Rotavirus-Specific Cytotoxic T Lymphocytes Cross-React with Target Cells Infected with Different Rotavirus Serotypes

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Splenocytes from adult C57BL6 (*H-2^b*) mice orally inoculated with nonmurine rotaviruses lysed syngeneic rotavirus-infected target cells. Cytotoxic T lymphocytes (CTLs) were responsible for this cytotoxic activity. Cytotoxic activity was (i) detected 7 days after primary oral inoculation; (ii) not detected in uninoculated animals; (iii) specific for rotavirus-infected target cells; (iv) eliminated by treatment with Thy 1.2-specific immunoglobulin M and complement; and (v) restricted at *H-2D^b*. In addition, rotavirus-specific CTLs cross-reacted with target cells infected with different human or animal rotavirus serotypes. Heterotypic protection against rotavirus challenge may be mediated by cross-reactive CTLs.

Diarrheal diseases are an important cause of disease and death worldwide. In Asia, Africa, and Latin America between 1978 and 1979, 3 billion to 5 billion cases of diarrhea accounted for 5 million to 10 million deaths (31). Since their initial identification in 1973, rotaviruses were found to be the most important cause of acute gastroenteritis of infants and young children both in the United States and in developing countries (2, 12). Because of the worldwide impact of these viruses, there is a great deal of interest in disease prevention by vaccine.

Several investigators (30; H. F. Clark, F. Borian, L. M. Bell, K. Modesto, A. Fieo, and S. A. Plotkin, *Pediatr. Res.* 21:323A) found that infants and young children were protected against challenge with virulent serotype 1 human rotaviruses after oral inoculation with bovine strains. However, bovine rotaviruses are serotypically distinct from serotype 1 human rotaviruses (10). The immunologic basis of this heterotypic protection is unknown. Heterotypic protection is not clearly explained by the humoral immune response.

Because the gastrointestinal tract is a rich source of cytotoxic T lymphocytes (CTLs) (6, 14), we are interested in the importance of CTLs in both homotypic and heterotypic protection against rotavirus challenge. We found that rotavirus-specific CTLs cross-reacted with target cells infected with different human or animal rotavirus serotypes. These findings may in part explain the immunologic basis of heterotypic protection against rotavirus-induced gastroenteritis.

MATERIALS AND METHODS

Animals. C57BL6 (*H-2^b*) female mice (8 to 12 weeks old) obtained from Charles River Breeding Laboratories, Inc. (Portage, Mich.) were housed in isolation units. Animals were bled on arrival by retro-orbital capillary plexus puncture, and sera were tested for rotavirus-specific antibodies by enzyme-linked immunoassay as previously described (28). Only seronegative animals were used in these studies.

Cells. Simian virus 40-transformed C57BL6 mouse embryo fibroblast cells (B6/WT-3) originally described by Prete et al. (25) were provided by Steven Jennings (The Milton S. Hershey Medical Center, Hershey, Pa.). These cells express high levels of cell surface *H-2K^b* and *H-2D^b* as determined by fluorescent flow cytometry (S. Jennings, personal communication). B6/WT-3 cells were grown in Dulbecco modified Eagle medium supplemented with 5% heat-inactivated (56°C, 30 min) fetal bovine serum, 0.075% NaHCO₃, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer 0.03% glutamine, 100 µg of streptomycin sulfate per ml, and 100 U of penicillin per ml (B6 medium).

Simian virus 40-transformed murine H-2 recombinant cells (K5RSV [*K^bD^d*] and KHTGSV [*K^dD^b*]) (24) and simian virus 40-transformed *H-2^d* cells (MKSA [*K^dD^d*]) were provided by Barbara Knowles (The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.). K5RSV and KHTGSV cells were grown in B6 medium, and MKSA cells were grown in B6 medium with 10% fetal bovine serum.

Fetal rhesus monkey kidney cells (MA-104) were grown in cell medium 199 (M199) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, 0.03% glutamine, and 20 µg of gentamicin sulfate per ml.

Virus. Simian rotavirus RRV strain 2 (MMU 18006) was obtained from Nathalie Schmidt (Viral and Rickettsial Disease Laboratory, Berkeley, Calif.). Simian rotavirus SA-11 was obtained from H. H. Malherbe (Gull Laboratories, Salt Lake City, Utah). The WA strain of human rotavirus and the OSU strain of porcine rotavirus were obtained from Richard Wyatt (National Institutes of Health, Bethesda, Md.). The bovine rotavirus strain NCDV, adapted to growth in tissue culture at the University of Nebraska, was provided by Robert Yolken (The Johns Hopkins University, Baltimore, Md.). Plaque-purified stocks of virus for use in these studies were prepared in MA-104 cells.

