Pathogenicity of Rotavirus in Rabbits

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The role of rotavirus in diarrheal disease of rabbits was investigated, and a model for human rotavirus infection was established. Orogastric inoculation of 8- and 12-week-old New Zealand White rabbits with a rabbit strain of rotavirus (L:ALA:84) resulted in fecal shedding of virus for 6 to 8 days from 2 to 5 days after inoculation. Most rabbits exhibited diarrhea, coincident with the onset of viral shedding, which persisted for 2 to 4 days. Diarrhea was characterized by soft or fluid stools and fecal staining of the perineum. Inoculation of 3-week-old rabbits resulted in a briefer period of viral shedding and diarrhea of a milder nature. Histopathologic examination during the period of viral shedding revealed a mild, nonsuppurative enteritis. Inoculated rabbits exhibited antibodies in serum to rotavirus by enzyme-linked immunosorbent assay. Sham-inoculated or uninoculated rabbits maintained in the same cage or the same room with inoculated rabbits acquired rotavirus infection. The mild diarrheal disease which resulted with a rotavirus isolate from severe field cases suggests that cofactors were involved.

Rotaviruses have been associated with diarrheal disease in rabbits from Great Britain (1), Europe (2, 8, 9), Japan (12), and the United States (5, 13). In nearly all reports, weanling rabbits 4 to 6 weeks old were affected. In endemically infected colonies, rotavirus infection caused low morbidity in weanling rabbits (5, 9). In two outbreaks (8, 13), high morbidity and mortality in rabbits 1 to 3 weeks old occurred, suggesting that rotavirus may have been recently introduced into the colonies. However, other enteric pathogens were also identified, making it difficult to elucidate the role of rotavirus in producing diarrheal disease. Hence, experimental studies were undertaken to examine the pathogenicity of rotavirus in specific-pathogen-free rabbits by using a rotavirus isolated from one of the outbreaks of severe disease in rabbits (13).

MATERIALS AND METHODS

Animals. The New Zealand White rabbits used in this study were from our own specific-pathogen-free colony, which has been free of rotavirus since its establishment in September 1984. Rabbits were monitored quarterly for the presence of rotavirus in the feces and rotaviral antibodies in the sera. For the experimental inoculations, rabbits were housed in conventional facilities separate from other rabbits and handled under specific-pathogen-free conditions. Rabbits were housed individually in stainless steel cages with wire floors. The lighting cycle was 12 h of illumination and 12 h of darkness. Commercial feed and water were available ad libitum. Room temperature was maintained at 20 to 22°C, relative humidity was 40 to 50%, and the room was ventilated at a rate of 12 air changes per hour. Individuals entering the room wore foot covers, gloves, masks, and coats, which were kept in the same room. All supplies and materials used were reserved for that room only. Three age groups of rabbits were used: two litters of 3-week-old rabbits which were being nursed by their dams, 8-week-old rabbits which had been weaned 2 weeks previously, and 12-week-old rabbits.

Inoculation and sampling. Rabbits were sedated with ketamine hydrochloride (30 mg/kg) and acepromazine (0.1 mg/kg) given intramuscularly. In 8- and 12-week-old rabbits, 2.0 ml of rotavirus was inoculated through an infant feeding tube (8 French [FR]; Cutter Laboratories, Inc., Berkeley, Calif.) into the stomach. In 3-week-old rabbits, 0.5 ml of rotavirus was inoculated through an infant feeding tube (5 FR; Cutter Laboratories) into the stomach. In the first experiment, eight 8-week-old rabbits were inoculated. Two rabbits received tissue culture media (sham inoculated), two rabbits received 2×10^4 fluorescent focus-forming units (ffu) of tissue culture virus, two rabbits received 2×10^7 ffu of tissue culture virus, and two rabbits received stool filtrate obtained from a 3-week-old diarrheic rabbit (13). Since there was no difference in the response of 8-week-old rabbits to virus in stool filtrate or tissue culture virus, all subsequent experiments employed the higher-titer tissue culture virus as the inoculum. Ten 12-week-old rabbits were inoculated with 2×10^7 ffu of tissue culture virus, while two rabbits were inoculated with tissue culture media (sham inoculated). Two does and their respective litters were used. Litter 65813 consisted of eight pups 16 to 18 days old and litter 06931 consisted of nine pups 20 to 21 days old. A total of 15 pups were inoculated with 5×10^6 ffu of tissue culture virus, while the does and 1 pup from each litter were not inoculated (sentinels). Before inoculation of one litter, three 10-week-old rabbits were placed in the room and remained uninoculated (sentinels). Rabbits 8 and 12 weeks old were caged individually, and sham-inoculated rabbits were located in a separate cage rack in the same room. Does and litters were housed in kindling cages until the litter was 6 weeks old (about 3 weeks after inoculation). Sham-inoculated and sentinel rabbits were handled before inoculated rabbits were. Fresh feces, expressed from the anus or rectal swabs (pups only), were obtained daily; and a clotted blood sample was collected before inoculation and weekly after inoculation.

