GUT MUCOSAL IMMUNIZATION WITH REOVIRUS SEROTYPE 1/L STIMULATES VIRUS-SPECIFIC CYTOTOXIC T CELL PRECURSORS AS WELL AS IgA MEMORY CELLS IN PEYER'S PATCHES

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The ability of a vertebrate to recognize microorganisms as foreign and eliminate them is critical for host survival. For pathogens that enter via the mucosal surface, studies have shown that the prevention of adherence or colonization decreases the likelihood of their causing disease (1). To limit colonization, both specific and nonspecific defense mechanisms that act at mucosal surfaces constitute the first line of defense against infectious agents (1, 2).

Since the discovery of IgA, much research has been aimed at determining the mechanism of stimulating the expression and secretion of this mucosally associated immunoglobulin. Peyer's patches (PP),[1] which constitute the organized lymphoid follicles found within the walls of the small intestine, contain a unique microenvironment that allows for the generation of an antibody response dominated by IgA-committed B cells. IgA-committed precursor cells, generated by interaction of antigen with the lymphoid elements present in PP, enter the circulation, mature, and eventually repopulate the lamina propria of mucosal surfaces with IgA-secreting plasmablasts (3–5). It appears that antigens that impinge on mucosal surfaces and interact with PP are especially efficient at generating an antibody response dominated by IgA (6). Recent data has suggested that T cell subsets are present in PP that are capable of influencing the isotype expression of B cells. Two such T cell populations have been described by McGhee and coworkers (7–10). The first population has been shown to recognize and interact with IgA-committed B cells to allow them to mature into IgA-secreting plasma cells (7, 8). The second population, described as IgA-specific contrasuppressor T cells, allow an IgA plaque-forming cell response to occur in the spleens of mice rendered tolerant by previous oral immunization (9, 10).

While PP contain T cells that appear important for the generation of an IgA

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[1] *Abbreviations used in this paper:* M cell, microfold cell; MLN, mesenteric lymph node; MOI, multiplicity of infection; pCTL, precursor cytotoxic T lymphocyte; PEC, peritoneal exudate cells; PP, Peyer's patch; PLN, peripheral lymph nodes.

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response, the generation of cytotoxic T cells (CTLs) to non–major histocompatibility complex (MHC)-encoded (nominal) antigens in PP has not been demonstrated. Since CTLs have been shown to be important in the resolution of viral infections (11–13), it is of interest to examine the ability of mucosal tissues to generate a CTL response. Such a response may be critical for the effective control of infections initiated at mucosal surfaces, because subsets of T cells may differ in their preferred recirculation pathways (14), and those primed in the mucosa may have a propensity to return there to aid in the resolution of infection. To analyze such mucosal priming, we have used reovirus serotype 1, strain Lang (reovirus 1/L) as a probe to determine whether murine PP are capable of supporting the generation of a virus-specific CTL response. Reovirus 1/L was chosen for these studies for the following reasons: (*a*) reovirus 1/L is a naturally occurring enteric virus that is stable within the gastrointestinal tract (15); (*b*) priming for reovirus-specific CTLs has been shown to occur in the murine spleen in response to intraperitoneal immunization with virus (16); and (*c*) reovirus 1/L preferentially binds to and is transported from the intestinal lumen into PP through microfold (M) cells (17, 18), thereby allowing interactions to occur within the patch between virus, antigen-presenting cells, and lymphocytes. Our initial findings (19) have shown that the enteric application of reovirus 1/L results in detectable virus-specific IgA antibodies in intestinal secretions. In this paper, we have examined the clonal B cell response generated in vitro by cells primed in vivo with reovirus 1/L. We found that a high proportion of B cell clonal precursors obtained from PP and the spleen after intraduodenal immunization are precommitted to IgA production when they are stimulated in vitro in the splenic focus assay. Therefore, reovirus 1/L appears to be an efficacious mucosal antigen.