Viral growth and infectivity titration by plaque assay were performed as previously described (21).

Cytotoxicity assays. (i) Immunization of animals. Mice were inoculated with 10⁷ PFU of tissue culture-derived rotaviruses by proximal esophageal intubation through a 10-gauge polyethylene tube. Mock-infected animals were inoculated

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with a preparation derived from uninfected MA-104 cells in an equivalent volume.

(ii) **Preparation of effector cells.** To determine the primary CTL response *in vivo*, we sacrificed mice 7 days after oral inoculation and removed their spleens. Single-cell suspensions were obtained by careful dissection with 19-gauge needles, and splenocytes were washed twice in B6 medium. Erythrocytes were lysed in AKC medium (0.16 M NH_4Cl , 0.01 M KHCO_3 , pH 7.2), and lymphocytes were washed twice in B6 medium and suspended in RPMI 1640 containing 10% fetal bovine serum, 10 mM HEPES, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.03% glutamine, and 3×10^{-5} M 2-mercaptoethanol (RPMI CM).

To determine the secondary CTL response after stimulation of lymphocytes *in vitro*, splenic lymphocytes obtained 4 weeks after oral inoculation were stimulated *in vitro* with the same virus at a multiplicity of infection of 2, placed at 37°C for 1 h, and added to uninfected lymphocytes at a responder-to-stimulator ratio of 2:1. Lymphocytes (10^7) were added to individual wells of a 24-well plate and placed at 37°C in 5% CO_2 for 5 to 7 days. Lymphocytes were washed once in RPMI CM.

(iii) **Determination of effector cell phenotype.** Lymphocytes at concentrations calculated to give the required effector-to-target ratios were suspended in Thy 1.2-specific immunoglobulin M (IgM) (HO-13-4; American Type Culture Collection, Rockville, Md.) (16) and incubated at 4°C for 1 h. Cells were centrifuged at $600 \times g$, suspended in agarose-adsorbed guinea pig complement (provided by Irv Weissman, Stanford University School of Medicine, Stanford, Calif.) diluted 1:5 in B6 medium and incubated for 1 h at 37°C. Cells were treated again with Thy 1.2-specific IgM and complement as above, incubated at 37°C for 1 h, and washed three times with RPMI CM.

(iv) **Preparation of target cells.** B6/WT-3, K5RSV, KHTGSV, and MKSA cells were grown to confluency in 96-well plates, washed twice with phosphate-buffered saline, and incubated with 25 μl of tissue culture-derived rotaviruses per well at a multiplicity of infection of 100 for 1 h at 37°C. Mock-infected target cells were inoculated with a preparation derived from uninfected MA-104 cells in an equivalent volume. Cells were washed once with phosphate-buffered saline, and 100 μl of serum-free K1 medium (Dulbecco modified Eagle medium-Ham's F-12, 10 mM HEPES, 10 mM selenium salts, 1.1 g of NaHCO_3 per liter, 25 ng of prostaglandin E1 per ml, 50 nM hydrocortisone, 10 μg of bovine pancreas insulin per ml, 5 μg of transferrin per ml, 5 pM triiodothyronine) was added per well. At 3 h after infection, 2.5 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, Ill.) was added to individual wells of a 96-well plate (2.5 μCi per 3×10^4 cells), and the cells were incubated for 1 h at 37°C and washed twice with phosphate-buffered saline.

(v) **^{51}Cr -release assays.** For primary *in vivo* responses, effector cells were added to target cells at ratios of 150:1, 50:1, 16.5:1, and 5.5:1. For secondary *in vitro* stimulations, effector cells were added to target cells at ratios of 50:1, 16.5:1, 5.5:1, and 1.8:1. Effector cells in 100 μl of RPMI CM were added to approximately 3×10^4 target cells in flat-bottomed polystyrene 96-well plates (Costar, Cambridge, Mass.). All assays were performed in triplicate. After a 4-h incubation at 37°C in 5% CO_2 , 50 μl of supernatant was removed and assayed for radioactivity in a gamma radiation counter. Target cells incubated with RPMI CM only (spontaneous release) or RPMI CM containing 1% sodium dodecyl sulfate (total release) were included in each assay. (Spontaneous/total release ratios were between 25 and 30%.) Per-