Virus. A rotavirus (L:ALA:84) isolated from the intestinal contents of preweanling rabbits which died of diarrheal disease in a specific-pathogen-free colony was used (14). Ma 104 cells were used to propagate the virus.

Enzyme-linked immunosorbent assay for rotavirus detection. A capture enzyme-linked immunosorbent assay was

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used for the detection of rotavirus in feces from rabbits (14). Half of a microELISA plate (Costar, Cambridge, Mass.) was coated with preimmune rabbit serum and half was coated with hyperimmune rabbit anti-rotavirus serum. Fecal suspensions were added to both halves of the plate in duplicate wells. The second antibody was antibody to rotavirus conjugated to horseradish peroxidase (Dako Corp., Santa Barbara, Calif.). The substrate was *ortho*-phenylenediamine. The reaction was stopped after 30 min and read on a spectrophotometer (Multiscan; Flow Laboratories, Inglewood, Calif.). Optical density readings of >0.1 , when negative control values were <0.05 , were regarded as positive for rotavirus.

Electrophoretotyping of rotavirus RNA. Electrophoretotyping of rotavirus RNA was performed as described by Thouless et al. (14). The RNA was extracted from fecal specimens with a phenol-chloroform mixture in the presence of 1% sodium dodecyl sulfate. It was electrophoresed with a Laemmli discontinuous system on a 10% polyacrylamide gel for 16 h at 10 mA. The bands of double-stranded RNA were stained with silver.

Serological assay. Rotavirus antibodies were detected by an enzyme-linked immunosorbent assay by using a tissue culture-adapted human rotavirus (combined subgroups 1 and 2) as antigen (14). Virus grown in Ma 104 cells was concentrated by polyethylene glycol precipitation and partially purified by rate zonal centrifugation through 45% (wt/vol) sucrose. Polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight at 4°C with 1 µg of virus per ml disrupted in 0.1% sodium dodecyl sulfate in carbonate bicarbonate buffer (pH 9.6). Half-log serum dilutions from $10^{-1.5}$ (1/32) to $10^{-5.0}$ (1/100,000) were made across the plate in phosphate-buffered saline–0.05% Tween–0.1 mg of gelatin per ml. Plates were incubated for 2 h at 37°C and washed three times with phosphate-buffered saline–0.05% Tween, and then horseradish peroxidase-conjugated, affinity-purified, goat anti-rabbit immunoglobulin G (IgG; Sigma Chemical Co., St. Louis, Mo.) was added. Plates were incubated for a further 2 h and washed three times with phosphate-buffered saline–0.05% Tween, and substrate was added. The reaction was stopped with 8 M H_2SO_4 after 30 min. The optical density of each well was read at 492 nm with a spectrophotometer (Multiscan; Flow Laboratories). An optical density value of 0.3 was used as the cutoff point for the antibody titer because this was the lowest value consistently on the linear part of the dilution curve. Known rotavirus-positive and -negative control sera were included in each assay. The positive serum used was a hyperimmune serum prepared by injecting purified rotavirus into a rabbit (14). The negative control serum came from our rotavirus-free rabbit colony and gave optical density values of <0.2 at a serum dilution of 1/32.

