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NOTES

Rotavirus-Specific Cytotoxic T Lymphocytes Appear at the Intestinal Mucosal Surface after Rotavirus Infection

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The gastrointestinal tract is constantly exposed to a variety of potentially invasive bacteria and viruses. The first line of defense of the host against these pathogens is the intestinal mucosal surface, which consists of epithelial cells, intraepithelial lymphocytes (IELs), mucus, and secretory immunoglobulins. Little is known about the function, memory, or trafficking of IELs after intestinal infection. We found that IELs obtained 6 days after oral inoculation of mice with the intestinal pathogen rotavirus (simian strain RRV) lysed rotavirus-infected target cells; cytotoxic T lymphocytes (CTLs) were responsible for rotavirus-specific cytotoxic activity. Rotavirus-specific cytotoxic activity by IELs was (i) eliminated by treatment with Thy1.2-specific immunoglobulin M plus complement, (ii) restricted by proteins encoded at the major histocompatibility complex, and (iii) absent in mock-infected animals. Oral inoculation of mice with RRV also induced rotavirus-specific CTLs in splenic and intestinal lymphocytes (mesenteric lymph nodes, Peyer's patch). Parenteral inoculation induced rotavirus-specific CTLs in splenic, intestinal (IELs, mesenteric lymph nodes, Peyer's patch), and nonintestinal lymphocytes (inguinal nodes). Therefore, presentation of rotavirus to the intestinal mucosal surface was not necessary to induce IELs with virus-specific cytotoxic activity. At 4 weeks after oral or parenteral inoculation of mice with RRV, rotavirus-specific CTL precursors appeared among splenic, Peyer's patch, inguinal, and mesenteric node lymphocytes, but not among IELs. IELs with rotavirus-specific cytotoxic activity may be generated from precursors at a site other than the intestinal mucosal surface. Part of the response of the host to enteric infection may include surveillance and lysis of virus-infected villus epithelial cells by IELs.

Infections of the intestinal tract are among the most prevalent causes of infant disease and death worldwide. Each year an estimated 3 to 5 billion cases of infectious diarrhea account for 5 to 10 million deaths (31). The devastating impact of intestinal pathogens has excited interest in host immunity at the intestinal mucosal surface. Most of this interest has focused on the importance of secretory immunoglobulin A (IgA) (18). However, the role of intestinal lymphocytes in intestinal infections has not been explored. Considering the fact that lymphocytes are abundant at the intestinal mucosal surface (4, 26), little is known about the function, memory, or trafficking of these cells after intestinal infection.

Intestinal lymphocytes are located either at the intestinal mucosal surface among intestinal epithelial cells (intraepithelial lymphocytes [IELs]), among lymphatic capillaries and connective tissue close to the epithelial surface but below the basement membrane (lamina propria lymphocytes), or within lymphatic nodules at the base of villus crypt epithelial cells (Peyer's patch [PP] lymphocytes). About 50 to 60% of IELs are T cells, most of which have surface markers consistent with the functions of cytotoxicity or suppression (4, 26). In addition, less than 10% of IELs are B cells (immunoglobulin positive), and 40 to 50% are large, granular lymphocytes of unknown function and novel phenotype ($Thy1^-$, $CD4^-$, $CD8^+$) (4, 25). In the lamina propria and PP, on the other hand, B cells outnumber T cells by ratios of 2:1 and 3:1, respectively; no large, granular lymphocytes reside in these compartments (19, 25).