We also report that a single intraduodenal application of reovirus 1/L is capable of generating detectable levels of virus-specific cytotoxicity in PP lymphocytes upon in vitro restimulation. Such effectors show the surface phenotype of CTLs (Thy-1^+, Lyt-2^+), are MHC-restricted, and virus specific. Furthermore, the local generation in the PP of the CTL response is suggested by the finding that the intraduodenal application of reovirus 1/L generates higher levels of virus-specific CTLs in the PP as compared to peripheral lymph nodes (PLN) 2 or 6 d after immunization. Virus-specific CTLs can be detected in PP 6 mo after a single enteric application of reovirus 1/L, indicating a long-lived memory response.

Materials and Methods

Mice. Male C3HeB/FeJ and CBA/J (H-2^k), and DBA/2J (H-2^d) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c (H-2^d) mice were bred in our animal facility. These mice were housed in isolators, and screened on a regular basis for reovirus antibody. Virally primed mice were kept physically isolated from all other experimental and stock mice. 6–12-wk-old mice were used in all experiments. Lymphoid tissues from three mice were pooled for each experiment.

Cell Lines. The following cell lines were used in these studies: L-929 (H-2^k) fibroblast cells, L-929 cells transfected with H-$2D^d$ (DM-1) and H-$2L^d$ (LM-1) genes (obtained from Drs. J. Weis and J. Seidman, Harvard Medical School, Boston, MA) (20), the B10.D2 simian virus 40–transformed kidney line KD2SV (obtained from Dr. B. Knowles, The

Wistar Institute, Philadelphia, PA) (21), and the A/Sn-derived Moloney virus-transformed T cell lymphoma Yac-1.

Viruses. Reovirus 1/Lang (1/L) and reovirus 3/Dearing (3/D) clones were originally obtained from Dr. Bernard Fields (Harvard Medical School, Boston, MA). We have recloned these parental stocks on L cell monolayers, and used gradient-purified third-passage stocks, titered by limiting dilution on L cell monolayers (22). These viral stocks manifest the characteristic dsRNA (double-stranded RNA) banding patterns associated with type 1/L or 3/D reovirus, as determined by polyacrylamide gel electrophoresis (23). Vaccinia virus (WR isolate) was kindly provided by Dr. J. Bennink (The Wistar Institute, Philadelphia, PA).

In Vitro Generation of Virus-specific CTLs from Spleen. Splenic CTLs were generated in vitro from mice primed in vivo with reovirus 1/L (24) or vaccinia virus (25) following previously published protocols. Cells were cultured in a 5% CO_2 atmosphere in complete medium consisting of RPMI-1640, 25 mM Hepes supplemented with 50 μg/ml gentamycin, 2 mM glutamine, 10% FCS (Gibco Laboratories, Grand Island, NY) and 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO).

In Vitro Generation of Virus-specific CTLs from Peyer's Patches. Enterically primed mice were immunized by the intraduodenal application of 3×10^7 PFU of reovirus 1/L suspended in 0.5% gelatin (J. T. Baker Chemical Co., Phillipsburg, NJ) dissolved in PBS (gel saline) after a small laparotomy (6). Control mice were subjected to the same surgical procedures and injected with gel saline alone. 1 wk after priming, a single-cell suspension of PP lymphocytes was obtained. 2×10^5 lymphocytes for bulk cultures, or varying numbers for limiting-dilution cultures, were added per well of a 96-well round-bottomed microtiter plate (Nunc, Roskilde, Denmark) in the presence of 5×10^4 virally pulsed peritoneal exudate stimulator cells (PECs). PECs were obtained from syngeneic mice that had received a single intraperitoneal injection of 1.5 ml of thioglycolate medium without indicator (BBL Microbiology Systems, Cockeysville, MD) 3–4 d before being harvested by peritoneal lavage with 10 ml of HBSS (Gibco Laboratories). Harvested PECs were virally pulsed at a multiplicity of infection (MOI) of one for 1 h, during which they were exposed to 1,600 rad of γ-radiation from a cobalt source. On the following day, Con A–conditioned medium at a final concentration of 10% was added and the cultures were harvested after 6 d for bulk cultures, or 8 d for limiting-dilution cultures. The preparation of conditioned medium has been previously described (26). Live lymphocytes were obtained from bulk cultures by Ficoll/Isopaque centrifugation (27).

