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Noninfectious Rotavirus (Strain RRV) Induces an Immune Response in Mice Which Protects against Rotavirus Challenge

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We found that female adult mice parenterally inoculated with noninfectious rotavirus (simian strain RRV) developed virus-specific neutralizing antibodies in the serum; newborn mice from these dams were protected against RRV-induced gastroenteritis. In addition, mice parenterally inoculated with noninfectious RRV developed virus-specific cytotoxic T-lymphocyte precursors in the spleen. Replication of rotavirus in intestinal epithelial cells was apparently not required to induce rotavirus-neutralizing antibodies or rotavirus-specific cytotoxic T lymphocytes. Parenteral immunization of infants and young children with noninfectious rotaviruses may induce an immune response which protects against rotavirus challenge.

Rotaviruses are the most important cause of infant and childhood gastroenteritis both in developed countries and in developing countries (2, 13). Because of the devastating impact of this pathogen, there is a great deal of interest in disease prevention by vaccine. Currently, two rotavirus candidate vaccine strains (WC3 and RRV) are being tested in large-scale trials of protective efficacy in infants and young children (3, 12). Strains WC3 and RRV are live rotaviruses originally isolated from the feces of a calf and a rhesus monkey, respectively (4, 26).

Rotaviruses cause disease by replicating in mature villus epithelial cells of the small intestine (25). Although nonhuman rotaviruses are probably less adapted to growth in the human intestine than are human strains, children orally inoculated with RRV shed infectious virus as long as 7 days after inoculation (14). In addition, children inoculated with RRV develop fever more frequently than placebo-inoculated controls (14). Therefore, immunization with rotavirus vaccines which do not replicate in the intestine (such as inactivated whole rotaviruses, purified rotavirus proteins, or rotavirus genes cloned into attenuated viral or bacterial vectors) may be useful.

In this study, we found that adult mice parenterally inoculated with noninfectious RRV developed RRV-specific neutralizing antibodies in the serum and RRV-specific cytotoxic T-lymphocyte (CTL) precursors in the spleen. In addition, newborn mice from dams parenterally immunized with noninfectious RRV were protected against RRV-induced gastroenteritis. Replication of rotavirus in intestinal epithelial cells was apparently not required to induce virus-specific humoral and cellular immune responses.

MATERIALS AND METHODS

Animals. Female C57BL6 mice (8 to 12 weeks old) were obtained from Charles River Breeding Laboratories (Portage, Mich.).

Cells. Simian virus 40-transformed C57BL6 mouse embryo fibroblast cells (B6/WT-3) grown as previously described (21) were provided by Steven Jennings (Louisiana State University, Shreveport). Fetal rhesus monkey kidney cells (MA-104) were grown as previously described (20).

Virus. Simian rotavirus RRV strain 2 (MMU 18006) was obtained from Nathalie Schmidt (Viral and Rickettsial Disease Laboratory, Berkeley, Calif.). Plaque-purified stocks of virus for use in these studies were prepared in MA-104 cells. Viral growth in MA-104 cells and infectivity titrations by plaque assay were performed as previously described (20).

Tissue culture stocks of RRV mixed with various concentrations of β-propiolactone (BPL) were maintained at 4°C for 72 h and then at 37°C for 3 h. Tissue culture stocks of RRV in a fluid layer approximately 1.0 mm thick (2.5 ml/60-mm-diameter petri dish) were exposed for 10 min at room temperature to UV light from a General Electric germicidal lamp (G30T8; 30W, 90 cm) placed directly above the petri dishes at a distance of 19.0 cm. BPL- and UV-treated RRV preparations were tested for viral infectivity and hemagglutination activity as previously described (10, 20).

Protection against rotavirus-induced diarrhea. (i) Immunization and handling of dams. Female C57BL6 mice (8 to 10 weeks old) were parenterally inoculated with either 10^7 PFU of RRV inactivated with 0.10% BPL plus UV irradiation or an equivalent volume of supernatant fluids from mock-infected MA-104 cells. On the day of inoculation, female mice were mated with C57BL6 males at a female-to-male ratio of 1:1.