The present study characterizes the murine intestinal and nonintestinal cytotoxic T-lymphocyte (CTL) response to the intestinal pathogen, rotavirus (simian strain RRV). We chose to study the murine CTL response to RRV infection for several reasons. First, rotaviruses are the most important cause of infant and childhood gastroenteritis both in the United States and in developing countries (1, 13). Although a number of investigators have examined serum and intestinal secretions for the presence of rotavirus-specific antibodies after rotavirus infection (14, 17), there is little information on the cellular immune response to rotavirus (30). To develop a successful rotavirus vaccine, it may be important to understand all aspects of the immune response associated with protection against challenge. Second, the intestinal surface is a rich source of potential virus-specific CTLs (4, 26). Because rotaviruses replicate in mature, small intestinal, villus epithelial cells (28), rotavirus-specific CTLs would probably have to act at the intestinal mucosal surface to limit production and spread of infectious virus. Third, simian rotavirus strain RRV is currently used by researchers at the National Institutes of Health in large-scale trials of protective efficacy in infants and young children (12). We recently developed a murine model for oral infection with tissue culture-adapted simian rotaviruses (21, 23, 24). We found that simian rotavirus replicated in intestinal epithelial cells and induced diarrhea in suckling mice; the clinical symptoms, small intestinal histopathologic changes, and type-

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TABLE 1. RRV-specific cytotoxic activity by splenic lymphocytes at various intervals after oral or parenteral inoculation of mice with infectious or noninfectious RRV

Mice inoculated with[a]	% Specific ^{51}Cr release by splenic lymphocytes[b] collected at various intervals (days) after infection				
	1	3	6	9	12
RRV PO	0	0	15	1	0
RRV IP	2	14	32	6	3
Noninfectious RRV IP	ND[c]	ND	2	ND	ND
Mock PO	ND	ND	2	ND	ND
Mock IP	ND	ND	3	ND	ND

[a] Adult C57BL/6 mice were inoculated (i) orally (RRV PO) or intraperitoneally (RRV IP) with 10^7 PFU of RRV, (ii) intraperitoneally with an equivalent quantity of RRV completely inactivated with 0.10% BPL and UV irradiation (Noninfectious RRV IP), or (iii) orally (Mock PO) or intraperitoneally (Mock IP) with supernatant fluids from mock-infected MA-104 cells.

[b] Splenic lymphocytes were obtained at various intervals after inoculation and tested for RRV-specific cytotoxic activity by ^{51}Cr-release assay at effector-to-target-cell ratios of 50:1.

[c] RRV-specific cytotoxic activity by splenic lymphocytes was not determined (ND) for certain time intervals after inoculation.

specific humoral immune responses were similar to those found in human rotavirus infection (21). Adult mice orally inoculated with simian rotaviruses develop a vigorous rotavirus-specific cellular and humoral immune response (20, 23), but do not exhibit signs of diarrhea.

RRV-specific cytotoxic activity among splenic lymphocytes peaked 6 days after oral or parenteral inoculation of mice with infectious RRV. To determine when splenic lymphocytes with RRV-specific cytotoxic activity appeared after RRV inoculation, adult C57BL/6 mice (obtained from Taconic Laboratories, Germantown, N.Y.) were inoculated (i) orally or intraperitoneally with 10^7 PFU of RRV, (ii) intraperitoneally with an equivalent quantity of RRV completely inactivated with 0.10% β-propiolactone (BPL) and UV irradiation, or (iii) orally or intraperitoneally with supernatant fluids from mock-infected MA-104 cells. Splenic lymphocytes obtained at various intervals after inoculation were tested for RRV-specific cytotoxic activity by ^{51}Cr-release assay, using RRV-infected B6/WT3 target cells at effector-to-target-cell ratios of 50:1 (Table 1). B6/WT-3 (*H-2*^{b} haplotype, simian virus 40-transformed murine embryo fibroblast) cells were provided by Steven Jennings (Louisiana State University Medical Center, Shreveport, La.) and grown as previously described (23). Simian rotavirus RRV strain 2 (MMU 18006) was obtained from Nathalie Schmidt (Viral and Rickettsial Disease Laboratory, Berkeley, Calif.) and was plaque purified in MA-104 cells; viral growth and infectivity titration by plaque assay were performed as previously described (22). To detect rotavirus-specific cytotoxic activity, B6/WT3 cells were infected with 100 PFU per cell of RRV or with supernatant fluids from mock-infected MA-104 cells for 1 h at 37°C. 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 [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 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) per well was added. At 4 h after infection, $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, Ill.) was added to each well (2.5 μCi per 3×10^4 cells) and incubated for 1 h at 37°C. Target cells were washed twice with phosphate-buffered saline, and effector cells were added to target cells for 4 h at 37°C. Percent ^{51}Cr release from RRV-infected target cells was defined as experimental release minus spontaneous release (no CTLs) divided by total release (in the presence of detergent) minus spontaneous release. Percent specific ^{51}Cr release was determined by subtracting percent ^{51}Cr release from mock-infected target cells from percent ^{51}Cr release from RRV-infected target cells. (Percent ^{51}Cr release by effector cells of mock-infected target cells was consistently less than 5%.) To ablate RRV infectivity, tissue culture stocks of RRV were exposed to 0.10% BPL at 4°C for 72 h and then were exposed at 37°C for 3 h. BPL-treated RRV in a fluid layer approximately 1.0 mm thick (2.5 ml per 60-mm-diameter Petri dish) was exposed for 10 min at room temperature to UV light from a germicidal lamp (30 W, 90 cm, G30T8; General Electric Co., Schenectady, N.Y.) placed directly above the Petri dishes at a distance of 19.0 cm. BPL- and UV-treated RRV preparations were tested for viral infectivity as previously described (22).