Cytotoxicity Assay. A standard ^{51}Cr-release assay, using various E/T ratios, was used to measure cell-mediated cytotoxicity (28). Assays were performed in 96-well V-bottomed microtiter plates (Nunc) in a 5% CO_2 incubator for 5 h. All assays were performed in triplicate.

Yac-1, P-815, or LPS blasts were labeled with ^{51}Cr by overnight incubation at 37°C with 200 μCi of Na^{51}Cr (Amersham Corp., Arlington Heights, IL) in complete medium. LPS blasts were obtained by Ficoll/Isopaque centrifugation (27) of splenocytes cultured for 2 d in the presence of 50 μg/ml of *Escherichia coli* serotype 0111:B4 LPS (Sigma Chemical Co.). L cells, the transfected L cell lines DM1 and LM1, or KD2SV cells were infected with reovirus at an MOI of five before overnight culture in T-25 tissue culture flasks (Costar, Cambridge, MA) at 31°C (L cells) or 37°C (transfected L cells or KD2SV cells) in the presence of 200 μCi of Na^{51}Cr (Amersham Corp.). Adherent targets were released by incubation with versene (EDTA) solution. Vaccinia virus–infected L cell targets were prepared as previously described (25).

Complement-mediated Cell Lysis. 30H12 (anti-Thy-1) (29) and 3.155 (anti-Lyt-2) (30) monoclonal antibodies were used to lyse effector cells before assay. Equal volumes of cultured lymphocytes, undiluted antibody (acid supernatant), and a 1:5 dilution of rabbit complement were incubated at 37°C for 45 min. Cells were washed twice before being assayed. Control cells were incubated with complement alone.

Limiting-dilution Analysis. Replicate microculture wells (n = 20), containing varying numbers of either PP or PLN lymphocytes obtained from mice intraduodenally stimulated with reovirus 1/L, were stimulated in vitro as described. After an 8-d culture period, the

contents of individual microtiter wells were resuspended and divided into two portions in V-bottomed 96-well microtiter plates (Nunc), and assayed for cytotoxic activity vs. either reovirus 1/L infected or uninfected L cells. Cultures were considered to demonstrate cytotoxicity to a particular target if the resultant ^{51}Cr release was three standard deviations above control levels. Individual microcultures, tested for cytotoxicity against infected and uninfected L cells, were considered to be virus specific if the ^{51}Cr release was two- to threefold higher against infected vs. uninfected L cell targets. The fraction of microcultures at particular input cell doses that did not generate virus-specific cytotoxicity was used to calculate the precursor CTL frequency by the maximum likelihood method (31). This calculation also generates a p value, which determines whether the calculated frequency is consistent with single-hit kinetics. Frequencies with an associated p value >0.05 are consistent with single-hit kinetics and are considered valid by the maximum likelihood calculation.

Clonal Assay for Reovirus-specific B Cells. Frequencies of reovirus-sensitive B cells were determined by the splenic focus assay (32). Splenocytes from BALB/c mice, immunized 10 wk previously by the intraperitoneal injection of 3×10^7 PFU of reovirus 1/L, or PP lymphocytes, and splenocytes from BALB/c mice immunized by the intraduodenal application of 3×10^7 PFU of reovirus 1/L 4 wk previously, were analyzed. Limiting numbers of cells were injected into the tail veins of reovirus 1/L-primed (3×10^7 PFU in CFA for >10 wk), lethally irradiated (1,600 rad) recipients. After 16 h, recipient spleens were removed and diced. The resultant fragments were distributed into 96-well flat-bottomed microtiter plates (Costar). Each fragment was challenged with 2×10^9 particles of UV-inactivated reovirus 1/L (33). Excess antigen was removed from culture on day 4 and supernatants were collected on days 7, 10, and 13 for analysis by RIA for reovirus-specific antibodies.