(ii) Infection of infant mice. Seven-day-old mice were orally inoculated with 10^6 PFU of RRV (approximately 10 times the dose at which 50% of inoculated animals develop diarrhea) (22). The pups were inspected daily for diarrhea by gentle palpation of their abdomens. At the time of inoculation of suckling mice, sera were collected from dams and from two suckling mice per litter by retroorbital capillary plexus puncture and decapitation, respectively.

Cytotoxicity assays. (i) Immunization of animals. Female C57BL6 mice (8 to 10 weeks old) were orally or parenterally inoculated with either 10^7 PFU of RRV, an equivalent quantity of RRV completely inactivated with 0.10% BPL plus UV irradiation, or an equivalent volume of supernatant fluid from mock-infected MA-104 cells.

(ii) Preparation of effector cells. Mice were sacrificed 4 weeks after oral or intraperitoneal inoculation with either 10^7 PFU of RRV, an equivalent quantity of infectious RRV inactivated with 0.10% BPL plus UV irradiation, or an equivalent volume of supernatant fluids from mock-infected MA-104 cells. Splenic lymphocytes were obtained and stim-

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TABLE 1. Reduction of rotavirus infectivity after treatment with BPL or UV irradiation or both[a]

BPL concn (%)	UV irradiation	HA titer[b]	Infectivity titer[c]
0	−	32	7.8
0	+	32	4.5
0.05	−	8	6.9
0.05	+	8	2.7
0.10	−	4	4.3
0.10	+	4	0
0.15	−	2	0

[a] Rotavirus strain RRV was treated with BPL or UV irradiation or both as described in Materials and Methods.
[b] Reciprocal of viral hemagglutination (HA) titer.
[c] Viral infectivity titers are expressed as $\log_{10}$ plaque-forming units per milliliter.

ulated in vitro with irradiated, RRV-infected splenic lymphocytes from uninfected animals as previously described (21).

(iii) Preparation of target cells and ^{51}Cr release assay. Preparation of B6/WT-3 target cells and ^{51}Cr release assays for detection of rotavirus-specific CTLs were performed as previously described (21).

PRN assay. The plaque reduction neutralization (PRN) assay used was a modification of the technique described by Matsuno et al. (15) and was performed as previously described (19).

RESULTS

Treatment of RRV with BPL or UV irradiation or both. To inactivate rotavirus infectivity and maintain immunogenicity of viral outer-capsid proteins, tissue culture stocks of RRV were treated with various concentrations of BPL or UV irradiation or both. Preparations were tested for hemagglutination activity and infectivity titer (Table 1). We found that treatment of RRV with either UV irradiation or 0.10% BPL resulted in approximately a 2,000-fold decrease in the infectivity titer. However, treatment of RRV with BPL altered the structural integrity of the viral outer capsid as indirectly reflected in an eightfold decrease in the viral hemagglutination titer. UV irradiation, on the other hand, did not result in a decrease in viral hemagglutination activity. Treatment of RRV with 0.10% BPL and UV irradiation completely inactivated viral infectivity.

Inoculation of adult mice with noninfectious RRV induces RRV-specific neutralizing antibodies and RRV-specific CTL precursors. To determine whether mice inoculated with noninfectious rotaviruses developed a rotavirus-specific humoral or cellular immune response, adult mice were orally or parenterally inoculated with infectious RRV, BPL-UV-inactivated RRV, or an equivalent volume of supernatant fluid from mock-infected MA-104 cells. At 4 weeks later, sera were tested for rotavirus-specific neutralizing antibodies by PRN assay and splenic lymphocytes were boosted in vitro with RRV and tested for the presence of CTL precursors by ^{51}Cr release assay (Table 2). We detected RRV-specific neutralizing antibodies and RRV-specific CTL precursors in mice orally or parenterally inoculated with infectious RRV. The titers of virus-specific neutralizing antibodies and levels of virus-specific cytotoxic activity did not significantly differ among animals inoculated orally with infectious RRV, parenterally with infectious RRV, or parenterally with noninfectious RRV. Parenteral but not oral inoculation of mice with noninfectious RRV induced rotavirus-neutralizing antibodies and rotavirus-specific CTLs.

