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## Development of a Murine Model to Study the Pathogenesis of Rotavirus Infection

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A murine model to study enteritis induced by bovine (BRV) and murine rotavirus (MRV) has been developed. The course of infection was determined by clinical symptoms of diarrhea and virus isolation as well as histopathological, immunohistochemical, and electron microscopic methods. Both isolates were able to replicate and produce clinical symptoms in neonatal mice. Rotavirus-free neonates were orally inoculated with MRV or BRV and observed over a 192-hr postinoculation (HPI) period. Following infection with  $10^4$  PFU of virus, diarrhea and maximal intestinal dysfunction, as measured by xylose absorption, did not occur until beyond 20 hr postinfection even though maximal virus production occurred at 10-15 HPI. Immunohistochemically and by electron microscopy we were able to demonstrate viral antigen and virus particles in the enterocytes of villous tips at 5-8 HPI. The appearance of diarrheal symptoms was dependent on the virus dose and the type of virus isolate inoculated. The disease could be induced with doses as low as  $1 \times 10^2$  PFU/mouse of BRV and  $1 \times 10^1$  PFU/mouse of MRV. On the basis of these results, MRV was found to be more virulent than BRV in this model. The model should prove useful for studies designed to assess rotavirus virulence genes and for vaccine protection studies. This work emphasizes the need for early sample collection for critical evaluation of any vaccine or antiviral agent using this model. © 1989 Academic Press, Inc.

### INTRODUCTION

Since the discovery of rotavirus in feces of calves suffering from diarrhea (Mebus *et al.*, 1969), it has been reported that rotaviruses are ubiquitous animal and human pathogens that cause significant diarrheal diseases in most mammalian and avian species (reviewed in Babiuk, 1984; Babiuk *et al.*, 1985; Flewett and Babiuk, 1984). Rotaviruses constitute a genus of the family Reoviridae and infectious viral particles are characterized by a 70-nm-sized double-shelled capsid surrounding an icosahedral core that contains 11 segments of double-stranded RNA. They have been associated with enteritis of young animals (under 1 month) and infants (6 months to 2 years) (Flewett and Babiuk, 1984). It has been estimated that over  $500 \times 10^6$  cases of diarrhea occur annually in humans, resulting either directly or indirectly in approximately  $10 \times 10^6$  deaths, of which approximately  $1 \times 10^6$  have been attributed to rotaviruses (reviewed in Babiuk, 1984). Although an accurate estimate of numbers of diarrheal cases in animals are not available, it is believed to be at least as high as in humans, thus indicating the economic importance of this disease. It has been estimated that approximately 5% of the calves born in North America die from diarrhea before they reach 1 month of age. On the basis of these findings it has been estimated that losses in the United States average \$250 million and on a world wide basis, \$1-7 billion annually (reviewed in Ratafia, 1987).

The significant role played by these viruses in acute neonatal enteritis has created a world-wide impetus to develop an appropriate vaccine (Both and Jennings, 1987; Chanock *et al.*, 1988; Jordan, 1988; Ratafia, 1987). No such vaccine

is yet available. In order to develop effective methods of prevention and control, efforts have been directed at developing animal models for rotavirus-induced diarrhea which would mimic human (6 months to 2 years) (Conner *et al.*, 1988) and animal (<1 month) (Eiden *et al.*, 1986; Eydeloth *et al.*, 1984; Gouvea *et al.*, 1986; Offit *et al.*, 1984; Offor *et al.*, 1985; Ramig, 1988; Riepenhoff-Talty *et al.*, 1982; Starkey *et al.*, 1986; Wolf *et al.*, 1981) situations. In order to test rotavirus vaccines developed in our laboratory we have developed a murine model for murine rotavirus (MRV) and bovine rotavirus (BRV) infections, in which rotavirus-induced diarrhea can be produced in the first week of the neonate's life, thus mimicking the time when disease is prevalent in natural cases. These studies were conducted to determine the effect of virus dose on the induction of clinical disease (diarrhea) and the kinetics of virus replication in the murine intestinal tract. This model should prove useful in studies aimed at identifying rotavirus genes (Offit *et al.*, 1986) involved in the pathogenesis of the disease and expression of immunogenicity of the virus.

## MATERIALS AND METHODS

### *Animals*

Rotavirus-free mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). In order to ensure that they were not previously exposed to rotavirus, they were bled from coccygeal vein on arrival. The sera were tested for rotavirus antibodies using an enzyme-linked immunosorbent assay described previously (Ijaz *et al.*, 1987b) and all mice were found to be seronegative. To maintain them rotavirus free, the animals were housed in isolation units throughout the experiment and allowed to give birth naturally.

### *Cells and Virus*

Bovine rotavirus isolate C-486 was cultured from the feces of diarrheic calves by a method described previously (Babiuk *et al.*, 1977). Mouse rotavirus was kindly provided by Dr. A. Cepica (Atlantic Veterinary College, University of P.E.I., Canada). Both virus isolates were propagated in confluent African green monkey kidney cells (MA-104) in 1  $\mu$ g of trypsin (Difco Laboratories, Detroit, MI) per milliliter, in the absence of fetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, NY). The cells were normally cultured in Eagle's minimal essential media (MEM) supplemented with 10% FBS. BRV and MRV grown in MA-104 cells and harvested were clarified by centrifugation at 1000g for 20 min. The viruses were concentrated from the clarified supernatant fluids by pelleting through a 40% sucrose cushion at 25,000 rpm in an SW28 rotor (Beckman) for 2.5 hr at 15°C. The virus pellet was resuspended in double distilled water and the amount of virus protein was estimated spectrophotometrically as described previously (Ijaz *et al.*, 1987b).

### *Plaque Assay*

A plaque assay for the quantitation of BRV and MRV was performed according to the method described previously (Ijaz *et al.*, 1985; Smith *et al.*, 1979). The cell monolayers in 12-well plates were washed three times with MEM to remove as much of the FBS as possible from the cultures. Every well was then inoculated with 0.25 ml of an appropriately diluted virus suspension and infection was al-

lowed to take place for 1 hr at 37°C in a CO<sub>2</sub> incubator. At the end of the incubation period, the inoculum was removed and each monolayer was overlaid with 1.0 ml of a medium containing MEM, 1.5% nobel agar, 25 µg/ml pancreatin, 2 mM glutamine, 100 µg/ml DEAE dextran. The plates were incubated at 37°C for 5 days and at that time the monolayers were fixed with 10% formalin-saline and stained with crystal violet and the plaques enumerated.