RIA. A solid-phase RIA was used to detect reovirus-specific antibodies. Reovirus 1/L was suspended at 4.8×10^{11} particles/ml in $NaHCO_3$ buffer, pH 9.5. 25 μl were added per well to 96-well vinyl microtiter plates (Costar) for 16 h at 4°C. These plates were then used to identify supernatants containing reovirus-specific antibodies with ^{125}I-labeled rabbit anti-mouse Fab or rabbit anti-mouse isotype scoring reagents generated in our laboratory (34).

Results

B Cell Response after Introduodenal Immunization with Reovirus Serotype 1/L. The generation of a reovirus-specific B cell response was investigated at the clonal level by means of splenic fragment cultures. We found that 4 wk after the intraduodenal application of reovirus 1/L, a high proportion of clones, obtained from either the spleen (61%) or PP (73%), were secreting IgA antibodies specific for reovirus (Table I). Further, the majority of these clones were synthesizing IgA exclusively. In comparison, mice stimulated by the intraperitoneal injection of reovirus 1/L produce a B cell response that is dominated by IgG-secreting clones (86%) with few clones exclusively making IgA (Table I). Although the isotype profile exhibited after intraduodenal immunization was markedly skewed toward IgA production, similar rises in frequencies of clonal precursors from normally low levels ($<1/10^6$) were observed regardless of the route of viral inoculation. These results demonstrate that, by the criterion of B cell priming, reovirus is an efficacious immunogen when applied intraduodenally.

Virus-specific Cytoxic Activity Is Found in PP after Intraduodenal Application of Reovirus Serotype 1. To investigate CTL responsiveness after mucosal challenge, an in vitro culture system, in which precursor CTLs (pCTLs) could expand into effector CTLs, was used. 6 d after the intraduodenal application of 3×10^7 PFU of reovirus 1/L, significant levels of cytotoxicity were generated in vitro from

TABLE I

Frequencies and Isotype Profile of Reovirus-specific Memory B Cells

Isotype expressed	Clones expressing given isotype in:		
	Spleen*	Spleen‡	PP‡
		%	
Some IgM	4.5	33	18
Some IgG	86	14	9
Some IgA	36	61	73
IgA only	9	50	64
Clones analyzed (n):	22	14	11
Frequency per 10^6 B cells§	23	31	33

* Donor mice were primed by intraperitoneal injection of 3×10^7 PFU of reovirus 1/L 10 wk before sacrifice.

‡ Donor mice were primed by the intraduodenal injection of 3×10^7 PFU of reovirus 1/L 4 wk before sacrifice.

§ Frequencies were calculated assuming 60% B cells were in PP, 40% in the spleen, and that 4% of donor cells lodge in the spleen of recipient mice.

