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Memory and Distribution of Virus-Specific Cytotoxic T Lymphocytes (CTLs) and CTL Precursors after Rotavirus Infection

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The gastrointestinal tract is constantly exposed to a variety of potentially invasive bacteria, viruses, and parasites. The first line of defense against these pathogens is the intestinal mucosal surface, which consists of epithelial cells, intraepithelial lymphocytes (IELs), mucus, and secretory immunoglobulins. In addition, the intestine is a rich source of lymphocytes located within Peyer's patches and the lamina propria. Little is known about the function, memory, trafficking, or origin of intestinal T lymphocytes after intestinal infection. We studied the murine cytotoxic T-lymphocyte (CTL) response to the intestinal pathogen rotavirus (simian strain RRV). Adult mice were inoculated orally or via the hind footpad with RRV; virus-specific cytotoxic activities in intestinal and nonintestinal lymphocyte populations were determined by ^{51}Cr release assays. In addition, virus-specific CTL precursor (CTLp) frequencies were determined by limiting-dilution analysis. IELs containing rotavirus-specific cytotoxic activity were detected after oral but not footpad inoculation and expressed α/β but not γ/δ cell surface protein; virus-specific CTLs did not appear to arise from CTLp among IELs. In addition, the site at which RRV was presented to the immune system determined the site at which RRV-specific CTLp first appeared. Frequencies of rotavirus-specific CTLp detected in Peyer's patches were 25- to 30-fold greater after oral than after footpad inoculation. However, regardless of the route of inoculation, rotavirus-specific CTLp were distributed throughout the lymphoid system 21 days after infection. Implications of these findings for vaccine design are discussed.

Infections of the intestinal tract are among the most prevalent causes of disease and death worldwide. Each year an estimated 3 billion to 5 billion cases of diarrhea account for 5 million to 10 million deaths (60). 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) (32). However, the role of intestinal T lymphocytes in intestinal infections has only recently been explored (10, 28, 29). Considering that T lymphocytes are abundant in Peyer's patches (PP), lamina propria (LP), and mesenteric lymph nodes (MLN) and at the intestinal mucosal surface (intraepithelial lymphocytes [IELs]) (7, 31, 47, 48), little is known about the function, memory, trafficking, or origin of intestinal T cells.

This 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, there is a great deal of interest in using the gastrointestinal tract as a means of inducing an immune response that will protect against diseases acquired parenterally. For example, adult volunteers orally inoculated with adenoviruses (13) or influenza viruses (59) are protected against diseases acquired via the respiratory tract. Similarly, raccoons orally inoculated with vaccinia virus vectors expressing rabies virus glycoprotein G are protected against parenteral rabies virus challenge (51). The degree to which lymphocyte memory among intestinal and nonintestinal T lymphocytes is determined by initial site of immunization remains unclear. Second, rotaviruses are the most important cause of infant and childhood gastroenteritis both in the United States and in developing countries (4, 22). Although a number of investigators have examined serum and intestinal secretions for the presence of rotavirus-specific antibodies after rotavirus infection (23, 30), there is little information on the cellular immune response to rotavirus (57). To develop a successful rotavirus vaccine, it may be important to understand all aspects of the immune response associated with protection against challenge. Third, simian rotavirus strain RRV has been used by researchers at the National Institutes of Health in large-scale trials of protective efficacy in infants and young children (21).

Several observations in adult and suckling mice support the hypothesis that rotavirus-specific CTLs may be important in protection against rotavirus challenge. Simian rotavirus strains replicate in intestinal epithelial cells and induce diarrhea in suckling mice (36, 38, 41); the clinical symptoms, small intestinal histopathologic changes, and type-specific humoral immune responses are similar to those found in human rotavirus infection. We found that IELs obtained 6 days after oral inoculation of mice with RRV lysed RRV-infected target cells; CTLs were responsible for RRV-specific cytotoxic activity (39). Passive transfer of Thy-1^+ $CD8^+$ IELs or splenic lymphocytes from rotavirus-inoculated mice ablates rotavirus shedding from the intestinal tract of mice with severe combined immunodeficiency syndrome (10). In addition, CTLs from adult mice inoculated with murine rotavirus passively protect suckling mice against murine rotavirus-induced disease (40). Lastly, the immunologic basis of protection against heterotypic challenge found after immunization of infants and young children with bovine rotavirus strain WC3 may in part be explained by rotavirus-specific CTLs (42).