#### *In Vitro Replication Kinetics of BRV and MRV*

MA-104 cells were infected with either BRV or MRV at a multiplicity of infection (MOI) of 1.0, 0.1, and 0.01 PFU/cell as described earlier (Carpio *et al.*, 1981). Following infection, the monolayer was washed to remove the unadsorbed virus. At various times postinfection, the entire culture was harvested by scraping the cells with a rubber policeman and sonicated to release intracellular virus. The virus was quantitated by plaquing on MA-104 cells as described above.

#### *In Vivo Experimental Design and Sampling Procedures*

Neonates, 7 days old, were used in all experiments. In order to determine the virus dose and kinetics of virus replication, groups of neonates (10/group/dilution) were inoculated with decreasing concentrations of each rotavirus isolate (10<sup>6</sup>, 10<sup>5.5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, 10<sup>1</sup> PFU/mouse). The preparations were administered by intubation of the stomach with a soft flexible plastic feeding tube. Trypan blue dye (GIBCO) was used as a marker to assess the accuracy of inoculation. Virus infection was evaluated clinically for 192 hr postinoculation (HPI). In order to determine the kinetics of BRV and MRV replication in neonatal mice, 10 mice from each group were sacrificed at different time intervals postinoculation up to 192 hr and their intestines were dissected out and stored at -70°C. For quantitation of the virus, each intestine was suspended in MEM (10% weight/volume), homogenized using a tissue homogenizer (Brinkman Instruments, Ontario, Canada), and centrifuged in an Eppendorf centrifuge. The supernatant was aliquoted and used for virus quantitations by the plaque assay.

#### *Xylose Absorption Test*

Xylose absorption tests were performed to measure small intestine dysfunction following infection as described previously (Ijaz *et al.*, 1987a). Briefly, animals were inoculated with BRV, MRV, or placebo (distilled water). At different times postinfection they were given D-xylose (100 µl of 5% w/v solution of D-xylose). Two hours after administration of xylose, they were sacrificed by decapitation and their blood was collected in heparinized hematocrit tubes. Plasma was collected and assayed for D-xylose concentration as described previously (Ijaz *et al.*, 1987a).

#### *Electropherotyping of Rotavirus RNA*

Electropherotyping of BRV and MRV RNA was performed as described earlier (Sabara *et al.*, 1982). Briefly, the virus isolated from intestinal samples was cultured in MA-104 cells, partially purified through 40% sucrose as described earlier, and RNA was extracted with a phenol-chloroform mixture. It was electrophoresed with a Laemmli discontinuous system on a 12.5% polyacrylamide gel for 39 hr at 20 mA. The bands of double-stranded RNA were visualized by ethidium bromide staining.

### *Isolation and Immunofluorescence Staining of Rotavirus-Infected Intestinal Epithelial Enterocytes*

Intestinal epithelial enterocytes were isolated by a procedure described previously (Riepenhoff-Talty *et al.*, 1982; Weiser, 1973). Briefly, at different times postinoculation of the virus, small intestines were removed, minced, and washed in phosphate-buffered saline (PBS). They were then placed in PBS containing 0.5 mM EDTA and agitated gently for 5 min at 37°C. This procedure resulted in a population of cells comprising approximately 90% epithelial enterocytes being released into the media. These cells were washed thoroughly, and cytospin preparations were made on clean glass slides using a Cytocentrifuge (Shandon Southern Instruments, Inc., SWEICKLEY, PA). The cells were fixed for 10 min in cold acetone, incubated with monoclonal antibody directed against virus protein-6 of BRV, and stained with a fluorescein-labeled goat anti-mouse IgG (Boehringer-Mannheim Biochemicals, Mannheim, West Germany).

### *Microscopic Analysis*

For histopathological examination of the infected tissue, samples of upper, middle, and lower small intestine were fixed in 4% buffered formaldehyde. For immunofluorescence, fresh segments of intestine were frozen in cryostat mounting media with liquid nitrogen and then kept at  $-70^{\circ}\text{C}$ . Six-micrometer frozen sections were cut in a cryostat and mounted on poly-L-lysine hydrobromide (MW  $>300,000$  Sigma Chemical Co., St. Louis, MO) 0.1% solution coated glass slides. The sections were allowed to air dry and then fixed for 5 min in cold acetone. They were washed for 5 min in PBS (0.1 M pH 7.6). Nonspecific adherence of anti-rotavirus antibody to the sections was blocked by pretreating the slides with 5.0% normal goat serum in PBS. The slides were incubated in a humid chamber for 30 minutes, excess normal serum was removed, and a 1/200 dilution of rabbit antibody to rotavirus in 1.0% normal goat serum in PBS was applied onto the sections. The slides were incubated a further 30 min in a humid chamber, followed by three washes for 5 min in PBS. A 1/200 dilution of fluorescein-labeled anti-rabbit IgG (Cappel, Malnein, PA) was applied to each section and the slides were incubated in a humid chamber for 30 min. The washing was repeated and the slides were finally mounted in buffered glycerol and examined by fluorescent microscopy (excitation 450–490 nm).

For transmission electron microscopy, samples of upper, middle, and lower small intestine were fixed for 2 hr in 3% glutaraldehyde in PBS (0.2 M pH 7.4) within 30 sec of decapitation. The glutaraldehyde-fixed specimens were postfixed in 2%  $\text{OsO}_4$  and embedded in Spurr resin. Ultrathin sections were stained in methanolic uranyl acetate (20%, w/v) followed by lead citrate and examined with the aid of a Philips Model 410 LS electron microscope at 60 kV (Coelho *et al.*, 1981).

## RESULTS

### *In Vitro Kinetics of Virus Replication*

To determine if there was a difference in the *in vitro* kinetics of replication by the two isolates, MA-104 cells were infected with either virus at a MOI of 1.0, 0.1, or 0.01 PFU/cell. Following infection with a high MOI of virus (Fig. 1) an increase in infectious virus could be detected at 4 or 5 HPI and peaked at 8 and 10 HPI for

