Extramucosal Spread and Development of Hepatitis in Immunodeficient and Normal Mice Infected with Rhesus Rotavirus

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The pathogenic profiles of two heterologous animal rotaviruses, rhesus rotavirus strain MMU 18006 and bovine rotavirus strain WC3, were evaluated in mice with severe combined immunodeficiency (SCID mice) and normal BALB/c mice. Control animals were inoculated with homologous murine strain EDIM 5099 or a tissue culture-adapted murine rotavirus. Heterologous infection with rhesus rotavirus resulted in hepatitis in 84% of SCID and 21% of BALB/c mice, with mortality rates of 27 and 0%, respectively. Surviving SCID animals developed chronic liver disease, while symptoms in BALB/c mice resolved in 2 to 4 weeks after onset. Histopathologic examination revealed a diffuse hepatitis with focal areas of parenchymal necrosis. Rotavirus was detected in liver tissue from 100% of 29 SCID and 85% (11 of 13) BALB/c animals tested by cell culture infectivity, immunofluorescence, or electron microscopy. No extramucosal spread of virus or hepatitis was observed after infection with heterologous bovine strain WC3 or homologous murine rotaviruses. This finding of a novel rotavirus-induced disease manifestation suggests altered tissue tropism in a heterologous host for a group of viruses previously shown to replicate exclusively in the gut mucosa. The implications of our observations suggest that in human vaccine trials utilizing heterologous rotavirus strains, special attention should be paid to children with immunodeficiency disorders, and screening for hepatic function should be included in vaccine protocols.

Rotavirus represents a major cause of severe diarrheal disease in human infants and other mammals, with significant morbidity as well as mortality, particularly in developing countries (4, 9). It has been estimated that rotavirus is responsible for 10⁶ deaths in children annually (10). The worldwide impact of these viruses has generated great interest in disease prevention by a specific vaccine. The application of the Jennerian concept, that cross-reactive replicating viral strains from heterologous hosts may cause silent infection in humans and induce resistance, has been most promising in the development of vaccines for human rotavirus infection (18). Currently, two candidate vaccine strains, rhesus rotavirus (RRV) strain MMU 18006 and bovine rotavirus strain WC3, are being tested in large-scale trials in infants and young children (1, 6). Strains MMU 18006 and WC3 are live rotaviruses originally isolated from the feces of a rhesus monkey and a calf, respectively (7, 30).

The pathogenesis of rotavirus disease involves an acute intestinal infection with virus replication exquisitely limited to the terminally differentiated mature enterocytes in the small intestinal villi (3). While different rotavirus types exhibit similar pathogenesis, they have distinct species specificities and only the homologous host represents the clearly permissive system. Notably, however, young children inoculated with RRV have been shown to shed infectious virus for up to 7 days after inoculation and to develop fever and loose stools more frequently than placebo-inoculated controls (20).

The rotavirus infection in the suckling mouse model, because of its similarity to the disease in human infants, has

been extensively used to study the pathogenic mechanisms and development of immunity to homologous rotavirus (8, 29). Recently, this model has also been shown to be applicable to study of the disease induced by nonmurine strains, such as human, simian, and bovine rotaviruses (2, 23, 25). However, most heterologous strains have been shown to replicate inefficiently in the intestines of suckling mice and to require an input dose 5 or 6 logs higher than that of mouse rotavirus in order to produce diarrhea consistently (12). With the use of high-titered rotavirus inoculum, this convenient animal model can be employed to study candidate vaccine strains.

In normal healthy infants, rotavirus produces an acute self-limiting diarrheal disease, whereas in children with immunodeficiency disorders, rotavirus results in a chronic infection (27). Similarly, we found a persistent murine rotavirus infection in mice with severe combined immunodeficiency (SCID mice) (26). These mice lack functional B and T cells (5). Following oral inoculation with murine rotavirus, the suckling SCID mice developed severe acute diarrhea, with low levels of virus replication in the intestine and viral shedding in the feces that continued indefinitely. However, no evidence of extramucosal spread or altered tissue tropism of homologous rotavirus was observed. The present studies were undertaken to determine the outcome of infection in SCID and immunocompetent mice inoculated with heterologous rotaviruses.