To explore the memory, distribution, and origin of intes-

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tinal and nonintestinal lymphocytes after rotavirus infection, adult mice were inoculated orally or via the hind footpad with RRV; virus-specific cytotoxic activities in intestinal and nonintestinal lymphocyte populations were determined by ^{51}Cr release assays. In addition, virus-specific CTL precursor (CTLp) frequencies were determined by limiting-dilution analysis. We found that the site at which RRV was presented to the immune system determined the site at which RRV-specific CTLp first appeared. However, regardless of the route of inoculation, rotavirus-specific CTLp were distributed throughout the lymphoid system 21 days after infection.

MATERIALS AND METHODS

Animals. C57BL/6 (*H-2*^{b}) female mice (8 to 12 weeks old) were obtained from Taconic Breeding Laboratories (Germantown, N.Y.).

Cells. Simian virus 40-transformed C57BL/6 mouse embryo fibroblast cells (B6/WT3), grown as previously described (38), were provided by Steven Jennings (Louisiana State University, Shreveport). Fetal African green monkey kidney cells (MA-104) were grown as described previously (37).

Virus. Simian rotavirus RRV strain 2 (serotype 3) was obtained from Nathalie Schmidt (Viral and Rickettsial Disease Laboratory, Berkeley, Calif.). Plaque-purified stocks of virus were prepared in MA-104 cells. Viral growth and infectivity titration by plaque assay were performed as described previously (37).

Immunization. Some mice were orally inoculated with 10^7 PFU of RRV by proximal esophageal intubation through a 10-gauge polyethylene tube. Other mice were subcutaneously inoculated with 5×10^6 PFU per hind footpad through a 30-gauge needle. Mock-infected animals were inoculated with a preparation derived from uninfected MA-104 cells in an equivalent volume.

Isolation of intestinal and nonintestinal lymphocytes. Spleens, MLNs, and inguinal lymph nodes (IngLNs) were removed, and single-cell suspensions were obtained by careful dissection with 21-gauge needles. Circulating lymphocytes were isolated by centrifugation through Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradients. IELs were isolated as previously described (39). To isolate LP and PP lymphocytes, small intestines were removed from the duodenum to the ileocecal junction and flushed of fecal material with Hanks balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS) and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (HBSS-FBS). The mesentery, adherent connective tissue, and fat were dissected from the intestines, and PP lymphocytes were removed from the antimesenteric border. Intestines were opened longitudinally, cut into 2- to 3-cm pieces, and washed in HBSS-FBS at room temperature. Intestines were washed twice at 37°C in HBSS containing 3.4×10^{-3} M EDTA (HBSS-EDTA), once in HBSS-EDTA containing 1 mM dithiothreitol, three times in HBSS-EDTA, and three times in HBSS-FBS. Intestines were washed twice for 40 min each time at 37°C with HBSS containing 20% FBS and 10 U of class III collagenase (Worthington Biochemical, Freehold, N.J.) per ml. Supernatants were collected, pooled, filtered through cotton, and centrifuged at $600 \times g$ for 10 min. Cells were suspended in RPMI 1640 (GIBCO, Grand Island, N.Y.), layered on top of 30 to 80% discontinuous Percoll gradients (Sigma Chemical, St. Louis, Mo.), and centrifuged at $600 \times g$ for 25 min at 4°C. Cells at the 55% Percoll interface were collected and washed twice in HBSS-FBS.

Distribution of infectious rotavirus in intestinal and nonintestinal tissues. Mice were inoculated orally or subcutaneously via the hind footpad with RRV as described above. Animals were sacrificed 1, 2, 3, 4, 5, or 6 days after infection, and spleens, PP, IngLNs, MLNs, and blood were collected and tested for the presence of infectious RRV by a viral plaque assay as previously described (37). Whole blood was frozen, thawed, and centrifuged at $12,800 \times g$ for 1 min. Supernatant fluids were tested for presence of infectious RRV as described above.