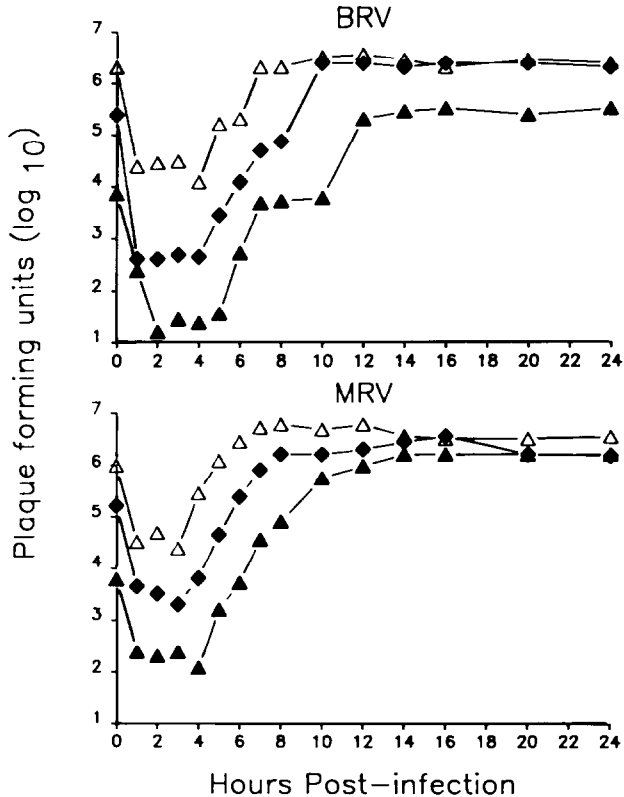


FIG. 1. Kinetics of rotavirus replication in MA-104 cells. Confluent monolayers of MA-104 cells were infected at an MOI of 1.0 ( $\Delta$ ), 0.1 ( $\blacklozenge$ ), or 0.01 ( $\blacktriangle$ ). At various times postinfection, the cells were removed and sonicated, and infectious virus was quantitated by the plaque assay.

MRV and BRV, respectively. However, when cells were infected with a lower MOI (0.1 or 0.01) appearance of increased levels of infectious virus was delayed until 5 and 6 HPI for MRV and BRV, respectively. At 0.1 and 0.01 MOI the virus peaked at 10 and 14 HPI for MRV and BRV, respectively.

#### *In Vivo Kinetics of Replication of BRV and MRV*

In order to be able to test for protection against rotavirus challenge in neonatal animals, a mouse model was developed. Initially, studies were performed to investigate the minimal amount of virus required to induce diarrhea in neonatal mice and the kinetics of replication of both BRV and MRV in murine enterocytes under *in vivo* conditions. Neonates at the age of 7 days were inoculated with either BRV or MRV in separate groups. Ten mice from each group were sacrificed at different time intervals postinoculation up to 192 hr. The results of a representative experiment are shown in Fig. 2. Samples taken within 1 hr postinoculation resulted in a titer virtually the equivalent to the inoculum (data not shown). By 5 HPI the virus titer had dropped (Fig. 2) suggesting that the virus either was inactivated by the intestinal contents or was taken up by enterocytes and converted to a noninfectious form. Of interest was the observation that as diarrhea began, the quantity of virus in intestinal contents dropped dramatically and was not detectable during the diarrheic phase (Fig. 2).

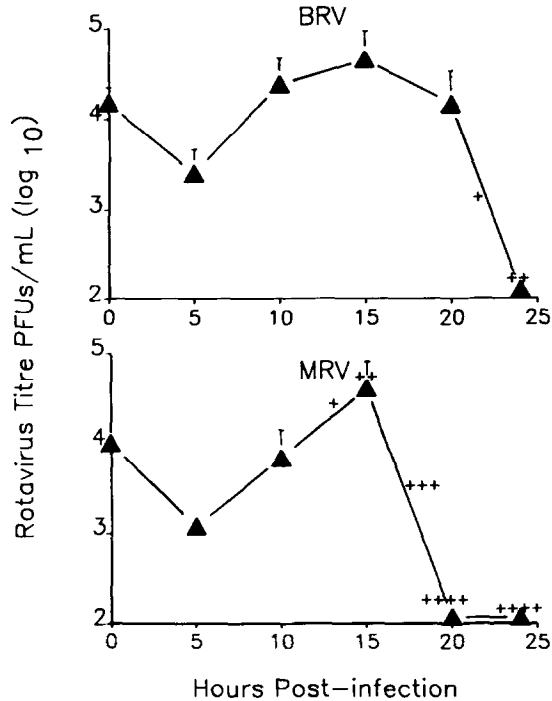


FIG. 2. Kinetics of rotavirus replication in neonatal mice. Animals were inoculated with  $1 \times 10^4$  of BRV or MRV whereas controls received mock-infected cell extract. At different time intervals, 10 mice from each group (virus inoculated and control) were sacrificed and their intestines dissected out. For virus quantitation they were suspended in 10% MEM (w/v), homogenized, and centrifuged in an Eppendorf centrifuge. The supernatant was aliquoted and used for the plaque assay. Each value represents the mean of 10 individual neonates. See Table I legend for clinical scores (+, ++, +++, +++++).

When higher virus doses (approximately  $10^6$  PFU) were used, intestinal virus did not reach the input titer during the peak of apparent virus replication, suggesting either that the replication was limited or that the high input virus resulted in rapid death and sloughing of the cells before they produced maximal levels of infectious virus (data not shown). A correlation was found between the rapidity with which intestinal functions decreased and the dose of the challenge virus (Fig. 3).

Clinically, the appearance of diarrheal symptoms was dependent on the virus dose and the type of virus isolate inoculated (Table I). In the case of BRV-inoculated animals infected with  $10^6$  PFU, the onset of diarrhea first occurred at 6 HPI. The symptoms were maximal at 14 HPI and persisted for 162 additional hr before subsiding. The onset of symptoms was delayed when neonatal mice were inoculated with the lower virus dose and the recovery was earlier than that of higher virus dose-inoculated groups. In all instances the mice inoculated with the MRV had more severe diarrhea than those inoculated with BRV. No clinical symptoms were observed when  $10^1$  PFU of BRV was inoculated, but it was evident in MRV-infected mice.

At necropsy, colon contents of control animals were found to be semisolid in consistency. In contrast, in diarrheic mice, the colon contents were liquid, mucoid, bright lemon yellow to amber to gray-green. In all diarrheic mice, the colon