MATERIALS AND METHODS

Animals. Young adult male and female CB-17 mice with SCID (original breeders were obtained from Fox Chase Center, Philadelphia, Pa.) were shipped in isolation cages from the University of Alabama at Birmingham and from the University of Pennsylvania at Philadelphia to the Children's

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Hospital of Buffalo. On arrival, the mice were transferred in a biological safety cabinet to microisolator cages. Immuno-competent BALB/c mice, the *Igh* congenic partner of the CB-17 strain with SCID, were purchased from rotavirus-free colonies of Charles River Breeding Laboratories, Inc., Portage, Mich. All water, food, and bedding were sterilized before use.

Cells and viruses. MA104 cells are a continuous line of rhesus monkey kidney cells and were grown in medium 199, as previously described (12). RRV strain MMU 18006 (30) was passaged until a high titer was attained (1.2×10^7 PFU/ml) and then plaque purified in MA104 cells. The bovine rotavirus strain WC3, isolated and characterized by F. Clark (Philadelphia, Pa.) (7), was passaged in MA104 cells (2.5×10^8 PFU/ml). Mouse rotavirus strain EDIM 5099 (originally obtained from R. Wyatt, National Institutes of Health, Bethesda, Md.) was grown in suckling mice, and the clarified intestinal homogenate was used as inoculum. The titer of the preparation used in these experiments was 2×10^7 50% infective doses per ml. Tissue culture-adapted EDIM strain EW(TC-EDIM) was grown and passaged in MA104 cells (1.4×10^5 PFU/ml) (12). RRV and murine rotaviruses were quantitated in the cell culture immunofluorescence test, and titers were expressed as fluorescent focus units (FFU) (33).

Mouse inoculation. All virus preparations were given orally to 1- to 3-day-old animals in a volume of 10 μ l by using a micropipetter with a small disposable tip. Sufficient time and suckling was allowed for the infant mice to swallow the liquid. Groups of SCID and BALB/c mice were organized into five groups to receive the following: (i) 10 μ l of undiluted RRV (final dose 1.2×10^5 PFU [10^5 FFU]), (ii) 10 μ l of a 1:100 dilution of RRV (final dose, 1.2×10^3 PFU [10^3 FFU]), (iii) 10 μ l of undiluted WC3 (final dose, 2.5×10^6 PFU), (iv) 10 μ l of undiluted EDIM (final dose, 10^3 FFU), (v) 10 μ l of undiluted TC-EDIM (final dose, 1.4×10^3 PFU [10^3 FFU]). Animals inoculated with murine rotaviruses were kept in separate facilities from those infected with heterologous strains.

The infant mice were assessed daily for diarrhea by gentle palpation of the abdomen and by noting the color and consistency of extruded feces.

In vivo neutralization. Neutralization experiments utilizing monoclonal antibodies specific for major neutralization antigens of RRV were carried out. The monoclonal antibodies against RRV VP4 (clone 2G4) and VP7 (clone 159) have previously been characterized (28). Fifty microliters of RRV stock (1.2×10^7 PFU/ml) was incubated with 50 μ l of an equal mixture of monoclonal antibodies for 1 h at room temperature. Eight 1-day-old SCID pups were inoculated with 20 μ l of the virus-antibody mixture, and eight control pups were inoculated with 10 μ l of the virus alone. Mice were checked daily for diarrhea, as described above.