Determination of lymphocyte surface markers by fluorescence-activated cell sorter analysis. The percentage of lymphocytes bearing the markers Thy-1.2, CD8, and Ig was determined by fluorescence-activated cells sorter analysis as previously described (11, 48). T-cell surface markers were detected by using rat anti-mouse Thy-1.2-specific IgM (33), rat anti-mouse CD8 (52) (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 (Cappel Laboratories, Malvern, Pa.).

CTLp frequency analysis. Intestinal and nonintestinal lymphocytes for use in these assays were collected as described above. Twenty replicates each of 1,000 to 400,000 cells were placed in individual wells of 96-well V-bottomed plates. In addition, 10^5 irradiated, syngeneic, splenic lymphocytes (antigen-presenting cells) were exposed to 5 PFU of RRV per cell and added to individual wells with putative effector cells. Putative effector cells and antigen-presenting cells were placed in wells each containing 100 μl of RPMI complete medium containing RPMI 1640, 10% FBS, 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; 8 μg of methyl-α-D-mannopyranoside (Sigma) and 10% supernatant fluids from concanavalin A-stimulated rat splenic lymphocytes were added. After 7 days, effector cells were tested against RRV- or mock-infected target cells by a ^{51}Cr release assay.

^{51}Cr release assay for detection of rotavirus-specific CTLs. Preparation of B6/WT3 target cells and ^{51}Cr release assays for detection of rotavirus-specific CTLs obtained 6 days after inoculation or after in vitro stimulation in limiting-dilution analysis were performed as previously described (42). Virus-specific ^{51}Cr release in limiting-dilution assays was defined as greater than 10% lysis of RRV-infected target cells and at least twofold-greater lysis of RRV-infected than of mock-infected target cells. CTLp frequencies were determined according to the maximum-likelihood method (15).

Determination of T-cell receptor molecules of lymphocytes with rotavirus-specific cytotoxic activity. Monoclonal antibodies directed against CD3 (2C11) (26) and T-cell receptor proteins α/β (H57597) (25) and γ/δ (3A10) (19) were provided by Susumu Tonegawa (Massachusetts Institute of Technology, Cambridge). Splenic lymphocytes or IELs were resuspended in RPMI complete medium containing a 1:8 dilution of supernatant fluids from monoclonal antibodies (expressed in tissue culture) and placed at 37°C for 1 h. ^{51}Cr release assays were performed as previously described (38).

RESULTS

Distribution of infectious RRV after oral or parenteral inoculation. Infectious RRV was detected in PP and MLNs 1 day after oral inoculation and in the spleen 2 days after oral

TABLE 1. Distribution of infectious RRV in intestinal and nonintestinal tissues after oral or footpad inoculation of mice with RRV[a]

Tissue	Route of inoculation	Viral infectivity titer (log_{10} PFU/g of tissue) on given day after infection				
		1	2	3	4	5
PP	p.o.	2.5	4.1	4.8	4.2	2.5
	s.c.	—[b]	—	—	—	—
MLN	p.o.	4.2	4.6	3.7	3.6	2.1
	s.c.	—	—	—	—	—
Spleen	p.o.	—	4.2	3.7	2.2	—
	s.c.	5.2	4.4	1.7	—	—
IngLN	p.o.	—	—	—	—	—
	s.c.	5.8	5.1	2.5	—	—
Blood	p.o.	—	—	—	—	—
	s.c.	—	—	—	—	—

[a] Adult C57BL/6 female mice were inoculated orally (p.o.) by proximal esophageal intubation with 10^7 PFU of RRV or subcutaneously (s.c.) via the hind footpad with 5×10^6 PFU of RRV per footpad. Animals were sacrificed at various intervals after infection, and PP, MLN, spleens, IngLN, and blood were tested for the presence of infectious RRV by plaque titration assay.