After either oral or parenteral inoculation of mice with infectious RRV, RRV-specific cytotoxic activity by splenic CTLs was greatest at 6 days after inoculation (Table 1). Parenteral inoculation induced cytotoxic activity among splenic lymphocytes earlier and at greater levels than that found after oral inoculation. Inoculation of mice with noninfectious RRV or with supernatant fluids from mock-infected MA-104 cells did not induce RRV-specific cytotoxic activity among splenic lymphocytes at 6 days after inoculation. Detection of peak rotavirus-specific cytotoxic activity approximately 6 days after oral or parenteral inoculation of mice with RRV is consistent with that observed after parenteral inoculation of mice with Sendai (5), vaccinia (10), or influenza viruses (2).

RRV-specific CTLs were detected at the intestinal mucosal surface 6 days after inoculation of mice with infectious RRV. To determine whether rotavirus-specific CTLs appeared at the intestinal mucosal surface after rotavirus infection, adult mice were inoculated orally or intraperitoneally with RRV or orally with supernatant fluids from mock-infected MA-104 cells. IELs were isolated 6 days after infection and tested for RRV-specific cytotoxic activity by measuring ^{51}Cr release from RRV-infected B6/WT3 cells (Table 2). To isolate IELs, small intestines were removed from the duodenum to the ileocecum and flushed of fecal material with 0.9% NaCl. The mesentery, adherent connective tissue, and fat were dissected from the intestines, and PP lymphocytes were removed from the anti-mesenteric border. Intestines were opened longitudinally, cut into 2- to 3-cm pieces, and washed in Hanks balanced salt solution containing 25 mM HEPES and 50 μg of gentamicin sulfate per ml (pH 7.4; 300 mosmol/liter). Small intestines were washed four times in Hanks balanced salt solution containing 10^{-4} M EDTA, and supernatants were collected, pooled, decanted through gauze, and centrifuged at 600 × *g* for 10 min. Cells were suspended in Hanks balanced salt solution plus 5% fetal bovine serum, filtered through nylon wool, centrifuged at 600 × *g* for 10 min, suspended in 30% Percoll in RPMI 1640 medium, and centrifuged at 600 × *g* for 10 min. Cells were suspended in RPMI 1640, layered on top of 30 to 80% discontinuous Percoll gradients, and centrifuged at 600 × *g* for 20 min at 4°C. Cells at the 55% Percoll interface were collected, washed twice in RPMI 1640, and suspended in RPMI 1640 containing 10% fetal bovine serum, 10 mM HEPES, 0.03% glutamine, and 3×10^{-5} M 2-mercaptoethanol. In addition, the percentage of lymphocytes bearing Thy1.2, CD8, and immunoglobulin was determined by fluorescence-activated

TABLE 2. Cytotoxic activity and surface markers of intestinal and nonintestinal lymphocytes 6 days after oral or intraperitoneal inoculation of mice with RRV