Histopathology. Tissue sections of the intestinal tract were collected from 12-week-old rabbits. Beginning with inocula-

tion, one rabbit was killed at 2- to 3-day intervals until 14 days after inoculation. Thereafter, one rabbit was killed at weekly intervals until 56 days after inoculation. Sections of the small intestine, cecum, colon, and mesenteric lymph nodes were fixed in 10% neutral-buffered Formalin.

RESULTS

Eight-week-old rabbits. All rabbits, except one, inoculated with tissue culture virus shed rotavirus from days 2 to 9 after inoculation (Table 1 and Fig. 1). One rabbit which received 2×10^4 ffu of tissue culture virus started shedding on day 4. Rabbits inoculated with stool filtrate and sham-inoculated rabbits shed rotavirus from days 4 to 9 after inoculation. All rabbits inoculated with rotavirus developed diarrhea during the period of viral shedding, except the one rabbit with delayed shedding of virus noted previously. Sham-inoculated rabbits did not develop diarrhea (Table 2 and Fig. 1). In rabbits inoculated with tissue culture virus, abnormal stools occurred 2 to 3 days after inoculation, whereas in rabbits inoculated with stool filtrate, abnormal stools were first noted 6 days after inoculation. In all affected rabbits, stools were abnormal for only 2 to 3 days. Diarrhea consisted of soft or fluid stools accompanied by fecal staining of the perineum. Anorexia occurred concurrently. Following the diarrhea, feces were scant, small, and hard. None of the rabbits had detectable antibodies to rotavirus in serum prior to and 7 days after inoculation. By 14 days after inoculation, all rabbits had antibodies to rotavirus. Three weeks after inoculation antibody titers were relatively similar in all rabbits (Table 3).

Twelve-week-old rabbits. Rotavirus was detected in the feces of inoculated rabbits 2 to 5 days after inoculation and continued to be detected for 6 to 7 days (Table 1). Shedding of rotavirus in sham-inoculated rabbits was detected 4 to 5 days after inoculation of other rabbits and persisted for 7 to 8 days (Table 2). All inoculated rabbits except one developed diarrhea during the period of rotaviral shedding. Diarrhea was present for 1 to 2 days and was similar in character to that observed in 8-week-old rabbits. Sham-inoculated rabbits also developed diarrhea of a similar character and duration. Gross examination of rabbits at necropsy revealed fluid and gas in the small intestine, cecum, and colon from 2 to 9 days after inoculation. During the period of rotaviral shedding, rabbits had histopathologic evidence of enteritis (Table 4). The inflammatory changes were characterized by a diffuse infiltration of low to moderate numbers of lymphocytes and plasma cells into the villi and lamina propria of the duodenum, jejunum, and ileum between 4 and 9 days after inoculation. Villous atrophy was observed in one rabbit that was inoculated 7 days earlier. Mild evidence of lymphoid reaction was found in the mesenteric lymph nodes. Lesions were not observed in the cecum or colon. None of the

TABLE 1. Response of rabbits to inoculation with rotavirus

Age (wk)	No.	Inoculum (titer [ffu])	No. with viral shedding	No. with antibody response	No. with diarrhea
12	10	Tissue culture virus (2×10^7)	9 ^a	6 ^b	8
8	6	Tissue culture virus (2×10^4 , 2×10^7), stool filtrate	6	6	5
3	15	Tissue culture virus (5×10^6)	15	15	8

^a One rabbit was killed before rotavirus was detected in the feces.

^b Four rabbits were killed before antibodies were detected in the serum.

FIG. 1. Detection of rotavirus in the feces of 8-week-old rabbits after inoculation. Diarrhea is indicated by filled boxes. Rabbits were inoculated with 2×10^7 ffu of tissue culture virus (high titer), 2×10^4 ffu of tissue culture virus (low titer), stool filtrate, or tissue culture media (control).

TABLE 3. Antibody titers in serum of rabbits following inoculation of rotavirus, as determined by enzyme-linked immunosorbent assay

Age (wk)	No.	Antibody titer in serum (negative log dilution) ^a	
		Mean $\pm$ SD	Range
12	6	4.3 $\pm$ 0.2	4.0–4.7
8	6	5.0 $\pm$ 0.3	4.6–5.5
3	15	3.9 $\pm$ 0.3	3.2–4.2

^a Three weeks after inoculation; these serum samples were assayed simultaneously.

rabbits had detectable antibodies prior to inoculation. By 7 days after inoculation, several rabbits had low antibody titers; however, by 14 days all rabbits had antibodies to rotavirus. Three weeks after inoculation, antibody titers approached those of the 8-week-old rabbits (Table 3).