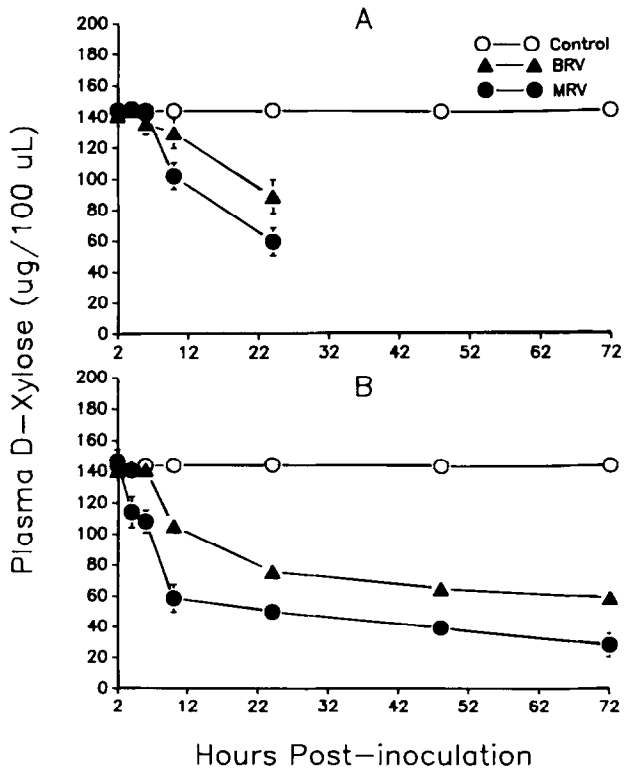


FIG. 3. Comparison of plasma D-xylose concentration between control and rotavirus-inoculated mice with  $10^4$  (A) and  $10^6$  (B) PFU/mouse of each isolate. At different times postvirus inoculation they were given D-xylose (100  $\mu$ l of 5% w/v solution of D-xylose). Two hours after D-xylose administration they were sacrificed and their blood was collected in heparinized hematocrit tubes. Plasma was collected by centrifugation of the blood-filled hematocrit tubes and was assayed for D-xylose concentration as described in the text.

and cecum were distended with gas. Infected animals also exhibited external soiling around the anal region.

#### *Xylose Absorption Test*

In order to monitor the rate of intestinal damage caused by BRV or MRV following infection of neonatal mice, a D-xylose absorption test was performed. The results of these experiments are shown in Fig. 3. In all cases, rotavirus-inoculated mice had plasma D-xylose concentrations lower than those of control mice. Also the groups inoculated with MRV revealed a decrease in xylose absorption which was maintained up to 72 HPI and was more rapid than that of animals inoculated with BRV. This was a reflection of the severity of damage caused by the MRV (Fig. 3).

None of the mock-infected animals showed clinical symptoms of diarrhea, the presence of malabsorption as determined by xylose absorption test, or the presence of infectious rotavirus as detected by the plaque assay and histopathological techniques.

#### *Histopathology and Immunohistochemical Examination of Virus-Infected Intestinal Tissue and Isolated Enterocytes*

Histopathological lesions consisting of enlarged and vacuolated cells with de-



TABLE I  
Diarrheal Score in 7-Day-Old Neonatal Mice Inoculated with Rotavirus

Time (HPI)	Bovine rotavirus dose (PFU/ml)						Murine rotavirus dose (PFU/ml)								
	$\times 10^6$	$\times 10^5$	$\times 10^4$	$\times 10^3$	$\times 10^2$	$\times 10^1$	$\times 10^5$	$\times 10^4$	$\times 10^3$	$\times 10^2$	$\times 10^1$				
0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	++++	+	-	-	-	-	+	-	-	-	-	-	-	-	-
14	+++++	++	-	-	-	-	+++	+	-	-	-	-	-	-	-
16	+++++	+++	-	-	-	-	++++	++	+	-	-	-	-	-	-
18	+++++	++++	-	-	-	-	+++++	+++	++	+	-	-	-	-	-
20	+++++	+++++	-	-	-	-	+++++	++++	+++	++	+	-	-	-	-
22	+++++	+++++	+	-	-	-	+++++	+++++	++++	+++	++	+	-	-	-
24	+++++	+++++	++	-	-	-	+++++	+++++	+++++	++++	+++	++	+	-	-
28	+++++	+++++	+++	+	-	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
32	+++++	+++++	++++	++	+	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
40	+++++	+++++	+++++	++++	++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
48	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
56	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
64	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
72	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
80	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
96	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
104	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
120	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
144	+++++	+++++	+++++	+++++	+++++	-	+++++	+++++	+++++	+++++	++++	+++	++	+	-
168	++++	++++	++++	++++	++++	-	++++	++++	++++	++++	++++	+++	++	+	-
192	---	---	---	---	---	-	---	---	---	---	---	---	---	---	-

Note. Clinical scores: (-) No signs of disease in live mice and on necropsy. (+) No external sign but semiliquid colon contents. (++) Diarrhea was present on palpation of the abdomen and colon was filled with liquid feces and gas. (+++) Externally, anal region soiled with feces and diarrhea present on palpation. (++++) On external examination, dried feces around anal region with closure of anal cavity, but on palpation diarrhea was present, fluid oozing out, severe dehydration, internal liquid content in colon and cecum and distention due to accumulation of gas.

struction of villus tips were observed in the samples taken as early as 5 HPI (MRV) and 8 HPI (BRV) (Fig. 4). Samples of intestine, taken at different times postvirus inoculation of  $10^4$  PFU/mouse (BRV or MRV), were cryosectioned and