TABLE 1. Primary rotavirus-specific CTLs are detected in murine spleens after oral infection with serotype 3 rotaviruses^a

Virus used to prime <i>in vivo</i>	% Specific ^{51}Cr release from B6/WT-3 cells infected with: ^b									
	WA (1) ^c		RRV (3)		SA11 (3)		OSU (5)		NCDV (6)	
	150 ^d	50	150	50	150	50	150	50	150	50
WA	-4	-6	-2	-3	0	-3	0	0	1	3
RRV	-3	-2	20	6	6	7	3	4	17	1
SA-11	1	-3	24	11	17	-7	2	-3	4	-5
OSU	-1	-4	2	-2	4	0	4	0	4	0
NCDV	-3	-5	-2	-4	1	-1	2	-3	3	2
Mock	NT ^e	NT	2	-2	NT	NT	NT	NT	NT	NT

^a Splenic lymphocytes (effector cells) from adult (8- to 12-week-old) C57BL/6 (*H-2^b*) female mice orally inoculated with heterologous host (non-murine) rotaviruses were tested in cytotoxicity assays. Splenic lymphocytes were obtained 7 days after oral inoculation with 10^7 PFU of WA (human), RRV (simian), SA-11 (simian), OSU (porcine), or NCDV (bovine) rotavirus. Lymphocytes were tested in 4-h ^{51}Cr -release assays against B6/WT-3 target cells (*H-2^b*) infected with rotaviruses at a multiplicity of infection of 100.

^b Percent specific release at indicated effector/target cell ratio. Positive CTL responses are shown in bold-face type. CTL responses from mock-infected mice against WA-, SA-11-, OSU-, and NCDV-infected targets were not tested. Target cells incubated with RPMI CM only (spontaneous release) or RPMI CM containing 1% sodium dodecyl sulfate (total release) were included in each assay. Percent specific release was calculated as described in the text.

^c Rotavirus strain used to infect B6/WT-3 cells; serotypes are indicated in parentheses.

^d Effector/target cell ratios used in 4-h ^{51}Cr -release assays.

^e NT, Not tested.

cent specific release was defined as follows: [experimental release (cpm) - spontaneous release (cpm)]/[total release (cpm) - spontaneous release (cpm)].

RESULTS

Primary CTL response *in vivo*. (i) **Serotype specificities.** Splenocytes with rotavirus-specific cytotoxic activity were found after oral inoculation of mice with nonmurine rotaviruses; rotavirus-specific cytotoxic activity was not detected after mock infection (Table 1). However, only mice inoculated with serotype 3 rotaviruses (SA-11, RRV) developed a primary cytotoxic response. Cytotoxic activity was not detected after oral inoculation of mice with rotavirus serotype 1, 5, or 6.

In addition, we found that primary splenic lymphocytes appear to contain polyclonal populations of both serotype-specific and cross-reactive CTLs. Lymphocytes from mice infected with SA-11 virus lysed only serotype 3 (SA-11, RRV)-infected target cells, whereas lymphocytes from RRV-infected animals lysed either serotype 3 (RRV)- or serotype 6 (NCDV)-infected targets.

(ii) **Effector cell surface markers.** We determined that T lymphocytes were responsible for rotavirus-specific cytotoxicity (Table 2). We found that RRV-specific cytotoxicity was eliminated after treatment of effector cells with Thy 1.2-specific IgM and complement.

(iii) **H-2 restriction.** Using simian virus 40-transformed H-2 recombinant cells, we determined that primary rotavirus-specific CTLs from *H-2^b* mice recognized target cells in association with *H-2D^b*, but not *H-2K^b* (Table 3). Lymphocytes from mice orally inoculated with RRV lysed RRV-infected B6/WT-3 (*K^bD^b*) or KHTGSV (*K^dD^b*) target cells, but not K5RSV (*K^bD^d*) or MKSA (*K^dD^d*) targets.

