacryl-

amide gels as previously described (12). ^{32}P -labeled plus-strand RNA of rotavirus strain EW was prepared in an *in vitro* polymerase reaction mixture by using purified single-shelled murine rotavirus as previously described (6). An electrophoretic analysis of the hybridization of single-stranded probe to double-stranded genomic RNA was performed as previously described (12).

Mouse inoculation. CD-1 mice (Charles River Breeding Laboratories, Inc., Portage, Mich.) that were 3 to 5 days old were inoculated orally by using a 24-gauge needle. On numerous occasions, mice from this breeding colony have been shown to be free of antibody to rotavirus (P. Offit, unpublished data). The dams used in these specific experiments were not tested for antibody to rotavirus. Individual litters were housed in separate microisolator cages (Lab Products, Inc., Maywood, N.J.). The mice were assessed daily for illness. The excretion of liquid or very soft yellow feces from the anus of a pup after gentle abdominal pressure was scored as diarrhea. Fecal staining around the anus was also scored as diarrhea. Following inoculation, pups were sacrificed daily by general anesthesia, and their intestines were tested in the ELISA. In all experiments uninoculated control litters were included. These control litters remained free of disease and infection.

RESULTS

The three strains of murine rotavirus which we studied (strains EB, EW, and EHP) were passaged 10 to 15 times in primary AGMK cell roller tubes. By the fourth passage each strain produced recognizable cytopathic effects which were primarily characterized by an increased granularity of the cells and a blurring of the cell margins. Complete destruction of the cell monolayer was not observed. The cell culture supernatants were antigen positive when they were tested in the ELISA. Strains EB and EW were plaque purified three

times in secondary AGMK cells (Fig. 1). Strain EHP was not plaque purified. Following plaque purification, strains EB and EW were passaged an additional 10 to 15 times in MA104 cell roller tubes. The cytopathic effects in MA104 cells resembled those observed in AGMK cells. The supernatants of MA104 cell roller tubes were antigen positive in the ELISA. Attempts to plaque murine strains EB and EW on MA104 cells were unsuccessful.

The genomic RNAs of MA104 cell-adapted murine strains EB and EW and of AGMK cell-adapted strain EHP were examined by using polyacrylamide gel electrophoresis (Fig. 2). The three strains differed in their overall electrophoretotypes, but all three had a characteristic migration pattern of gene segments 10 and 11. These two gene segments migrated closely together compared with rhesus rotavirus.

In order to evaluate the genetic relatedness of the three murine rotavirus strains, ^{32}P -labeled plus-strand RNA from strain EW rotavirus was hybridized to double-stranded genomic RNAs from itself and from the other two murine strains (Fig. 3). The three strains were related but clearly not genetically identical. Under identical hybridization conditions, the strain EW probe did not hybridize to detectable

FIG. 2. Polyacrylamide gel electrophoresis of genomic RNAs from three strains of murine rotavirus and one strain of simian rotavirus. Lane 1, simian rotavirus strain RRV; lane 2, murine rotavirus strain EB; lane 3, murine rotavirus strain EHP; lane 4, murine rotavirus strain EW. A 12% gel was electrophoresed at 15 mA for 15 h as previously described (13). RNA was stained with ethidium bromide (8).

FIG. 3. Autoradiography showing hybridization of double-stranded genomic RNAs from three strains of murine rotavirus to single-stranded ^{32}P -labeled RNA from murine rotavirus strain EW. Lane 1, Strain EW probe plus strain EB double-stranded RNA; lane 2, strain EW probe plus strain EHP double-stranded RNA; lane 3, strain EW probe plus strain EW double-stranded RNA. The conditions used for hybridization and electrophoresis have been described previously (7).

degrees with a variety of other mammalian rotaviruses (Fig. 4).

The serotype specificities of strains EW and EB were tested by a neutralization assay (Table 1). Strain EW was not related to human serotypes 1 and 3. In a one-way cross, hyperimmune antisera to bovine rotavirus strain NCDV and porcine rotavirus strain OSU did not neutralize strain EW (data not shown). Murine rotavirus strain EB appeared to be similar, if not identical, to two serotype 3 rotaviruses, strains RRV and SA-11 (Table 1). A VP7-specific antibody with serotype 3-specific neutralizing activity neutralized strain EB efficiently (Table 1) (10).