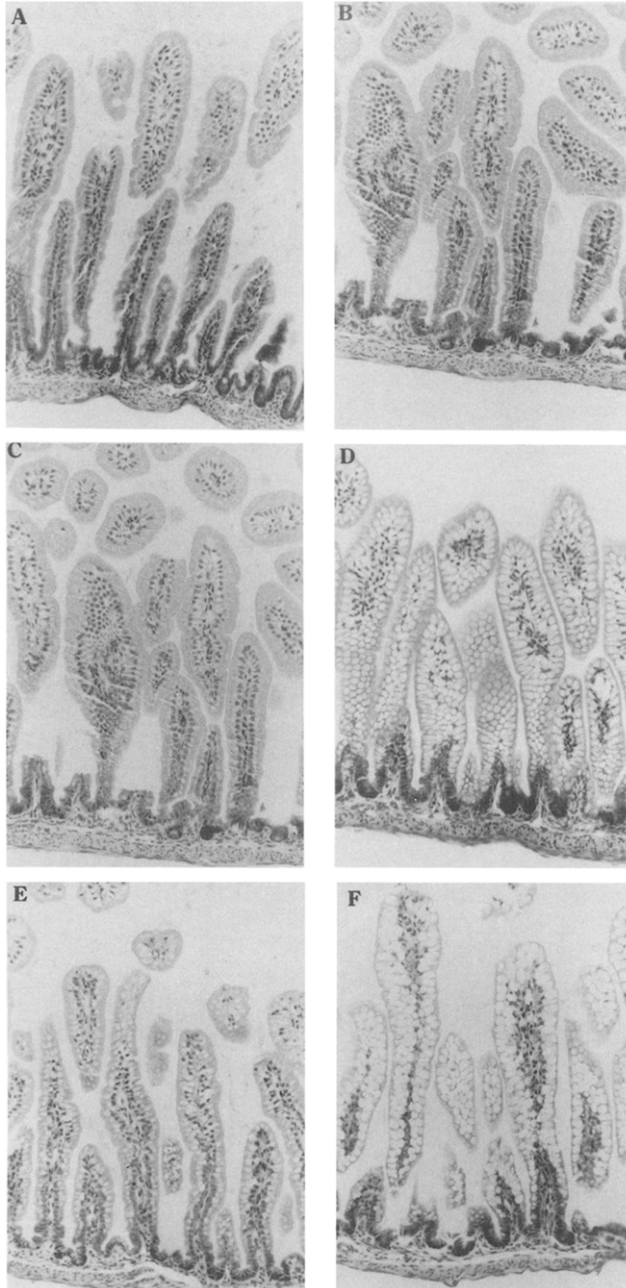


FIG. 4. Hematoxylin–eosin-stained sections of middle small intestine from BRV, MRV, and mock-infected mice prepared 5 and 8 HPI. (A and B) section from mock-infected mice prepared at 5 (A) and 8 (B) HPI. (C and D) Sections prepared from BRV-infected mice at 5 (C) and 8 (D) HPI. (E and F) Sections of MRV-infected mice prepared at 5 (E) and 8 (F) HPI. Magnification  $\times 240$ .

examined for the presence of viral antigens by indirect immunofluorescence (Fig. 5). Intestinal tissue taken from control animals showed no immunofluorescence whereas samples taken 2 HPI (BRV or MRV) showed the presence of virus antigen in the tips of the villi. This was found to increase as the infection proceeded until cells were sloughed. Furthermore, antigen was present earlier if

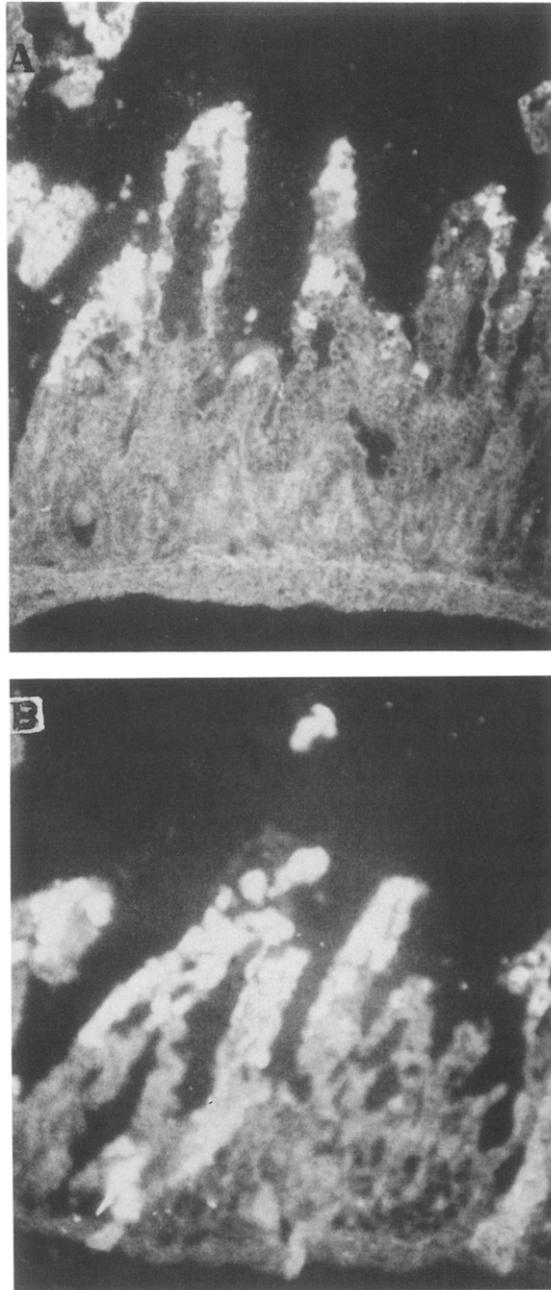


FIG. 5. Cryosections of small intestine of infant mice-infected rotavirus obtained at 5 HPI and stained with immunofluorescence reagents as described in the text A, BRV; and B, MRV.

higher multiplicities of infection were used. Virus antigen could also be demonstrated in the cytoplasm of isolated enterocytes. Fluorescence was regularly detected in material collected early in infection with the disappearance of antigen being correlated with sloughing of infected cells. A representative pattern of immunofluorescent staining of isolated virus-infected enterocytes is presented in Fig. 6.

#### *Electron Microscopic Studies*

Electron microscopy of small intestine from orally infected mice with  $4 \times 10^8$  PFU revealed extensive vacuolation in the cytoplasm of infected cells (Fig. 7) and the presence of single and double shelled particles (Figs. 8 and 9). The virus particles were seen on or between microvilli as well as within the cytoplasm (Fig. 9).

#### *RNA Migration Pattern of BRV and MRV Isolated from Intestinal Homogenates*

Due to the rapidity with which diarrhea occurred following infection, it was important to confirm that the mice were not suffering from diarrhea as a result of accidental infection from extraneous sources. This was achieved by analyzing the RNA of the recovered virus by PAGE. The rotavirus isolates recovered from intestinal suspension were identified as BRV and MRV by the characteristic electrophoretic pattern of their RNA genomes (Fig. 10).

### DISCUSSION

This study demonstrates that both homologous (MRV) and heterologous (BRV) rotavirus isolates can infect and replicate in the murine model (anti-rotavirus antibody-free mice) and infectious virus production peaks within 10–14 HPI leading to clinically yellowish diarrhea a few hours later. The MRV was much more virulent than other human or animal rotaviruses used in this model. Clinical symptoms observed using BRV or MRV in the present study were found to be in accord with EDIM as reported elsewhere (Kraft, 1982) with the only difference being that they occurred much earlier in this study than what has been reported for EDIM and other rotavirus isolates using a mouse model (Kraft, 1982; Offit *et al.*, 1984).

The observation that maximum virus production in the intestine occurred before the onset of diarrhea suggests that the virus-infected cells are sloughed and rapidly removed from the intestine resulting in diarrhea and intestinal dysfunction. This is in agreement with a proposed pathogenesis of rotavirus-induced disease. The diarrhea continues until the intestinal enterocytes are regenerated. The observation that higher doses of input virus induce diarrhea quicker indicates that more cells are killed more rapidly and to regenerate them takes longer. This suggestion is fully supported by the input multiplicity of infection and the kinetics of virus replication and induction of disease (Table I, Fig. 2).