Secondary CTL response after *in vitro* stimulation. **Serotype specificities.** Rotavirus-specific CTL precursors stimulated *in vitro* were detected after oral inoculation of mice

TABLE 2. T cells are responsible for rotavirus-specific cytotoxic activity^a

Treatment of splenocytes before CTL assay	% Specific ⁵¹ Cr release ^b
No treatment	
150 ^c	32
50.....	32
Complement alone	
150.....	30
50.....	32
Anti-Thy 1.2 + complement	
150.....	10
50.....	3

^a Splenic lymphocytes (effector cells) were obtained from adult (8- to 12-week-old) C57BL/6 (*H-2^b*) female mice 7 days after oral inoculation with 10⁷ PFU of RRV (simian) rotavirus. After treatment with Thy 1.2-specific IgM and complement, lymphocytes were tested in 4-h ⁵¹Cr-release assays against B6/WT-3 target cells (*H-2^b*) infected with RRV at a multiplicity of infection of 100.

^b Percent specific release at indicated effector/target cell ratio. Positive CTL responses are shown in bold-face type. Target cells incubated with RPMI CM only (spontaneous release) or RPMI CM containing 1% sodium dodecyl sulfate (total release) were included in each assay. Percent specific release was calculated as described in the text.

^c Effector/target cell ratios used in 4-h ⁵¹Cr-release assays.

with serotype 1, 3, 5, or 6; cytotoxic activity was not detected after RRV stimulation of lymphocytes from mock-infected animals (Table 4). Rotavirus-specific CTL precursors stimulated in vitro broadly cross-reacted with targets infected with different rotavirus serotypes (Table 4). In addition, NCDV-infected target cells were best lysed by NCDV-stimulated lymphocytes (Table 4). Therefore, CTLs obtained after secondary stimulation in vitro appear to contain polyclonal populations of both serotype-specific and cross-reactive CTLs.

DISCUSSION

Splenocytes with rotavirus-specific cytotoxic activity (Tables 1 and 4) were found after oral inoculation of mice with

TABLE 3. Primary rotavirus-specific CTLs from *H-2^b* mice recognize RRV-infected target cells in association with *H-2D^b* but not *H-2K^b* antigens^a

RRV-infected target cells	% Specific ⁵¹ Cr release ^b
B6/WT-3 (<i>K^bD^b</i>)	
150 ^c	41
50.....	42
K5RSV (<i>K^bD^d</i>)	
150.....	4
50.....	7
KHTGSV (<i>K^dD^b</i>)	
150.....	25
50.....	18
MKSA (<i>K^dD^d</i>)	
150.....	1
50.....	3

^a Splenic lymphocytes (effector cells) were obtained from adult (8- to 12-week-old) C57BL/6 (*H-2^b*) female mice 7 days after oral inoculation with 10⁷ PFU of RRV (simian) rotavirus. Lymphocytes were tested in 4-h ⁵¹Cr-release assays against B6/WT-3 (*K^bD^b*), K5RSV (*K^bD^d*), KHTGSV (*K^dD^b*), or MKSA (*K^dD^d*) target cells infected with RRV at a multiplicity of infection of 100.

^b Percent specific release at indicated effector/target cell ratios. Positive CTL responses are shown in bold-face type. Target cells incubated with RPMI CM only (spontaneous release) or RPMI CM containing 1% sodium dodecyl sulfate (total release) were included in each assay. Percent specific release was calculated as described in the text.

^c Effector/target cell ratios used in 4-h ⁵¹Cr-release assays.

TABLE 4. Rotavirus-specific CTL precursors stimulated in vitro broadly cross-react with target cells infected with different rotavirus serotypes^a

Virus used to prime in vivo and boost in vitro		% Specific ⁵¹ Cr-release from B6/WT-3 cells infected with:									
		WA (1)		RRV (3)		OSU (5)		NCDV (6)		Mock	
1°	2°	50	16	50	16	50	16	50	16	50	16
WA	WA	64	39	64	38	49	24	57	27	24	8
RRV	RRV	49	26	72	60	55	35	46	29	17	6
OSU	OSU	44	25	81	55	80	64	66	36	32	11
NCDV	NCDV	75	44	94	69	74	58	82	71	31	14
Mock	RRV	NT ^b	NT	10	6	NT	NT	NT	NT	NT	NT

^a See Table 1 footnotes for explanation of data.

^b NT, Not tested.

nonmurine rotaviruses. CTLs were responsible for cytotoxic activity in accord with criteria established by Zinkernagel and Doherty (35). Cytotoxic activity was (i) detected 7 days after primary oral inoculation (Table 1); (ii) not detected in uninoculated animals (Tables 1 and 4); (iii) specific for rotavirus-infected target cells (Tables 1 and 4); (iv) eliminated by treatment with Thy 1.2-specific IgM and complement (Table 2); and (v) restricted by antigens of the major histocompatibility complex (Table 3).