Source of lymphocytes	Inoculation of mice with[a]	% Lymphocytes with surface markers			% Specific ^{51}Cr release at effector-to-target-cell ratios of	
		Thy1	CD8	Immunoglobulin	33:1	11:1
IEL	RRV PO	72	73	2	20	14
	RRV IP	65	52	ND[b]	14	6
	Mock	65	61	5	1	1
PP	RRV PO	23	5	78	30	12
	RRV IP	17	8	ND	16	5
	Mock	27	8	76	0	0
MLN	RRV PO	48	15	53	30	10
	RRV IP	56	17	ND	36	20
	Mock	49	18	52	0	0
Spleen	RRV PO	41	13	61	33	13
	RRV IP	32	10	ND	51	33
	Mock	44	13	49	0	0
Inguinal lymph node	RRV PO	49	18	57	2	0
	RRV IP	55	19	ND	11	4
	Mock	61	25	47	0	0
B-cell lymphoma		1	1	99		
T-cell hybridoma		99	2	7		

[a] Adult C57BL/6 mice were inoculated either orally (RRV PO) or intraperitoneally (RRV IP) with 10^7 PFU of RRV or orally (Mock) with supernatant fluids from mock-infected MA-104 cells. Six days later animals were sacrificed and PP, MLN, inguinal lymph node, and splenic lymphocytes and IELs were removed and tested for rotavirus-specific cytotoxic activity in a ^{51}Cr-release assay and for T- and B-cell surface markers by fluorescent flow cytometry.

[b] B-cell surface markers were not determined (ND) for all lymphocyte populations.

cell sorter analysis (Table 2) as previously described (6, 26). T-cell surface markers were detected by using rat anti-mouse Thy1.2, rat anti-mouse CD8 (both provided by Charles Hackett, the Wistar Institute, Philadelphia, Pa.), and fluorescein isothiocyanate-conjugated, affinity-purified, mouse anti-rat IgG F(ab')2 (Pell-Freez Biologicals, Rogers, Ariz.). B-cell surface markers were detected by using fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Capell Laboratories, Malvern, Pa.). B-cell lymphomas and influenza virus-specific T-cell hybridomas were provided by Charles Hackett.

RRV-specific cytotoxic activity was detected among IELs 6 days after oral or parenteral inoculation of mice with infectious RRV. Rotavirus-specific cytotoxic activity was not detected among IELs from mock-infected mice. The percentages of T and B cells found among IELs were consistent with those found by other investigators (19, 26) and did not differ among infected and uninfected mice.

Rotavirus-specific cytotoxic activity by IELs was major histocompatibility complex (MHC)-restricted and ablated by treatment with Thy1.2-specific IgM plus complement. IELs were tested against MHC-compatible target cells [$H\text{-}2^b$] and MHC-incompatible target cells [$H\text{-}2^d$, $H\text{-}2^s$, and $H\text{-}2^k$] at effector-to-target-cell ratios of 33:1. Simian virus 40-transformed $H\text{-}2^s$ (PSJLSV) and $H\text{-}2^d$ (MKSA) cells were provided by Barbara Knowles (the Wistar Institute, Philadelphia, Pa.). Murine fibroblast $H\text{-}2^k$ (L) cells were provided by Donald Rubin (the Veterans Administration Medical Center, Philadelphia, Pa.). The percentage of ^{51}Cr released from $H\text{-}2^b$, $H\text{-}2^d$, $H\text{-}2^s$, and $H\text{-}2^k$ target cells was 20, 4, 2, and 1%, respectively. At an effector-to-target-cell ratio of 33:1, the percentage of ^{51}Cr released from RRV-infected target cells by IELs which were either untreated, treated with complement alone, or treated with anti-Thy1.2 plus complement was 15, 19, and 2%, respectively. Treatment of lymphocytes with anti-Thy1.2 plus complement was performed as previously described (23).