TABLE 2. Detection of RRV-specific neutralizing activity in serum and RRV-specific cytotoxic activity by splenic lymphocytes after inoculation of mice with infectious and noninfectious preparations of RRV[a]

Immunization (route)	PRN titer[b]	% Specific ^{51}Cr release at an effector-target ratio of:		
		50:1	17:1	6:1
RRV (oral)	6,500	44	28	17
RRV (parenteral)	11,600	65	67	41
Mock (parenteral)	<100	−4	5	0
Noninfectious RRV (oral)	<100	0	−1	0
	<100	−2	0	0
Noninfectious RRV (parenteral)	2,500	40	16	10
	5,600	73	17	12

[a] Adult C57BL6 mice were orally or parenterally inoculated with 10^7 PFU of RRV, an equivalent quantity of RRV inactivated with BPL plus UV irradiation (noninfectious RRV), or an equivalent volume of supernatant fluid from mock-infected MA-104 cells (Mock). At 4 weeks later, the animals were sacrificed and sera and splenic lymphocytes were obtained. The sera were tested for RRV-specific neutralizing activity by PRN assay, and the splenic lymphocytes were boosted in vitro with irradiated, RRV-infected antigen-presenting cells and tested for RRV-specific cytotoxic activity by ^{51}Cr release assay. The data represent inoculations of two animals each with noninfectious RRV orally and noninfectious RRV parenterally and one animal each with infectious RRV orally, infectious RRV parenterally and mock-infected MA-104 cells parenterally.
[b] The data are expressed as the reciprocal of the serum dilution showing a 50% reduction in the mean plaque count of RRV.

Suckling mice are protected against RRV-induced gastroenteritis after inoculation of dams with noninfectious RRV. To determine whether noninfectious RRV induced antibodies which passively protected suckling mice against rotavirus-induced diarrhea, adult female mice were parenterally inoculated with noninfectious RRV or supernatant fluid from mock-infected MA-104 cells and their newborns were challenged with RRV. We found that noninfectious RRV induced rotavirus-neutralizing antibodies in dams and that newborns orally challenged with RRV were protected against disease (Table 3); newborns from dams parenterally inoculated with supernatant fluids from mock-infected cells were not protected against challenge.

DISCUSSION

We detected rotavirus-specific neutralizing antibodies in sera of dams parenterally inoculated with noninfectious RRV. Newborns to which neutralizing antibodies from these dams were passively transferred were protected against RRV challenge. Therefore, replication of rotavirus in intestinal epithelial cells was not required to induce an immune response which passively protected suckling mice against rotavirus challenge. Protection against RRV challenge was most likely mediated by passively transferred rotavirus-specific immunoglobulin G present at the intestinal surface. It is unlikely that passively transferred rotavirus-specific helper T cells or CTLs were responsible for protection against challenge. These conclusions are based on the following findings. (i) We previously found that oral administration of milk from dams parenterally immunized with simian rotavirus (strain SA11) protected suckling mice against SA11 challenge; protective activity was detected in the rotavirus-specific immunoglobulin G fraction (17). (ii) In cross-fostering studies, we found that passively acquired rotavirus-specific intestinal but not circulating antibodies protected against challenge (17). (iii) Parenteral but not oral transfer of splenic lymphocytes obtained 6 days after paren-

TABLE 3. Protection of suckling mice against RRV-induced diarrhea by parenteral inoculation of dams with noninfectious RRV[a]