MA104 cell-adapted murine rotavirus strains EW and EB were inoculated per os into litters of 3- to 5-day-old CD-1 mice (Table 2). For comparison, suckling mice were also inoculated with two simian rotavirus strains (strains SA-11 and RRV). Previous studies have shown that high doses of both of these strains cause diarrhea in mice (19; Offit, unpublished data). Both of the heterologous simian strains and both of the homologous murine strains produced diarrhea of comparable severity in the suckling mice. However, the number of tissue culture infective doses required to produce diarrhea was between 10^5 - and 10^6 -fold less for

FIG. 4. Autoradiography showing hybridization of single-stranded ^{32}P -labeled RNA from murine rotavirus strain EW to genomic double-stranded RNAs from bovine, human, simian, and murine rotavirus strains. Lane 1, Strain EW probe plus double-stranded genomic rotavirus strain EW RNA; lane 2, strain EW probe plus double-stranded genomic bovine rotavirus strain NCDV RNA; lane 3, strain EW probe plus double-stranded genomic human rotavirus strain Wa RNA; lane 4, strain EW probe plus double-stranded genomic simian rotavirus strain SA-11 RNA. The conditions used for hybridization and electrophoresis have been described previously (7).

the murine strains than for the simian rotavirus strains (Table 2).

The extents of viral replication with the different rotavirus strains were studied by using the ELISA (Table 3). Intestines from mice infected with either strain SA-11 or strain RRV rotavirus did not contain sufficient viral antigen to be detected by this solid-phase assay. On the other hand, both murine rotavirus strains were detectable in mouse intestines for at least 8 days following inoculation.

In order to evaluate viral shedding, two separate 4-day-old pups per litter were inoculated with doses of rotavirus that caused diarrhea in more than 90% of the litter (strain SA-11, 5×10^6 TCID₅₀ per mouse; strain RRV, 5×10^7 TCID₅₀ per mouse; strain EB, 1×10^3 TCID₅₀ per mouse). The remaining pups in the litters were not inoculated. The spread of disease to the uninoculated litter mates was studied. In litters inoculated with the two simian strains, the uninoculated litter mates did not develop diarrhea despite the fact that the two inoculated pups developed typical illness. In the litter inoculated with murine rotavirus strain EB, all of the uninoculated pups developed diarrhea 4 to 5 days after the initial inoculation. These pups had rotavirus antigen in their intestines when they were tested by the ELISA (data not shown).

DISCUSSION

We successfully adapted three strains of murine rotavirus to grow in cell culture. We found that initial viral passage in primary monkey kidney cells was critical to successful cell culture adaptation of the three murine rotavirus strains. Similar findings have been described for some human rotavirus strains (29). The reason for the greater sensitivity of primary kidney cells for growth and plaquing of the murine rotaviruses is unclear. Since the restriction of growth of human rotaviruses is encoded on the fourth gene, it seems likely that this gene is also responsible for the fastidious nature of the murine strains (13). Following cultivation in primary cells, it was relatively easy to adapt these rotaviruses to growth in continuous monkey kidney cells. However, none of the murine strains was able to replicate in cell cultures to titers much higher than 10^5 TCID₅₀/ml. This relatively restricted growth in cell cultures is quite similar to the growth of many human rotavirus isolates (31).

The three murine strains were obtained at different times and from different geographic locations. We found that the three strains produced similar, but not identical, genomic patterns on RNA electrophoresis gels. This finding is in keeping with the electrophoretic heterogeneity found among rotavirus strains isolated from a number of other species (22, 23). The three strains had a characteristic migration pattern of genes 10 and 11, which has been demonstrated previously (25). A preliminary study of the genetic relatedness of these three strains indicated that the murine viruses are more closely related to each other than they are to several other mammalian rotaviruses. More extensive genetic studies of a number of human strains produced a similar conclusion; i.e., the human strains were closely related to each other but not closely related to other mammalian strains (7). This type of analysis might be interpreted as indicating that in the field interspecies reassortment between rotavirus strains occurs infrequently.

TABLE 1. Serotypic analysis of two tissue culture-adapted strains of murine rotavirus

Antiserum used	Plaque reduction neutralization or tube neutralization titers against the following viruses: ^a				
	Strain EW	Strain EB	Strain RRV	Strain SA-11	Strain WA
Hyperimmune guinea pig anti-EW	3,200	100	<200	ND ^b	<200
Hyperimmune guinea pig anti-EB	100	25,000	6,400	6,400	ND
Hyperimmune guinea pig anti-RRV	100	≥25,600	≥25,600	≥12,800	<200
Hyperimmune guinea pig anti-SA-11	<200	≥6,400	≥6,400	≥6,400	<400
Hyperimmune guinea pig anti-WA	<100	<100	<200	<200	≥25,600
Monoclonal anti-RRV VP7 (159)	<400	≥51,200	≥51,200	≥51,200	<400

^a Titers were determined in several different experiments.

^b ND, Not done.