[b] —, Infectious RRV was not detected at 0.7 PFU log_{10}/g of tissue; this was true for all determinations at 6 days after infection.

inoculation of adult mice with 10^7 PFU of RRV (Table 1). Infectious virus was detected in both PP and MLN 1, 2, 3, 4, and 5 days and in the spleen 2, 3, and 4 days after inoculation. In PP, infectious virus was detected 3 days after inoculation at titers 100-fold greater than those obtained 1 or 5 days after inoculation. RRV was not detected in the IngLN or blood after oral inoculation. All animals were healthy and well-appearing throughout these experiments.

Progressively decreasing quantities of infectious RRV were detected in the IngLN and spleen 1, 2, and 3 days after subcutaneous inoculation via the hind footpad with 5×10^6 PFU of RRV (Table 1). RRV was not detected in PP, MLN, or blood after footpad inoculation.

Cytotoxic activity and surface markers of intestinal and nonintestinal lymphocytes 6 days after inoculation of mice with RRV. Adult mice were inoculated orally with 10^7 PFU of RRV or inoculated subcutaneously with 5×10^6 PFU of RRV via the hind footpad. Six days after inoculation, intestinal and nonintestinal lymphocyte populations were collected and tested immediately for cytotoxic activity in a ^{51}Cr release assay at effector-to-target ratios of 33:1 and 11:1 (Table 2). The greatest levels of RRV-specific cytotoxic activity were detected in the LP, spleen, and MLN after either oral or footpad inoculation. On the other hand, RRV-specific cytotoxic activity was detected at the intestinal mucosal surface among IELs only after oral inoculation and among IngLN lymphocytes only after parenteral inoculation. Low levels of virus-specific cytotoxic activity were detected in blood and PP after either oral or footpad inoculation.

In addition, lymphocytes were tested for the percentage of cells bearing T- and B-cell surface markers by fluorescent flow cytometry. The percentages of intestinal and nonintes-

TABLE 2. Cytotoxic activities and surface markers of intestinal and nonintestinal lymphocytes 6 days after oral or subcutaneous inoculation of mice with RRV[a]

Source of lymphocytes	Route of inoculation	Lymphocytes (%) with surface markers			Specific ^{51}Cr release (%) at effector-to-target ratios of:	
		Thy-1	CD8	Ig	33:1	11:1
IEL	RRV p.o.	70	75	2	19	8
	RRV s.c.	69	73	3	2	2
	Mock p.o.	65	61	2	1	1
LP	RRV p.o.	63	34	23	33	16
	RRV s.c.	69	30	17	36	19
	Mock p.o.	56	17	40	0	0
PP	RRV p.o.	20	8	76	9	2
	RRV s.c.	29	14	70	7	2
	Mock p.o.	21	7	76	0	0
MLN	RRV p.o.	50	18	51	20	13
	RRV s.c.	55	24	35	22	6
	Mock p.o.	55	19	36	1	0
Spleen	RRV p.o.	41	20	48	39	27
	RRV s.c.	40	21	50	33	14
	Mock p.o.	38	19	57	3	0
IngLN	RRV p.o.	45	19	50	2	0
	RRV s.c.	41	17	55	14	10
	Mock p.o.	46	18	47	1	1
Circulating	RRV p.o.	50	19	41	9	2
	RRV s.c.	58	23	30	8	4
	Mock p.o.	50	11	35	2	1

[a] Adult C57BL/6 female mice were inoculated either orally (RRV p.o.) with 10^7 PFU of RRV, subcutaneously via the footpad with 5×10^6 PFU of RRV (RRV s.c.), or orally (Mock p.o.) with supernatant fluids from mock-infected MA-104 cells. Six days later, animals were sacrificed and lymphocytes from the indicated sources 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.

TABLE 3. Ablation of rotavirus-specific cytotoxic activity by monoclonal antibodies directed against T-cell receptor molecules[a]

Source of lymphocytes	^{51}Cr release (%) by lymphocytes treated with:			
	No antibody	Anti-CD3	Anti-α/β	Anti-γ/δ
IEL	15	1	1	14
Spleen	25	2	9	23

[a] Adult C57BL/6 female mice were inoculated orally by proximal esophageal intubation with 10^7 PFU of RRV. Animals were sacrificed 6 days after inoculation. Splenic lymphocytes and IELs were removed, treated with monoclonal antibodies directed against either CD3 or T-cell receptor heterodimers (α/β or γ/δ), and tested for cytotoxic activity in a ^{51}Cr release assay at effector-to-target ratios of 50:1.

tinal lymphocytes bearing the surface marker Thy-1, CD8, or Ig were similar to those previously described (7, 31, 47, 48) and did not significantly differ among RRV- or mock-infected mice.