Seven-day-old neonate mice were selected to study the kinetics of virus replication for the following reasons. First it has been demonstrated (Reipenhoff-Talty *et al.*, 1982) that maximum receptors for rotaviruses are present at 7 to 11 days of age. Second, 7-day-old mice are easier to handle than 1-day-old mice. Third, one of our goals is to investigate the potential of lactogenic immunity to protect mice from diarrhea. Results of previous experiments (Ijaz *et al.*, 1987b) revealed that the highest titer of anti-rotavirus neutralizing antibodies is present at 7 to 9 days

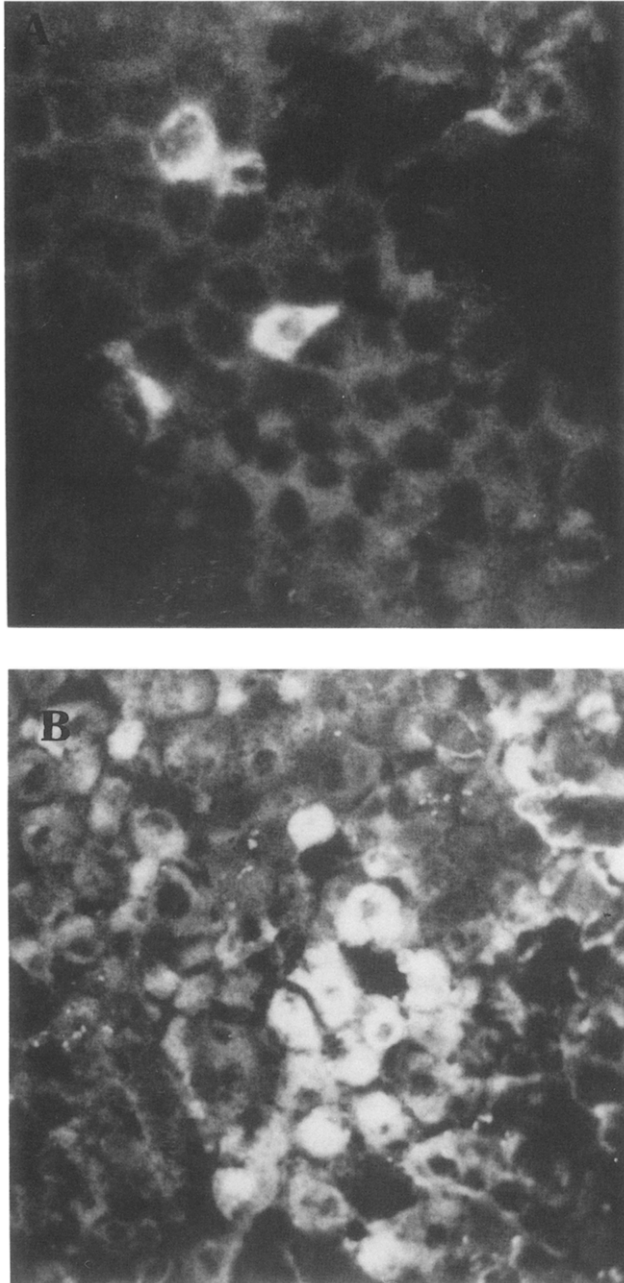


FIG. 6. Localization of rotavirus antigen in the cytoplasm of enterocytes isolated from A, BRV-infected (5 HPI); and B, MRV-infected (5 HPI) suckling mice.

postparturition in mice. All these features make it ideal to use this animal model in protection studies.

The virus titer in intestinal tissue sample taken 1 HPI never exceeded the inoculated virus dose which indicates that these isolates were undergoing the initial phases of replication cycle. This observation is consistent with previous

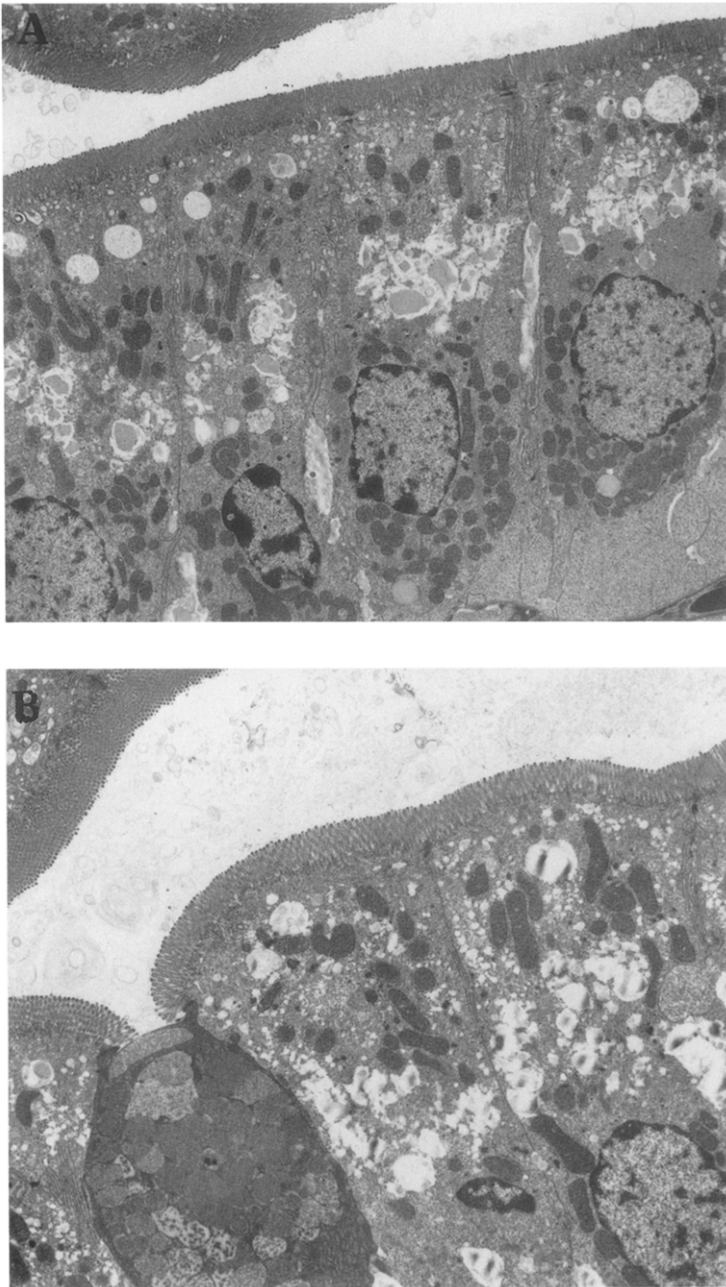


FIG. 7. Transmission electron micrograph of (A) mock-infected mice and (B) MRV. Note the accumulation of small vacuoles in virus-infected cells. Magnification  $\times 3840$ .