Rotavirus-specific CTLs may be important in either amelioration of acute infection or prevention of reinfection. Because rotaviruses cause disease by replicating in mature intestinal epithelial cells (28), rotavirus-specific CTLs would probably have to act at the intestinal mucosal surface to limit production and spread of infectious virus. IELs have been reported by others to exhibit natural killer activity (15, 25, 26) and alloantigen-specific cytotoxic T-cell activity (7, 15). However, a precise function for IELs in either infection or disease has not been demonstrated. The surveillance and lysis of virus-infected intestinal epithelial cells by IELs may be an important part of the host response to infection.

The finding of MHC restriction by rotavirus-specific intraepithelial CTLs is consistent with that previously reported for RRV-specific splenic CTLs; cytotoxic activity by splenic lymphocytes from C57BL/6 mice ($H\text{-}2^b$) was restricted at $H\text{-}2D^b$ (23). $Thy1^-$, $CD4^-$, $CD8^+$ large granular lymphocytes, which compose up to 50% of IELs, were apparently not responsible for RRV-specific cytotoxic activity. The findings of inducible, MHC-restricted cytotoxic activity by $Thy1^+$ IELs are consistent with the criteria for virus-specific CTLs established by Zinkernagel and Doherty (33).

Presentation of viral antigens to the intestinal mucosal surface was not required to elicit virus-specific CTLs at the intestinal surface. Rotavirus-specific CTLs were detected among IELs 6 days after parenteral inoculation of mice with RRV (Table 2). Rotaviruses inoculated intraperitoneally are most likely processed and presented by peritoneal antigen-presenting cells to lymphocytes with homing receptors for intestinal or nonintestinal high endothelial venules (HEVs). Alternatively, infectious RRV inoculated intraperitoneally may enter the bloodstream and infect the basolateral surface of intestinal villus epithelial cells. However, we were unable to detect infectious RRV in intestinal homogenates of parenterally inoculated animals (data not shown).

RRV-specific cytotoxic activity was detected among intestinal and nonintestinal lymphocytes 6 days after oral or parenteral inoculation of mice with infectious RRV. To determine the relative rotavirus-specific cytotoxic activity among intestinal and nonintestinal lymphocytes 6 days after rotavirus infection, adult mice were inoculated orally or intraperitoneally with RRV or orally with supernatant fluids from mock-infected MA-104 cells. Splenic, inguinal lymph node, mesenteric lymph node (MLN), and PP lymphocytes were isolated and tested for RRV-specific cytotoxic activity by measuring ^{51}Cr release from RRV-infected B6/WT3 cells (Table 2). Single-cell suspensions of PP lymphocytes were obtained as previously described (16). In addition, the percentage of lymphocytes bearing Thy1.2, CD8, and immunoglobulin was determined by fluorescence-activated cell sorter analysis (Table 2). Oral inoculation of mice with infectious RRV induced RRV-specific cytotoxic activity among intestinal (MLN, PP) and splenic lymphocytes. Parenteral inoculation induced RRV-specific cytotoxic activity among intestinal (MLN, PP) and splenic as well as nonintestinal (inguinal node) lymphocytes. Rotavirus-specific cytotoxic activity was not detected among lymphocytes from mock-infected mice. The percentages of T and B cells found among PP, MLN, splenic, and inguinal node lymphocytes were consistent with those found by other investiga-

tors (19, 26) and did not differ among infected and uninfected mice.