T-cell receptor molecules of IELs and splenic lymphocytes with rotavirus-specific cytotoxic activity. IEL and splenic lymphocytes were obtained 6 days after oral inoculation of mice with RRV. To determine T-cell receptor molecules associated with RRV-specific cytotoxic activity, lymphocytes (at effector-to-target ratios of 50:1) were incubated with monoclonal antibodies directed against either CD3 or T-cell receptor protein α/β or γ/δ (Table 3). Cytotoxic activity by IELs and splenic lymphocytes was ablated by treatment with anti-CD3 or anti-α/β but not by anti-γ/δ monoclonal antibody.

Frequencies of rotavirus-specific CTLp among intestinal and nonintestinal lymphocytes after oral or footpad inoculation. CTLp frequencies of intestinal and nonintestinal lymphocytes obtained after oral or footpad inoculation were determined by limiting-dilution analysis and normalized to the percentage of lymphocytes bearing Thy-1 and CD8 surface molecules (Table 4). CTLp were detected only in PP and MLN 3 days after oral inoculation and only in the spleen and IngLN 3 days after footpad inoculation.

Six days after oral inoculation, CTLp were detected in PP at frequencies approximately 25- to 30-fold greater than those obtained after footpad inoculation (Table 4). Conversely, 6 days after footpad inoculation, CTLp were detected in IngLN at frequencies approximately sevenfold greater than those obtained after oral inoculation. CTLp frequencies obtained in MLN, LP, spleen, and blood 6 days after oral inoculation were similar to those obtained in the same site after footpad inoculation. CTLp were not detected among IELs 6 days after oral or footpad inoculation.

CTLp frequencies obtained in PP, LP, MLN, spleen, IngLN, and blood 21 days after oral inoculation were similar to those obtained in the same site 21 days after footpad inoculation (Table 4).

DISCUSSION

We found that adult mice orally inoculated with RRV did not develop diarrhea. These findings are consistent with the observation that although adult animals orally inoculated with homologous or heterologous host strains often develop a vigorous rotavirus-specific humoral and cellular immune response (35, 38, 53), adult animals rarely develop acute gastroenteritis (53). Similarly, although rotaviruses are an important cause of acute gastroenteritis in infants and young children, rotaviruses are a rare cause of severe gastroenteritis in adults (2, 22, 24, 62). The increased incidence of rotavirus disease in the young of humans and other animals is not clearly explained by lack of previous exposure. These findings are more likely related to decreased expression of rotavirus receptors on the surface of villus epithelial cells in adults as compared with infants (49).

TABLE 4. Frequencies of RRV-specific CTLp 3, 6, or 21 days after oral or subcutaneous inoculation of mice with infectious RRV[a]

Source of lymphocytes	Route of inoculation	Frequencies (per 10^6 cells) at various intervals after infection					
		Day 3		Day 6		Day 21	
		Thy-1	CD8	Thy-1	CD8	Thy-1	CD8
LP	p.o.	ND[b]	ND	100	185	266	494
	s.c.	ND	ND	162	373	360	742
PP	p.o.	4	10	180	450	22	50
	s.c.	—[c]	—	7	14	28	70
MLN	p.o.	9	25	170	472	25	80
	s.c.	—	—	62	142	74	279
Spleen	p.o.	—	—	122	250	100	220
	s.c.	10	27	182	348	118	224
IngLN	p.o.	—	—	9	21	20	56
	s.c.	7	16	59	141	16	42
Circulating	p.o.	—	—	10	26	10	21
	s.c.	—	—	10	26	12	25

[a] Adult C57BL/6 female mice were inoculated either orally (p.o.) with 1×10^7 PFU of RRV or subcutaneously (s.c.) via the footpad with 5×10^6 PFU of RRV. Animals were sacrificed 3, 6, or 21 days later, and PP, MLN, IngLN, spleens, and circulating lymphocytes were removed and tested for CTLp frequencies by limiting-dilution analysis. Frequencies were calculated according to the maximum-likelihood method. In addition, lymphocytes were tested for T-cell surface markers by fluorescent flow cytometry (data not shown). CTLp frequencies were normalized to the percentage of Thy-1$^+$ or CD8$^+$ cells within a given lymphocyte population.