Virus used to immunize dams	Mouse	PRN titer of maternal and neonatal sera on day of neonatal challenge[b]	No. of neonatal mice with diarrhea/no. of mice challenged with RRV
Mock	Dam	<100	6/6
	Suckling 1	<100	
	Suckling 2	<100	
	Dam	<100	8/8
	Suckling 1	<100	
	Suckling 2	<100	
Noninfectious RRV	Dam	2,500	0/4
	Suckling 1	500	
	Suckling 2	800	
	Dam	1,250	0/6
	Suckling 1	300	
	Suckling 2	1,000	
	Dam	3,000	0/5
	Suckling 1	2,400	
	Suckling 2	1,800	

[a] Adult female C57BL6 mice were parenterally inoculated with RRV inactivated with 0.10% BPL plus UV irradiation (noninfectious RRV) or supernatant fluids from mock-infected MA-104 cells (Mock). At the time of immunization, female mice were mated with C57BL6 males. Seven-day-old mice from immunized dams were inoculated with 10^6 PFU of RRV. Sera from dams and two suckling mice per litter were collected at the time of newborn mouse inoculation and tested for RRV-specific neutralizing activity by PRN assay. The pups were inspected daily for diarrhea by gentle palpation of their abdomens.

[b] The data are expressed as the reciprocal of the serum dilution showing a 50% reduction in the mean plaque count of RRV.

teral inoculation of adult mice with RRV protected newborns against challenge with murine rotavirus strain JMV (data not shown). Presumably, rotavirus-specific lymphocytes acquired orally do not migrate efficiently through the mucus layer to the intestinal cell surface.

The rotavirus outer capsid consists of two proteins, vp4 and vp7 (5). Rotavirus attachment to target cells is mediated by vp7 (6, 24). Virus entry into the cytoplasm, on the other hand, is a function of vp4 (6, 11). Both vp4 and vp7 evoke antibodies which neutralize viral infectivity in vitro (8, 16) and protect against rotavirus challenge in vivo (7, 18). We found that RRV infectivity was completely ablated by treatment with 0.15% BPL or 0.10% BPL plus UV irradiation. However, treatment of RRV with BPL also altered the structural integrity of outer-capsid proteins as indirectly reflected in decreased viral hemagglutinating activity. Despite this alteration of rotavirus outer-capsid proteins, parenteral inoculation of mice with BPL-treated RRV evoked virus-specific neutralizing antibodies. These findings are similar to those previously described for noninfectious preparations of poliovirus (23) and rabies virus (29). However, oral inoculation of mice with BPL-treated RRV did not evoke a detectable virus-specific immune response. Processing and presentation of rotaviruses by intestinal antigen-presenting cells located in the intraepithelial layer, lamina propria, or Peyer's patch presumably occurs only after virus entry through either mature villus epithelial cells or membranous epithelial cells. Alteration of vp7 and vp4 by BPL probably decreased the amount of virus which attached to and entered intestinal epithelial cells. However, a sufficient quantity of noninfectious rotavirus can be presented to the intestinal surface for recognition by the immune system. Ijaz et al. found that mice inoculated orally three times with approximately 50 μg of purified, noninfectious bovine rotavirus per inoculation developed rotavirus-specific antibodies in milk detected by enzyme-linked immunosorbent assay; studies of protection against rotavirus challenge were not performed (9).

We previously detected rotavirus-specific CTL precursors in spleens of adult mice orally inoculated with infectious RRV (21). However, parenteral inoculation of adult mice with noninfectious RRV also induced virus-specific CTL precursors in the spleen (Table 2). Therefore, viral replication was not necessary to induce rotavirus-specific CTL precursors. Virus-specific CTL precursors have previously been detected in splenic lymphocytes after parenteral inoculation of mice with noninfectious preparations of respiratory syncytial virus (1), influenza virus (28), and Sendai virus (27). The importance of rotavirus-specific CTLs in protection against gastroenteritis remains unknown.

Parenteral inoculation of adult mice with noninfectious RRV induced RRV-specific neutralizing antibodies in serum and RRV-specific CTL precursors in the spleen. Replication of rotavirus in intestinal epithelial cells was not required to induce virus-specific immunity. These findings may be relevant to the development of a successful rotavirus vaccine.

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