Specimen collection. Groups of anesthetized mice were sacrificed by heart puncture at timed intervals postinfection. Serum specimens were collected for cell culture isolation and, in some animals, for determination of mouse hepatitis virus antibody. The livers were excised, and biopsies were taken for histopathological and ultrastructural studies. Additional segments of liver tissue were embedded in O.C.T compound (Tissue-Tek; Miles Laboratories, Inc., Elkhart, Ind.) and frozen in isopentane-liquid nitrogen. Five-micrometer-thick sections were cut manually with a Cyrocut Microtome (American Optical Corp., Buffalo, N.Y.) and then placed on gelatin-coated slides and air dried. The slides were stored at -20°C until used for immunofluorescence staining.

TABLE 1. Development of hepatitis after inoculation with RRV or mouse rotavirus in suckling SCID and BALB/c mice

Virus type, inoculating dose (FFU), and mouse strain	No. (%) of mice				
	Tested	With hepatitis	Dead	Virus positive in liver/no. tested	
				IFA	CCI
RRV (10^5)					
SCID	49	41 (84)	13 (27)	16/19 (84)	20/22 (91)
BALB/c	92	19 (21)	0	8/11 (72)	5/7 (71)
RRV (10^3)					
SCID	21	12 (57)	2 (10)	7/7 (100)	7/7 (100)
BALB/c	32	2 (6)	0	2/2 (100)	1/2 (50)
WC3 (10^6)					
SCID	21	0	0	0/5	0/7
BALB/c	26	0	0	0/13	0/11
EDIM (10^3)					
SCID	17	0	0	0/9	NA ^a
BALB/c	35	0	0	0/14	NA
TC-EDIM (10^3)					
SCID	11	0	0	0/4	0/4
BALB/c	15	0	0	0/9	0/10

^a NA, Not applicable.

One segment of liver was homogenized in 2 ml of sterile phosphate-buffered saline per gram of tissue in a tissue grinder (Thomas Scientific, Swedesboro, N.J.). The homogenate was sonicated and clarified three times by low-speed centrifugation. All sera and tissue preparations were stored at -70°C until tested or shipped in dry ice for cell culture isolation at the VA Hospital, Palo Alto, Calif., and for mouse hepatitis virus antibody determination by enzyme-linked immunosorbent assay (24) at Charles River. All samples were sent out under code.

Virus isolation and identification. Five microliters of liver homogenate in 1 ml of medium 199 (GIBCO Laboratories, Grand Island, N.Y.) with 0.5 μ g of trypsin (type IX; Sigma Chemical Co., St. Louis, Mo.) per ml was inoculated into each well of a 24-well tissue culture plate (Costar, Cambridge, Mass.) containing a monolayer of MA104 cells. The plate was incubated for 1 h at 37°C . The inoculum was removed, and the monolayer was washed twice with medium 199. After being washed, each well was fed with 1 ml of medium 199 with 0.5 μ g of trypsin. The monolayers were checked each day for the appearance of cytopathic effect. The supernatant fluid from tissue cultures exhibiting cytopathic effect was tested for rotavirus antigen by a standard detection enzyme-linked immunosorbent assay employing preimmune and hyperimmune goat antisera as the capture antibodies and guinea pig hyperimmune antibody as the detection antibody (12). Selected viral isolates were identified by polyacrylamide gel electrophoresis (14). Random samples of liver tissue homogenates were tested for growth of bacteria by using conventional methods and for other viruses by inoculation into HEP-2, primary rhesus monkey kidney, and human embryonic fibroblast cell lines.

IFA. Cryostat tissue sections were fixed in cold acetone for 10 min. After rehydration in phosphate-buffered saline, the sections were incubated with hyperimmune guinea pig anti-murine rotavirus serum for 30 min. After being washed three times in phosphate-buffered saline, the slides were incubated with fluorescein-conjugated goat anti-guinea pig immunoglobulin G (Accurate Chemical and Scientific Co., Westbury, N.J.). The slides were then washed, air dried, counterstained with Evans blue (Sigma), and covered with a cover slip and phosphate-buffered saline-glycerol. The spec-

FIG. 1. Hematoxylin- and eosin-stained section of liver from a SCID mouse 24 days after oral inoculation with RRV showing three necrotic foci (large arrow) and an increased number of inflammatory cells (small arrow). Magnification, $\times 250$.

ificity of the immunofluorescence assay (IFA) was controlled by appropriate blocking assays by using either gnotobiotic pig antirotavirus serum (kindly provided by L. Saif, Ohio State University, Wooster) incubated on the slide before staining with IFA reagents or antiserum incubated with 10^6 PFU of RRV before use in the staining protocol. The slides were examined with a BH-2 Olympus microscope equipped with a mercury vapor bulb.