[b] Not done.

[c] RRV-specific CTLp was not detected at a frequency of $0.1/10^6$ cells; this was true for all determinations for IELs.

Although adult mice did not develop gastroenteritis, infectious RRV was detected in PP and MLN up to 5 days after oral inoculation (Table 1). Detection of infectious virus in PP for 5 days is unlikely to represent simple passage of RRV through the small intestine but is more likely consistent with the hypothesis that RRV is replicating at the intestinal mucosal surface. Three observations support this hypothesis. First, infectious RRV was detected in PP 3 days after inoculation at titers 100-fold greater than those obtained 1 or 5 days after inoculation. Second, infectious virus was not detected 8 h after oral inoculation of adult animals with 10^7 PFU of bovine rotavirus strain NCDV (data not shown). We previously found that simian rotaviruses are better adapted to growth in the murine intestinal tract than is bovine strain NCDV (34). Finally, adult animals of different species have been found to shed rotavirus for several days without symptoms of gastroenteritis (8, 49).

Although rotaviruses have consistently been found to replicate only in intestinal villus epithelial cells (54), the appearance of infectious RRV in spleens of animals 2, 3, and 4 days after oral inoculation (Table 1) is consistent with several previous observations. Dharakul and co-workers detected rotavirus antigens by immunofluorescence in spleens of suckling mice up to 20 days after oral inoculation with murine rotavirus (9). In addition, Uhnoo and others detected infectious RRV in livers of suckling mice after oral inoculation; infectious RRV was not detected in livers of suckling mice orally inoculated with murine rotavirus or with bovine rotavirus strain WC3 (58). These findings suggest that simian rotavirus strain RRV may be more likely than homologous host strains to spread to sites distant to the intestinal mucosal surface when orally inoculated into mice. Because rotavirus infections are a rare cause of death in developed countries, and human rotavirus strains are difficult to grow in tissue culture, studies that examine the distribution of infectious rotavirus in nonintestinal tissues of infants and young children have not been performed.

The site at which RRV was presented to the immune system determined the site at which RRV-specific CTLp first appeared. RRV-specific CTLp were detected initially in PP and MLN after oral inoculation and in IngLN and spleen after footpad inoculation (Table 4). These findings are consistent with those of other investigators. Keyhole limpet hemocyanin (KLH)-specific helper T cells were first detected in PP after intra-PP inoculation of mice with KLH (12). Similarly, KLH-specific B cells were first detected in the ipsilateral IngLN of mice inoculated via the footpad with antigen (1).

Frequencies of RRV-specific CTLp detected in PP 6 days after oral inoculation of mice with RRV were approximately 25- to 30-fold greater than those obtained after footpad inoculation (Table 4). This finding is due either to generation of greater frequencies of CTLp at the site of inoculation or to selective recruitment of RRV-specific circulating CTLp to the intestine during intestinal infection. A number of investigators found that lymphoblasts initially generated in PP will leave PP and travel sequentially to the MLN, thoracic duct, and blood, finally returning to PP after several days (12, 46, 56). This trafficking of PP lymphocytes back to PP is mediated by specific binding molecules on PP lymphocytes (homing receptors) which attach to receptors (vascular addressins) on specialized capillary endothelial cells (high endothelial venules [HEV]) (3). The appearance of larger numbers of RRV-specific CTLp in PP 6 days after oral than after footpad inoculation is consistent with the observation that RRV replicates at the intestinal mucosal surface after oral but not footpad inoculation (Table 1). Influenza-specific CTL and T_h cells and lymphocytic choriomeningitis virus-specific CTL are selectively recruited to sites of active inflammation (6, 14, 27, 61). Recruitment of antigen-specific circulating lymphocytes to sites of infection may be mediated by increased blood flow (42, 43) or enhanced expression of vascular addressins on HEV by products of the inflammatory response (20) or both. However, we found that the frequencies of RRV-specific CTLp detected in the IngLN after footpad inoculation of mice with RRV were approximately sevenfold greater than those obtained after oral inoculation. It is unclear whether RRV replicates in IngLNs after footpad inoculation (Table 1). To determine the importance of virus replication on recruitment of virus-specific lymphocytes, we will study the CTL response of animals orally or parenterally inoculated with noninfectious RRV or rotavirus strains that are less adapted to growth in the murine intestinal tract.