TABLE 2. Infectivity titration of selected murine and simian rotavirus strains in suckling mice

Virus strain inoculated	Species of origin	TCID ₅₀ administered per 3-to 5-day-old pup ^a	% Of pups with diarrhea on day:								
			0	1	2	3	4	5	6	7	8
SA-11	Simian	5 × 10 ⁶	0	64	89	29	60	0			
		5 × 10 ⁵	0	0	0	0	0	0			
		5 × 10 ⁴	0	0	0	0	0	0			
EB	Murine	2.5 × 10 ⁴	0	0	88	83	100	33	NO ^b	NO	50
		2.5 × 10 ²	0	0	100	80	25	67	NO	NO	100
		2.5 × 10 ⁰	0	0	0	100	75	100	NO	NO	50
		2.5 × 10 ⁻²	0	0	0	0	0	0	NO	NO	0
EW	Murine	1.0 × 10 ⁴	0	0	0	75	83	40	100		
		1.0 × 10 ³	0	0	40	0	100	100			
		1.0 × 10 ²	0	0	11	58	40	66	0		
Rhesus rotavirus	Simian	5 × 10 ⁷	0	90	75	50	50	0			
		5 × 10 ⁶	0	0	14	20	33	0			
		5 × 10 ⁵	0	0	0	0	0	0			
		5 × 10 ³	0	0	0	0	0	0			
Control medium 199		0	0	0	0	0	0		0		

^a Doses were administered orally in 100 µl of medium 199 to single litters of suckling mice (9 to 16 pups).

^b NO, Not observed.

The serotypes of two of the murine rotaviruses were determined by using a neutralization assay. Of note is the fact that murine rotavirus strain EB appeared to be similar, if not identical, to several serotype 3 rotaviruses. Serotype 3 rotavirus strains have now been isolated from humans, monkeys, dogs, cats, horses, and mice (11). Murine rotavirus strain EW was not related to several previously defined rotavirus serotypes. Whether strain EW is serotypically distinct or related to other mammalian strains that were not studied by us remains to be determined. In previous studies, we showed that murine rotavirus strain EW (EDIM) could not be classified as a member of either subgroup 1 or subgroup 2 (9). On the other hand, murine rotavirus strain EB is a subgroup 1 virus (data not shown). Thus, the murine strains resemble isolates from other species (human, bovine, porcine) in their display of serologic heterogeneity.

Both of the tissue culture-adapted murine strains remained virulent for suckling mice. The percentages of pups with illness following strain SA-11, EB, and EW inoculations appeared to cycle over time (Table 2). Whether this cycling represented multiple rounds of replication or a physiologic response to infection remains to be seen. There have not been many studies of the pathogenicity of rotavirus strains following adaptation to cell culture. Some porcine strains appear to lose virulence following cultivation (3), but bovine strain NCDV was virulent during its early cell passage history (18).

The nature of the homologous murine rotavirus infections differed in several important respects from heterologous

rotavirus infections. Diarrhea could be produced by both groups of virus, but the murine strains were far more efficient at producing illness. In fact, it took between 10⁵ and 10⁶ times as much simian virus as murine virus to cause illness. Since the simian strains are capable of entering mouse enterocytes and initiating viral protein production, it is unlikely that the difference between these strains is determined primarily at the level of cell surface binding (19). From previously published studies, it is hard to determine whether the defect present in heterologous murine infections lies at the level of infectious virus production or at the level of viral spread. In any case, the data presented here clearly demonstrate a high level of host range restriction for murine infection. The molecular basis of this restriction can now be investigated by using genetic techniques (8, 12, 13).

Recently, two distinct strains of heterologous animal rotaviruses (strains NCDV and RRV) were evaluated for use as live vaccines for human rotavirus infection (13a, 28). Implicit in the choice of these animal strains as vaccine candidates was the assumption that they would be relatively attenuated in a heterologous host (humans). Our findings clearly support this assumption. In fact, infection of suckling mice with heterologous strain RRV, a human vaccine candidate, appears to be similar in several respects to infection of human infants with this simian rotavirus. In both mice and humans strain RRV replication is restricted, and viral shedding is not detectable by an ELISA. Very high-inoculum titers can cause illness in both species, but spread to other susceptible infants is restricted. Analysis of the genetic basis

TABLE 3. Shedding of rotavirus antigen in suckling mouse intestines following inoculation of murine or simian rotaviruses

Virus strain inoculated	Dose (TCID ₅₀ per mouse)	Optical density at 405 nm in a rotavirus ELISA of the intestinal homogenate on day:										
		0	1	2	3	4	5	6	7	8	9	10
RRV	5 × 10 ⁷	0.1 ^a	0.1	0.1	0.1	0.1	0.1	ND ^b	ND	ND	ND	ND
SA-11	5 × 10 ⁶	0.1	0.1	0.1	0.1	0.1	0.1	ND	ND	ND	ND	ND
EB	2.5 × 10 ⁴	0.1	0.76	0.77	0.66	1.10	1.00	ND	ND	0.50	ND	ND
EW	1 × 10 ⁴	0.1	0.1	0.33	0.46	0.49	0.64	0.33	ND	ND	ND	0.36

^a Each value is the average for two intestinal homogenates, each of which was tested in duplicate. 0.1, ≤0.1.

^b ND, Not done.

of host range restriction by using murine and simian or murine and bovine viral reassortants might define the molecular basis of attenuation of the candidate heterologous human rotavirus vaccines.

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