Three-week-old rabbits. Rotavirus was initially detected in the feces of inoculated rabbits 2 to 8 days after inoculation and continued to be detected for 2 to 10 days (Table 1 and Fig. 2). The duration of viral shedding was directly related to the onset of shedding; rabbits shedding sooner after inoculation were more likely to shed longer. The uninoculated littermates had a similar pattern of viral shedding. Does initially shed virus 4 to 7 days after inoculation of pups (Table 2 and Fig. 2). Three uninoculated rabbits in the same room with one litter began to shed virus 9 to 12 days after inoculation of pups (Fig. 2). The lower titers of virus found in pups was attributed to specimen collection. As pups were not producing solid feces, specimens were obtained with rectal swabs. At 3 to 12 days after inoculation, three of seven rabbits in one litter (65813) and five of eight rabbits in the other litter (06931) had either soft feces or fecal staining of the perineum for 1 to 3 days (Table 1). One doe developed soft feces, while the other doe and the two uninoculated pups (one from each litter) remained unaffected. All three uninoculated rabbits developed soft feces of 1 to 4 days duration during the period of rotaviral shedding (Table 2). All inoculated rabbits had antibodies to rotavirus by 14 days after inoculation. At 3 weeks after inoculation, antibody titers were lower in 3-week-old rabbits than in those in the other two age groups (Table 3). All rabbits shedding rotavirus developed antibodies.

Rotavirus RNA was extracted from the feces of experimentally infected rabbits, sham-inoculated rabbits, and uninoculated sentinel rabbits maintained in the same room. The electropherotypes of the inoculum and viruses recovered from all rabbits were identical (Fig. 3; RNA from experimentally infected rabbits is not shown). Hence, the experimentally inoculated rotavirus rather than a rotavirus that was unintentionally introduced infected the control rabbits.

TABLE 2. Response of sham-inoculated and uninoculated rabbits exposed to rotavirus

Age (wk)	No.	Sham inoculation	Location ^a	No. with viral shedding	No. with antibody response	No. with diarrhea
12	2	Yes	Same room	2	2	2
8	2	Yes	Same room	2	2	0
3	2	No	Same cage	2	2	0
Adult ^b	2	No	Same cage	2	2	1
10	3	No	Same room	3	3	3

^a In relation to rabbits inoculated with rotavirus.

^b Nursing does.

TABLE 4. Pathologic findings in 12-week-old rabbits after inoculation of rotavirus

Tissue	Histopathologic findings	Response on the following days after inoculation ^a :						
		0	2	4	7	9	11	14
Duodenum	Edema	—	—	+	—	—	—	—
	Inflammation	—	—	+	—	+	—	—
	Villous atrophy	—	—	—	—	—	—	—
Jejunum	Edema	—	—	—	+	—	—	—
	Inflammation	—	—	+	+	+	—	—
	Villous atrophy	—	—	—	—	—	—	—
Ileum	Edema	—	—	—	—	—	—	—
	Inflammation	—	—	+	+	+	—	—
	Villous atrophy	—	—	—	+	—	—	—

^a Values are for one rabbit on each day.