We found that RRV-specific CTLp were detected in the MLN and LP but not PP after hind footpad inoculation even though infectious RRV was not detectable at any of those sites. HEV in MLN have been shown to express vascular addressins for both PP and PLN homing receptors (55). Circulating rotavirus-specific CTLp generated in IngLN after footpad inoculation may bind to PLN vascular addressins in MLN. Similarly, it may be that HEV in LP express vascular addressins capable of attracting CTLp derived from PLN stimulation. Consistent with this hypothesis, Fuhrman and Cebra found that cholera toxin-specific plasmablasts were detected in LP in equal numbers after oral or parenteral inoculation of mice with cholera toxin (16).

We found that the frequencies of RRV-specific CTLp in spleen did not differ after oral or footpad inoculation. Because infectious RRV was detected in the spleen after either oral or footpad inoculation, RRV-specific splenic CTLp may have been generated initially in the spleen after presentation of RRV to splenic lymphocytes by splenic antigen-presenting cells. Alternatively, virus-specific CTLp may have originated in the PP or IngLN after oral or footpad inoculation, respectively, and trafficked to the spleen from the circulation (45). Trafficking of lymphocytes to the spleen involves mechanisms distinct from those controlling homing to PLN or PP (45).

Six days after infection, rotavirus-specific CTLs were detected among IELs after oral inoculation, among IngLN after hind footpad inoculation, and among LP, MLN, and spleen after oral or footpad inoculation (Table 2). The origin of CTLs in the IEL compartment is unclear. RRV-specific CTLp were not detected among IELs after oral inoculation despite the presence of CTLs at that site. In addition, although in mice a large percentage of T lymphocytes at the intestinal surface express γ/δ heterodimers (5), we found that RRV-specific CTLs at the intestinal surface expressed α/β heterodimers (Table 3). Therefore, rotavirus-specific CTLs in the IEL compartment may arise from CTLp at a site or sites other than the intestinal epithelial surface. Guy-Grand and coworkers postulated that at least some IELs may arise from precursors in PP and migrate via the blood to the LP and IEL compartment (17, 18). Our findings are at variance with those of London and coworkers, who detected reovirus-specific CTLp in the IEL compartment 1 week after intraduodenal inoculation of mice with reovirus (28).

Regardless of the route of inoculation, RRV-specific CTLp were distributed throughout the lymphoid system 21 days after infection. The greatest frequencies of RRV-specific CTLp were detected in LP after either oral or

footpad inoculation. These findings are at variance with those of London and coworkers, who found that the frequency of reovirus-specific CTLp was significantly greater in PP than PLN 6 months after intraduodenal inoculation of mice with reovirus (29). Whereas rotaviruses replicate in mature villus epithelial cells located in the distal villus, reovirus antigens are taken up by membranous epithelial cells overlying PP (63). The degree to which these differences contribute to observed differences in relative CTLp frequencies remains to be determined. Our findings are consistent with the hypothesis that oral inoculation of some live viruses may be an effective means of inducing CTL memory at sites distant to the intestine. Similarly, parenteral inoculation may be sufficient to induce CTL memory among intestinal lymphocyte populations. The extent to which our results reflect or require virus replication remains to be determined. However, the importance of site of inoculation in inducing lymphocyte memory among intestinal and nonintestinal lymphocytes may depend on the relative importance of resident as compared with recirculating virus-specific CTLp in generating virus-specific CTLs. The importance of resident RRV-specific lymphocytes is probably best studied by determining CTLp frequencies in intestinal lymphocyte populations after orally rechallenging mice with RRV 3 to 4 months after oral or parenteral immunization.

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