Light and electron microscopy. Consecutive sections of liver were fixed in buffered 10% Formalin solution (Lyne Laboratories, Stroughton, Mass.), embedded in paraffin, and subsequently stained with hematoxylin and eosin and used for histopathological studies. Adjacent sections of liver were fixed in 3% glutaraldehyde (EMS, Fort Washington, Pa.), processed, and stained with uranyl acetate and lead citrate for ultrastructural studies. The specimens were examined with a transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Following oral infection with RRV (10^5 FFU), 100% of SCID and 90% of BALB/c mice exhibited diarrhea characterized by watery yellow feces within 48 h. The diarrhea resolved by day six. Significantly, however, 41 of 49 (84%) SCID mice and 19 of 92 (21%) BALB/c mice developed icterus, clay- to white-colored feces, and dark yellow urine 7 to 22 days postinfection (Table 1). The mean incubation

periods for the development of hepatitis in the SCID and BALB/c mice were 11.4 and 8.6 days, respectively. Diarrhea occurred in approximately 80% of mice given a low dose (10^3 FFU) of RRV, and 57% of SCID and 6% of BALB/c mice exhibited symptoms of liver disease identical to animals given the high dose. The incubation period for clinical hepatitis in SCID mice fed 10^3 FFU of RRV was prolonged (mean, 14.5 days) compared with that in SCID mice fed the high dose of RRV (11.4 days). Fifteen of 53 symptomatic SCID mice died 4 to 32 days (mean, 11.8 days) after the onset of hepatitis, whereas no fatalities were recorded in the BALB/c mice. The surviving SCID animals exhibited a wasted appearance and remained stunted in growth with persistent hepatitis, as demonstrated both clinically and pathologically, for up to 95 days. On the other hand, the clinical hepatitis resolved in BALB/c mice in 2 to 4 weeks.

Evidence of virus replication in the liver was found in 100% of infected SCID mice, either by IFA (23 of 26) or cell culture infectivity (CCI) (27 of 29), for as long as 95 days postinoculation (Table 1). The virus was recovered from the livers of 11 of 13 (85%) BALB/c mice when tested within 7 to 30 days after infection. The remaining two animals were found to be negative for rotavirus in liver tissue in spite of the development of hepatitis. Five BALB/c mice that recovered from the disease exhibited no RRV in liver samples 4 to 11 weeks after the onset of symptoms. Seventeen of 70 RRV-inoculated SCID animals and 103 of 124 BALB/c mice did not develop hepatitis, and no virus was recovered from

FIG. 2. Electronmicrographs of lead citrate- and uranyl acetate-stained sections of liver from SCID mice sacrificed 24 days after inoculation with RRV showing virus-containing vacuolated areas (magnification, $\times 3,220$) with inset (arrow) showing virus budding (magnification, $\times 27,600$) (A); virus particles in the process of budding from membranous structures in a liver cell and enveloped particles (large arrow), complete virions, and empty capsids (small arrow) (magnification, $\times 54,600$) (B); and virus budding (small arrows) in a hepatocyte and virus clump (large arrow: inset [magnification, $\times 60,000$]) near lysosomes in a Kupffer cell (magnification, $\times 3,500$) (C).

60 of these animals tested by IFA or CCI. Virus could not be detected in samples of lung, kidney, spleen, or brain from RRV-infected SCID or BALB/c mice.