The detection of rotavirus-specific CTLs among splenic and intestinal (PP, MLN) but not inguinal node lymphocytes 6 days after oral inoculation of mice with RRV could not be explained by relative differences in the number of $Thy1^+$ or $CD8^+$ lymphocytes at those sites. Rather these findings are most likely explained by gut-associated lymphocyte trafficking. Guy-Grand and co-workers (9) found that T cells, arising from precursors in PP lymphocytes, migrated via MLNs and blood to the lamina propria and intraepithelial compartments. This trafficking of lymphocytes back to the intestine is mediated by binding to specific molecules (homing receptors) located on HEVs (29). The recent isolation and characterization of two monoclonal antibodies which bind to HEVs in the lamina propria, PP lymphocytes, and MLNs, but not to peripheral lymph nodes (e.g., inguinal), provides a molecular basis for this phenomenon (29). There are several possible explanations for the detection of rotavirus-specific CTLs in intestinal and splenic but not inguinal node lymphocytes after oral inoculation. (i) Rotaviruses, replicating in mature intestinal epithelial cells, may be taken up by membranous epithelial cells (M cells) overlying PP lymphocytes in a manner analogous to that of reoviruses (32), or they may cross the basement membrane and enter the lamina propria. CTLs, initially generated in PP lymphocytes or the lamina propria, would migrate to draining MLNs and return to the circulation, at which point they would be detected in the spleen. Lymphocytes then would circulate to HEVs in the lamina propria and emigrate across the basement membrane to the intraepithelial layer. Alternatively, rotavirus-specific CTLs, initially generated in the intraepithelial layer, may pass back into the lamina propria and migrate to MLNs and the spleen. These hypotheses are at variance with observations by Jung and co-workers (11), who recently found that alloantigen-activated lymphocytes lost homing receptors associated with binding to peripheral node HEVs. Antigen-activated T lymphocytes may therefore, at least transiently, become part of a sessile, nonrecirculating pool. However, the loss of peripheral node homing receptors observed after alloantigen stimulation of lymphocytes in vitro may not occur with intestinal homing receptors after virus stimulation of lymphocytes in vivo. (ii) Rotaviruses may infect lymphocytes in the intraepithelial layer or PP lymphocytes; infected lymphocytes may then migrate to MLNs and the spleen in which they would induce virus-specific CTLs. (iii) Rotavirus-specific proteins, protein fragments, or infectious virus may enter the blood or lymphatics, migrate to MLNs and spleen, and induce rotavirus-specific CTLs at those sites. However, we found that after oral inoculation of adult mice with RRV, infectious virus was detected in the intestines, but not in the liver, spleen, brain, kidneys, or blood (data not shown).

RRV-specific CTL precursors appeared among PP lymphocytes, MLNs, and splenic and inguinal node lymphocytes, but not among IELs 4 weeks after oral or parenteral inoculation of mice with infectious or noninfectious RRV. To determine whether CTL precursors appeared among intestinal and nonintestinal lymphocytes after RRV inoculation, adult C57BL/6 mice were inoculated (i) orally or intraperitoneally with 10^7 PFU of RRV, (ii) intraperitoneally with an equivalent quantity of RRV completely inactivated with 0.10% BPL and UV irradiation, or (iii) orally with supernatant fluids from mock-infected MA-104 cells. Animals were sacrificed 4 weeks later, and PP lymphocytes, MLNs, inguinal lymph nodes, splenic lymphocytes, and IELs were removed and stimulated in vitro with irradiated, RRV-infected, syngeneic splenic lymphocytes as previously described (23). At 7 days after in vitro stimulation, lymphocytes were tested for RRV-specific cytotoxic activity in a ^{51}Cr-release assay (Table 3). Approximately 35% of PP, inguinal node, MLN, and splenic lymphocytes and 10% of IELs were recovered 7 days after in vitro stimulation. Oral or parenteral inoculation of mice with infectious RRV or parenteral inoculation of mice with noninfectious RRV induced RRV-specific CTL precursors in PP, splenic, MLN, and inguinal lymph node lymphocytes, but not among IELs. Inoculation of mice with supernatant fluids from mock-infected MA-104 cells did not induce RRV-specific CTL precursors among splenic, intraepithelial, PP, MLN, or inguinal node lymphocytes.