DISCUSSION

The role of rotavirus infection in the pathogenesis of diarrheal disease in rabbits was investigated. Orogastric inoculation of 8- and 12-week-old rabbits with a rabbit strain of rotavirus resulted in shedding of virus for about 1 week beginning 2 to 5 days after inoculation. Virus recovered after inoculation had the same electropherotype as the virus that was inoculated, as did virus recovered from sham-inoculated and sentinel rabbits. While all infected rabbits developed a IgG response in serum to rotavirus, variability in antibody response among age groups was found when sera obtained 3 weeks after inoculation was assayed simultaneously. Most rabbits developed diarrhea coincident with the onset of viral shedding. Diarrhea was mild, characterized by soft or fluid stools which persisted for 2 to 4 days. Three-week-old rabbits had a briefer period of viral shedding, and only 50% exhibited diarrhea. Viral shedding may have been less effectively monitored in these young rabbits, as a rectal swab was obtained rather than a fecal pellet. The decreased incidence of diarrheal disease was in contrast to results presented in previous reports (8, 13), which showed high morbidity and mortality in 1- to 3-week-old rabbits. The rotavirus strain used in this study was an isolate from a severe outbreak (13). Stool filtrate from a dead rabbit in the outbreak was not more pathogenic than tissue culture-adapted virus. This suggests that factors other than virulence of the particular rotavirus isolate were involved in the

severity of infection. Copathogens or resistance of rabbits, resulting either from hereditary factors or from animal husbandry practices such as diet, hygiene, or stress, may be involved. There have been only two previous reports of experimentally induced rotavirus infection in rabbits (3, 10). Petric et al. (10) reported a milder syndrome in two 4-week-old rabbits which may have been due to low virus titer in the inoculum (1:4 by counterimmunoelectrophoresis) but was more likely due to the fact that the rabbits were the progeny of a seropositive doe. Although no preexisting antibodies were detected by complement fixation, it has been shown by immunofluorescence and enzyme-linked immunosorbent assay that maternally acquired antibodies persist until 8 weeks of age (4, 5). In contrast, Castrucci et al. (3) reported more severe disease in five 4- to 5-week-old rabbits when a high-titered, tissue culture-adapted rabbit rotavirus was used. Further studies are required to elucidate the role of rotavirus in the occasional severe diarrheal disease outbreaks which occur in rabbits.

Similarities in various aspects of rotavirus infection suggest that rabbits could be used as a model for human rotavirus infection. Young rabbits acquire maternal antibodies transplacentally while in utero (5). Passive antibodies persist for up to 2 months of age in young rabbits and appear

FIG. 2. Detection of rotavirus in the feces of rabbits after inoculation of a 3-week-old litter.

FIG. 3. Comparison of the electropherotypes of RNA from rabbit rotavirus L:ALA:84, which was used as the inoculum, and rotaviruses recovered from the feces of a sham-inoculated rabbit and a sentinel rabbit maintained in the same room. RNA was extracted with phenol and electrophoresed for 16 h at 10 mA in a 10% polyacrylamide gel. Bands were stained with silver. Lane A, Inoculum; lane B, sham inoculated; lane C, sentinel.

to protect them from severe rotaviral disease. This closely parallels the situation in human infants, who acquire mild rotavirus infections in the neonatal period while they are protected by maternal antibodies. More severe infections begin to occur when children are 5 to 6 months old. Unweaned rabbits are difficult to raise apart from their dams, so it is fortuitous that 8- to 12-week-old seronegative rabbits are the best age to use as a model for rotavirus infection. While diarrheal disease may be mild, and hence, not a dependable indicator of infection, virus shedding and antibody response are readily monitored. Also, histopathological and immunopathological examinations can define the extent of virus replication and intestinal pathogenesis. The model of rabbit rotavirus infection could be used for monitoring antiviral agents or cross protection by different rotavirus strains.

Another interesting aspect of this study was the occurrence of infection in sham-inoculated or uninoculated rabbits. Uninoculated does and offspring housed with the experimentally infected litters became infected, as expected. However, uninoculated rabbits which were housed in a separate rack in the room and handled before the inoculated rabbits also became infected. They are unlikely to have been infected by fomites by the usual fecal-oral route and may have been infected by airborne virus. Airborne transmission has been described for epizootic diarrhea of infant mice, in which uninoculated mice housed in separate cages in the same room developed diarrhea about 2 days after the appearance of diarrhea in experimentally infected mice (6). Spread of the virus that causes epizootic diarrhea of infant mice was prevented by the use of filter bonnets on the cages (7). It has also been reported that aerosolized epizootic diarrhea of infant mice virus can be efficiently transmitted to susceptible mice (11). Transmission by aerosol would explain why it is difficult to eliminate rotavirus from an infected rabbitry and to maintain a colony free of rotavirus.

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