reports (Eydelloth *et al.*, 1984; Gouvea *et al.*, 1986; Kraft, 1982; Offit *et al.*, 1984; Ramig, 1988; Starkey *et al.*, 1986; Wolf *et al.*, 1981). However, the peaks of infectious virus occurred earlier than in other studies, suggesting that the rate of replication of both the isolates is much faster than other human and animal rotavirus isolates used in mouse models. Among the two isolates used, MRV has been

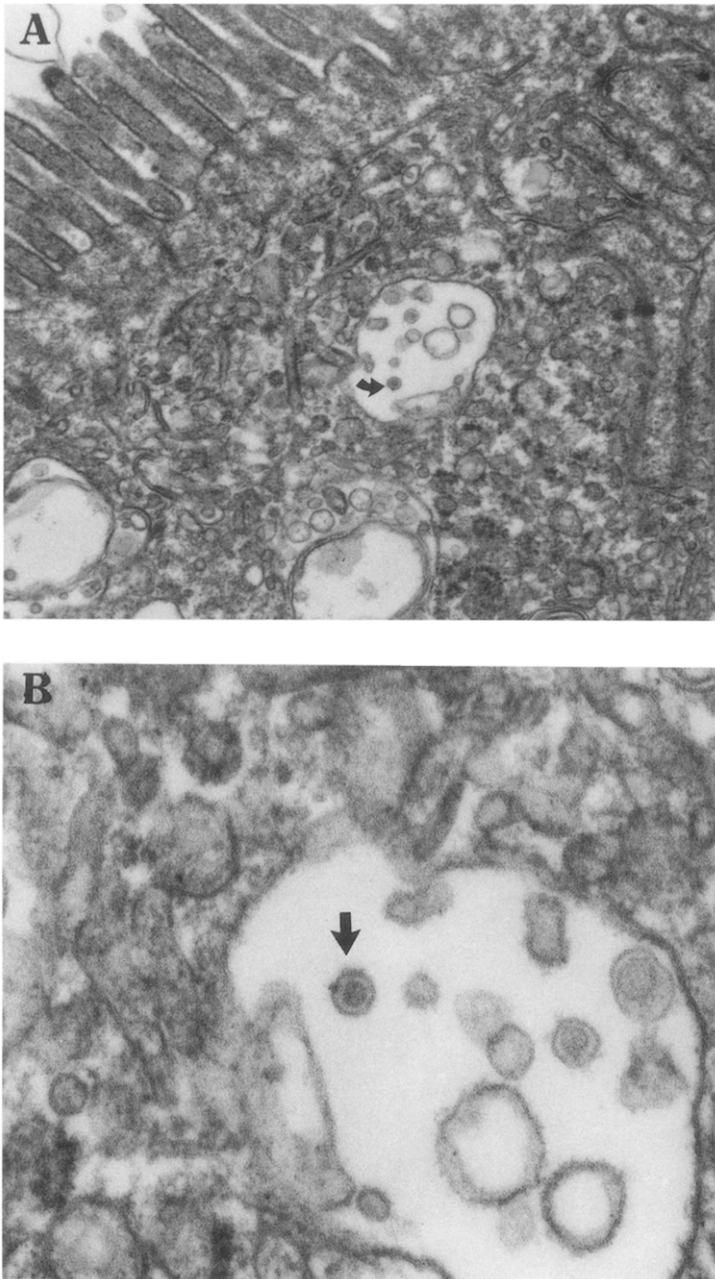


FIG. 8. Transmission electron micrograph showing an area of cytoplasm of an enterocyte with clumps of MRV particles, both incomplete and complete particles (arrow) are seen. Magnification: A,  $\times 38,400$ ; B,  $\times 121,600$ .

found to be more virulent than BRV in this model, as was evident clinically and by the use of the D-xylose absorption test. These differences are consistent with our earlier *in vitro* and *in vivo* studies comparing these two isolates (Sabara *et al.*, 1989).

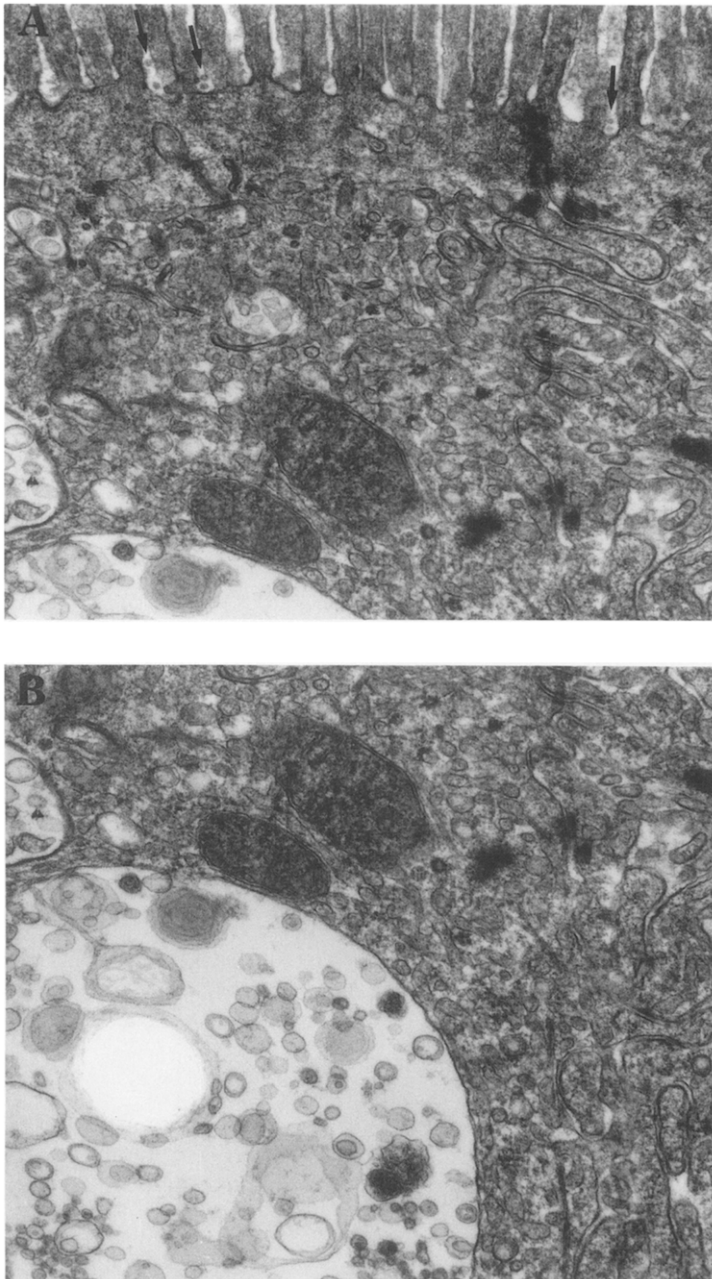


FIG. 9. Transmission electron micrograph showing BRV particles in cytoplasm (A) and in between microvilli (B) of an enterocyte. Magnification  $\times 38,400$ .