Macroscopically, liver tissue appeared pale and yellow in acutely ill animals, while it was darker and of hard consistency in the chronically infected mice. Histopathologic examination revealed an acute hepatitis with a marked infiltration of inflammatory cells and a temporal progression to necrotic foci. At 7 days postinfection, diffuse collections of mononuclear and polymorphonuclear leukocytes were scattered throughout hepatic lobules, involving all zones but predominantly in the portal areas. At 11 days after inoculation, foci of necrosis, leukocyte infiltration, clumps of mononuclear cells, and occasional multinucleated cells were observed. Subsequently, 24 days postinfection, extensive necrotic areas were found (Fig. 1). In addition, there was also a marked hyperplasia of Kupffer cells and development of fibrosis with an accumulation of periportal retrothelial nodules, inflammatory cells, and reticulin fibers.

Electron microscopic examination of ultrathin sections of the liver collected 7 to 24 days after infection demonstrated distinctive areas of virus replication. The virus was found budding from membranous structures in vacuolated cells resembling hepatocytes in SCID mice after 24 days of infection (Fig. 2). Both enveloped particles and complete

and incomplete nonenveloped viral particles were seen in the cytoplasmic vacuoles (Fig. 2). The viral budding was observed exclusively in the hepatocytes, whereas Kupffer cells contained virus particles scattered in the cytoplasm or in membrane-bound lysosomes (Fig. 2). Virus was more abundant and could be detected more readily in liver samples from SCID mice compared with those from BALB/c mice. Cryostat sections of liver stained by IFA demonstrated rotavirus antigen predominantly around the veins (Fig. 3), both in single cells and in large foci, as early as 7 and as late as 95 days after infection. Infectious rotavirus was recovered from liver homogenates in MA104 cell culture monolayers, and its identity was confirmed by a specific enzyme-linked immunosorbent assay. Polyacrylamide gel electrophoresis performed on virus isolated from the livers of infected mice demonstrated an RNA electropherotype identical to that of RRV. In addition, 16 of 17 serum specimens from symptomatic SCID mice tested by CCI were positive. None of 11 BALB/c mice tested showed evidence of viremia.

The possibility of an adventitious contaminating agent in the RRV preparation employed for these studies was ruled out by the absence of development of hepatitis or diarrhea in SCID mice fed the mixture of RRV and neutralizing monoclonal antibodies against VP4 and VP7. No virus could be isolated from their liver homogenates. All control pups

FIG. 3. Frozen section of liver stained with guinea pig anti-rotavirus serum followed by fluorescein-conjugated goat anti-guinea pig immunoglobulin G. Rotavirus antigen detected by fluorescent-antibody staining surrounding a portal vein in the liver from a SCID mouse 12 days after RRV inoculation.

suffered diarrhea, and seven of eight developed hepatitis. Rotavirus could be consistently detected in the livers of symptomatic animals. Furthermore, random liver samples tested for growth of bacteria were found to be sterile and no other viruses could be identified by CCI or by electron microscopy. Convalescent sera from 10 BALB/c mice, 5 with hepatitis and 5 asymptomatic, assayed for antibodies to mouse hepatitis virus were all negative.

Studies carried out following oral inoculation with the bovine strain WC3 are presented in Table 1. Although WC3 was administered at a higher dose (2.5×10^6 PFU) than RRV (1.2×10^5 PFU), none of the animals developed diarrhea or hepatitis. Virus could not be detected in the livers by IFA or CCI and could not be isolated from the feces.

In animals inoculated with the homologous strains of murine rotavirus, there was no evidence of development of hepatitis and no detection of virus in the liver, although 100% of these animals developed a diarrheal disease in a predictable manner. No mortality was observed in these animals; however, the SCID mice developed a persistent rotavirus infection, as reported previously (25).