TABLE 3. Detection of CTL precursors among intestinal and nonintestinal lymphocytes from mice inoculated with infectious or noninfectious RRV

Source of lymphocytes	Inoculation of mice with[a]	% Specific ^{51}Cr release at effector-to-target-cell ratios of		
		20:1	6.7:1	2.2:1
IEL	RRV PO	1	0	0
	RRV IP	0	1	2
	Noninfectious RRV	0	0	0
PP	RRV PO	44	29	12
	RRV IP	17	17	4
	Noninfectious RRV	10	3	3
	Mock	3	2	0
MLN	RRV PO	67	61	33
	RRV IP	58	51	50
	Noninfectious RRV	22	19	7
	Mock	2	1	0
Spleen	RRV PO	53	49	29
	RRV IP	52	50	54
	Noninfectious RRV	39	20	7
	Mock	5	2	1
Inguinal lymph node	RRV PO	61	45	30
	RRV IP	42	56	34
	Noninfectious RRV	31	13	4
	Mock	1	0	0

[a] Adult C57BL/6 mice were inoculated (i) orally (RRV PO) or intraperitoneally (RRV IP) with 10^7 PFU of RRV, (ii) intraperitoneally with an equivalent quantity of RRV completely inactivated with 0.10% BPL and UV irradiation (Noninfectious RRV), or (iii) orally with supernatant fluids from mock-infected MA-104 cells (Mock). Then 4 weeks later, animals were sacrificed and PP, MLN, inguinal lymph node, and splenic lymphocytes and IELs were removed and stimulated in vitro with irradiated, RRV-infected, syngeneic splenic lymphocytes as described in the text. At 7 days after in vitro stimulation, lymphocytes were tested for RRV-specific cytotoxic activity in a ^{51}Cr-release assay.

We were unable to detect rotavirus-specific CTL precursors at the intestinal surface 4 weeks after inoculation of mice with RRV. Rotavirus-specific CTLs which appear acutely after infection may be generated from precursors at a site or sites other than the intestinal surface (e.g., PP lymphocytes). This fact is consistent with the finding that most (approximately 70%) of the IELs are short-lived (27). Our findings are at variance with those of Ernst and coworkers, who detected alloantigen-specific CTL precursors among IELs (7). Rotavirus-specific CTL precursors generated after intestinal infection and stimulated in vitro with irradiated, RRV-infected syngeneic cells may be present at a frequency lower than that obtained after in vitro stimulation of IELs with irradiated, allogeneic cells. Alternatively, our inability to detect virus-specific CTL precursors at the intestinal surface may be due to (i) a lack of specific in vitro growth factors required by CTL precursors located at the

intestinal mucosal surface, (ii) the presence of suppressor T cells among IELs, or (iii) an impairment of CTL precursor function during the extensive procedure used to isolate IELs. We found that splenic lymphocytes from RRV-infected mice treated in a manner analogous to IELs during isolation retained CTL precursor activity (data not shown).

Detection of rotavirus-specific CTL precursors among intestinal and nonintestinal lymphocyte populations did not appear to be influenced by the site of inoculation. RRV-specific CTL precursors were detected after oral or parenteral inoculation of mice with RRV in PP, MLN, splenic, and inguinal node lymphocytes. However, London et al. found that the frequency of reovirus-specific CTLs was greater in PP than in peripheral lymph nodes after intraduodenal inoculation (16). To more accurately determine the degree to which route of immunization determines the frequency of rotavirus-specific CTLs at a particular site, we will perform limiting dilution assays on intestinal and nonintestinal lymphocyte populations after either oral or parenteral inoculation of mice with RRV.

Viral replication at the intestinal surface was not necessary to induce virus-specific CTL precursors among intestinal lymphocytes. Parenteral inoculation of mice with noninfectious RRV elicited RRV-specific CTL precursors among PP and MLN lymphocytes. These findings are consistent with those observed for antigen-specific B cells after parenteral inoculation of toxoids or inactivated virus. Antigen-specific B cells are detected in PP lymphocytes after either intraduodenal administration of cholera toxin or intraperitoneal administration of cholera toxoid (8). In addition, poliovirus-specific antibodies are detected by ELISA in nasopharyngeal secretions after either oral administration of live, attenuated poliovirus or parenteral administration of inactivated poliovirus (3).

Since their initial description, the function, memory, and trafficking of IELs after enteric infection has remained obscure. Surveillance and lysis of virus-infected intestinal epithelial cells by IELs may be an important part of the response of the host to infection.

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