Although it is unlikely that CTLs are important in the amelioration of a primary rotavirus infection (5), CTLs may be important in protection against reinfection for several reasons. First, heterotypic protection against rotavirus infection is not clearly explained by the humoral immune response. Several investigators (30; Clark et al., *Pediatr. Res.* 21:323A) found that infants and young children were protected against severe gastroenteritis caused by serotype 1 human rotaviruses after oral inoculation with the bovine strain NCDV or WC-3. However, NCDV and WC-3 are serotypically distinct from serotype 1 human rotaviruses (10). The immunologic basis of this heterotypic protection is unknown. We found that rotavirus-specific CTLs (Table 1) and CTL precursors (Table 4) cross-reacted with target cells infected with different rotavirus serotypes. Cross-reactive CTLs may in part explain the immunologic basis of heterotypic protection. Second, the intraepithelial and lamina propria lymphocyte compartments are rich sources of CTLs (6, 14, 22, 23). We previously found that only rotavirus-specific antibodies at the intestinal mucosal surface protected against disease; high titers of circulating antibodies did not protect (20). Rotavirus-specific CTLs may also need to be present at the intestinal mucosal surface to protect against challenge. Third, CTLs limit viral replication and prevent disease in a number of viral infections (7, 13, 15, 27, 32, 33, 36). We are currently studying whether rotavirus-specific CTLs passively protect suckling mice against homotypic or heterotypic rotavirus challenge.

Rotavirus replication in the intestinal tract appears to be important in eliciting a rotavirus-specific CTL response. Although rotavirus-specific CTL precursors were elicited after oral inoculation with all strains tested (Table 4), only mice inoculated with serotype 3 rotaviruses developed a primary CTL response (Table 1). This finding is consistent with the observation that serotype 3 rotaviruses are better adapted to growth in murine intestines than are non-serotype 3 strains (1, 19). To determine the importance of viral replication in eliciting rotavirus-specific CTLs, we are currently studying the CTL response after oral inoculation with

homologous host (murine) or β -propiolactone-inactivated rotaviruses.

We found that rotavirus-specific CTLs obtained 7 days after oral inoculation were primarily serotype specific (Table 1). However, CTL precursors from these animals broadly cross-reacted with target cells infected with different rotavirus serotypes after in vitro stimulation (Table 4). Apparently, both serotype-specific and cross-reactive CTLs or CTL precursors were generated after primary rotavirus inoculation. Consistent with our observations, both serotype-specific and cross-reactive CTLs are found after influenza A virus (3), vesicular stomatitis virus (26), and reovirus (8) infection. We are currently investigating the capacity of adoptively transferred, primary rotavirus-specific CTLs to protect against homotypic or heterotypic challenge. In addition, we will determine the rotavirus structural specificities of serotype-specific and cross-reactive CTLs.

Major histocompatibility complex-associated antigens may be important determinants of susceptibility to rotavirus infection. Rotavirus-specific CTLs from *H-2^b* mice recognized infected target cells in association with *H-2D^b* but not *H-2K^b* (Table 3). Consistent with these findings, polyclonal CTLs directed against lymphocytic choriomeningitis virus (34), vaccinia virus (4), influenza A virus (4), herpes simplex virus (11), and alphaviruses (18) recognize infected target cells in association with either *K* or *D* locus antigens. We are currently investigating the relationship between *H-2* haplotype and susceptibility to rotavirus infection.

Rotavirus-specific CTLs were detected in the spleens of orally inoculated mice (Tables 1 and 4). However, rotaviruses replicate only in mature villus epithelial cells of the small intestine (29); viral antigen has never consistently been detected in the blood or sites distant from the small intestine (17). There are two likely hypotheses to explain these observations. Lymphocytes rarely remain confined to a single compartment (9). After enteric infection, rotavirus-specific CTLs originating in the intestine (intraepithelial and lamina propria lymphocytes) may migrate to distant sites (e.g., the spleen). Alternatively, intestinal lymphocytes may themselves become infected with rotaviruses, travel to distal sites, and stimulate rotavirus-specific CTLs. We are currently evaluating the kinetics of the CTL response in intestinal (intraepithelial, lamina propria, Peyer's patch, mesenteric nodes) and nonintestinal (spleen, inguinal and cervical nodes) lymphocyte compartments after oral or parenteral rotavirus infection.

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