DISCUSSION

The observation of particular importance in the present study is the development of hepatitis with viral replication in the liver following orally induced infection with heterologous rhesus rotavirus. The etiological role of RRV in liver disease was documented only in RRV-inoculated animals with clin-

ical symptoms of hepatitis by our findings of infectious virus by cell culture isolation, replicating viral particles by electron microscopy, and viral antigen by specific IFA. Furthermore, the disease was not seen in animals fed live virus previously neutralized by RRV-specific monoclonal antibodies. The hepatitis was severe in SCID mice, resulting in death in over one-fourth of infected animals. The extraintestinal spread of virus or viral replication in the liver was conspicuously absent following infection with heterologous bovine strain WC3 and homologous mouse rotaviruses.

It is not known whether the tropism for liver tissue and the hepatovirulence of RRV in the mouse model observed here is a characteristic unique to this particular strain of rotavirus. In preliminary studies in our laboratory using another serotype 3 simian strain (SA-11), mild hepatitis developed in SCID mice and virus was detected by CCI and IFA in the liver. Support for increased virulence in mice of serotype 3 viruses also comes from data showing that only serotype 3 of human rotaviruses consistently produces diarrhea in CD-1 mice (2). Furthermore, the bovine and murine rotaviruses used in this study failed to induce liver disease. These results suggest that hepatotropism may be determined, at least in part, by the serotype of the virus. While serotype specificity of rotaviruses has been defined by the outer capsid viral glycoprotein VP7 (17), there is recent evidence that another outer capsid protein, VP4, contains type-specific neutralization determinants (21, 28). VP4 has also been implicated in gastrointestinal tract virulence (22) and other biological

functions such as hemagglutination, protease-enhanced plaque formation, and growth restriction in cell culture (16). It has been well documented that in order for VP4 to function in infectivity, it requires cleavage by trypsin (11). The mechanism by which VP4 cleavage occurs during replication in the liver in the absence of pancreatic proteases remains to be determined. It is also unclear whether host range restriction or tissue tropism is the result of several genes or a single gene product such as VP4. In reoviruses, also members of the *Reoviridae* family, the viral hemagglutinin coded for by the S1 gene is responsible for the differences in neurovirulence between reovirus types 1 and 3 and also determines cell and tissue tropism (36). However, studies have also shown that the M2 gene segment of reovirus type 3 is responsible for relative virulence among strains of reovirus type 3, illustrating the multigenic nature of neurovirulence in these viruses (15).

In addition to tropic differences among viruses, our studies suggest that hepatovirulence and the patterns of liver damage produced by RRV are related to the immunocompetence of the host. The intact immune system may have a significant influence in limiting the accessibility of the virus to other susceptible targets. The severity and chronicity of liver disease in profoundly immunocompromised animals may imply that specific antibody and T-cell-mediated immunity are necessary to reduce the viral infectious load and subsequent spread and replication of virus in the liver. However, it should be pointed out that rotavirus replication in the liver and the development of hepatitis were also observed in normal immunocompetent mice even though the disease was less severe and the animals recovered from infection without any fatalities.

Despite the high frequency of rotavirus infection in human infants, extraintestinal localization of infectious virus has not been examined in any carefully conducted epidemiologic study. Clinically apparent hepatitis in association with rotavirus infection has not been described elsewhere, although there are several reports of elevated transaminase levels (alanine aminotransferase and aspartate aminotransferase) and one of hepatomegaly in children (19, 31, 32). Pale, fatty feces have also been associated with rotavirus infection (34). A single report exists in which rotavirus-like particles were found in a hepatic abscess (13). Heterologous rotavirus infection in children as a result of inoculation with any of the bovine or rhesus rotavirus candidate vaccine strains has not been reported to result in overt liver disease (1, 6, 18, 35). Although the relevance of these studies in mice to humans is unknown, the implications of our results suggest that screening of hepatic function should be included in vaccine trial protocols and that special attention should be paid to children with immunodeficiency disorders. In addition, these studies demonstrate that the exquisite susceptibility of the SCID mouse makes this animal model an excellent system in which heterologous rotaviruses could be studied.

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