The results of the present study on the infection and replication of both homologous and heterologous rotavirus in a murine model confirm and extend the reports of others (Eydelloth *et al.*, 1984; Gouvea *et al.*, 1986; Kraft, 1982; Offit *et al.*, 1984; Ramig, 1988; Starkey *et al.*, 1986; Wolf *et al.*, 1981). However, our results differ from previous reports in several respects. (1) Most other studies



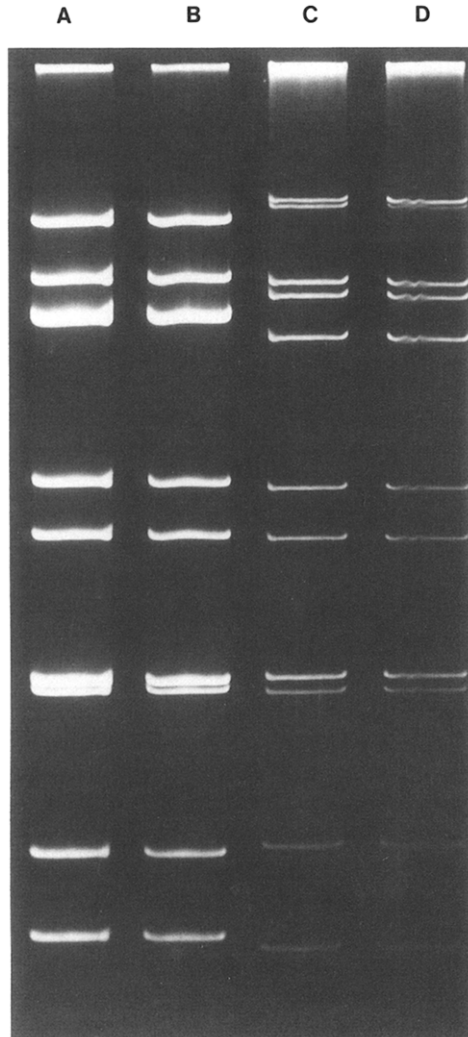


FIG. 10. Polyacrylamide gel electrophoresis of dsRNA of (a) BRV, (b) BRV, recovered from the intestinal homogenate of infected mice, (c) MRV, and (d) MRV recovered from the intestinal homogenates of infected mice. Electrophoresis was carried out as described under Materials and Methods.

used approximately  $10^6$  PFU/mouse of both heterologous as well as homologous rotavirus to demonstrate the replication and disease symptoms and that lower doses of virus would not produce disease in mice. In our study, we not only demonstrated the replication, but also production of clinical symptoms at doses as low as  $10^1$  PFU/mouse (Table I). (2) Clinically, diarrhea was observed much earlier than in other studies (Eydelloth *et al.*, 1984; Gouvea *et al.*, 1986; Kraft, 1982; Offit *et al.*, 1984; Ramig, 1988; Starkey *et al.*, 1986; Wolf *et al.*, 1981). If high doses were used, symptoms were observed as early as 4 HPI and continued up to 192 HPI. In other studies it took about 24–48 hr following infection to induce clinical symptoms. (3) The length of clinical symptoms and the time required to recover from infection were longer than those reported by other investigators. (4) The biphasic nature of infectious rotavirus or rotavirus antigen production as

reported in some studies using mice was not seen in the present study. The drop in infectious rotavirus titer at 20 HPI could be seen as a result of accelerated virus clearance associated with the start of diarrheal symptoms and loss of enterocytes bearing rotavirus specific receptors required for infection to continue. No infectious virus was recovered between 24 and 192 hr postinfection, despite the presence of diarrhea symptoms. These differences can all be explained by the high virulence for mice of the specific isolates used in this study.

The pathogenesis of rotavirus infection has also been investigated in other animal models. These include gnotobiotic calves (Mebus *et al.*, 1969; Tzipori *et al.*, 1980), lambs (Snodgrass *et al.*, 1977; Tzipori *et al.*, 1980), and piglets (Crouch and Woode, 1978; Gaul *et al.*, 1982; Hall *et al.*, 1976; Leece *et al.*, 1978; Theil *et al.*, 1978; Tzipori *et al.*, 1980). The virus tropism in these animals, like the murine model described here, is limited to enterocytes lining the upper regions of intestinal villi. However, the pathology is extremely severe in piglets in which complete erosion of jejunal villi has been reported (Theil *et al.*, 1978). The denudation of the upper regions of affected villi has been observed in these species leaving lamina propria exposed. This type of pathology was not observed in this study with either BRV or MRV infection. Similarly infections by other animal and human rotaviruses in murine models have not been reported to cause such severe pathological lesions (Coelho *et al.*, 1981).

In earlier experiments, the virulence of these two rotavirus isolates was also compared in our calf model using a ligated intestinal loop technique (Sabara *et al.*, 1989). The results revealed that in cattle MRV was also more virulent since it induced more fluid in the intestinal loops than did BRV (Sabara *et al.*, 1989). The differences in the virulence of BRV and MRV were further confirmed under *in vitro* conditions (Sabara *et al.*, 1989). A comparison of the kinetics of the protein synthesis revealed that no BRV proteins were identifiable at 2 HPI, whereas in the case of MRV, several virus-specific proteins were present at this time. These results correlate with our *in vitro* observations of the cytopathogenic effect (CPE) in MA-104 cells infected with MRV, CPE occurs almost immediately after virus adsorption to the cells, whereas for BRV, CPE was not observed until about 8 hr after adsorption (unpublished data).

It has been demonstrated that BRV and MRV used in this study differ from each other as far as their VP4 is concerned. Analysis of BRV and MRV VP4 by carboxypeptidase digestion revealed only one region of heterogeneity between these two viruses in the area spanning amino acids 307 and 407. The difference between BRV and MRV has been further manifested on the basis of their sensitivity to chelating agents (Sabara *et al.*, 1989). The observed difference in the virulence of these two viruses under *in vivo* conditions may be a consequence of the observed differences in the outer shell proteins of MRV when compared to BRV. More specifically, the difference in the primary structure of the VP4 (amino acid 307 and 407) may be one of the factors contributing to the increased virulence of MRV.

The results of the above-mentioned *in vitro* studies taken together with our *in vivo* data tend to support the replication kinetics of both BRV and MRV in the model used in the present study. The development of this model for rotavirus has provided a tool which will be useful for studying the pathogenesis of the virus as well as facilitating the *in vivo* evaluation of different anti-viral agents and vaccines produced against these viruses.

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