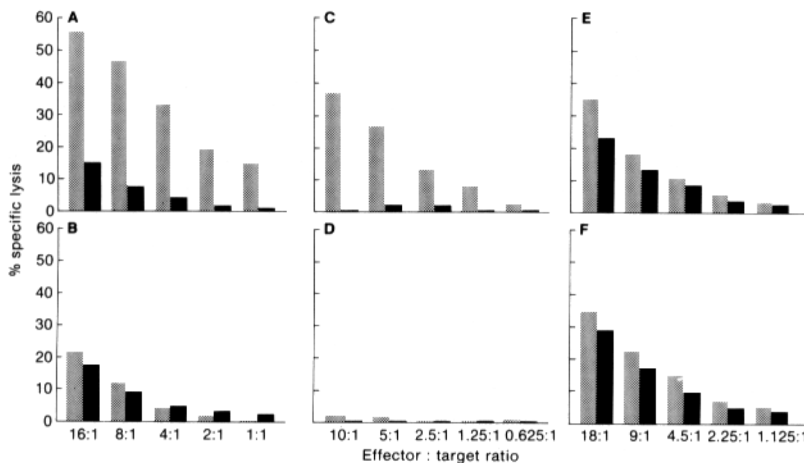

FIGURE 1. Cytotoxic activity of PP lymphocytes. PP lymphocytes obtained from C3H mice primed intraduodenally with reovirus (*a, c, e*) or control mice (*b, d, f*) were stimulated in vitro with reovirus 1/L and conditioned medium (*a, b*), reovirus 1/L alone (*c, d*) or conditioned medium alone (*e, f*). After 6 d of culture, cytotoxic activity was tested using uninfected L cells (*solid bars*) or reovirus 1/L-infected L cells (*stippled bars*) as targets.

PP lymphocytes (Fig. 1*A*) that were comparable to those obtained from parenterally immunized splenocytes (data not shown). PP cells from mice that had received an intraduodenal application of gel saline without virus did not produce a virus-specific cytotoxic response (Fig. 1*B*). However, PP cells from both primed and control mice produced detectable levels of cytotoxicity to uninfected L cells (Fig. 1, *A* and *B*). This cytotoxicity against L cells was dependent on the addition of conditioned medium to the culture system and not reovirus 1/L. PP cells from primed mice stimulated without the addition of conditioned medium produced virus-specific cytoxicity without detectable levels of cytotoxicity to uninfected L

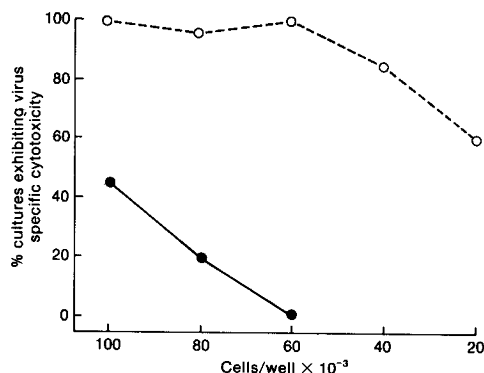

FIGURE 2. T cell help is required for the generation of virus-specific CTLs in in vitro cultures. Replicate microcultures seeded with the indicated numbers of input cells/well were stimulated in vitro in the presence (○) or absence (●) of 10% conditioned medium. After 8 d of culture, individual microculture wells were tested for virus-specific cytotoxic activity.

cells, while control PP lymphocytes produce cytotoxicity to neither infected nor uninfected L cells (Fig. 1, *C* and *D*). Conversely, primed or control PP lymphocytes cultured in the presence of conditioned medium alone produced substantial levels of cytotoxicity to L cells, regardless of viral infection (Fig. 1, *E* and *F*). Primed or control PP lymphocytes cultured in the absence of conditioned medium and virus produced no detectable levels of cytotoxicity to either infected or uninfected L cells (data not shown). Therefore, reovirus-specific cytotoxic effector cells can only be generated in vitro from PP that have been stimulated in vivo with reovirus.

Virus-specific Helper Activity Is Found in PP after Intraduodenal Application of Reovirus Serotype 1/L. Addition of an exogenous source of interleukin 2 (conditioned medium) increased the level of virus-specific lysis generated in nonlimiting cultures, although its presence was not an absolute requirement (Fig. 1, *A* vs. *C*). However, in limiting-dilution cultures, the generation of a virus-specific response was shown to be absolutely dependent upon factors present in conditioned medium. Individual microcultures seeded with limiting numbers of primed PP lymphocytes were assayed for the generation of virus-specific cytotoxic activity. Low input numbers of responding cells (6×10^4 cells/well) did not generate virus-specific cytotoxicity when cultured in the absence of conditioned medium. However, 100% of such microcultures generated virus-specific cytotoxicity when cultured in the presence of 10% conditioned medium (Fig. 2). The ability of nonlimiting (bulk) cultures of primed PP to generate virus-specific cytotoxic activity without the addition of helper factors indicates that virus-responsive helper cells are present in PP 6 d after intraduodenal stimulation.

Characterization of the Cytotoxic Potential of PP Lymphocytes. Because we have observed cytotoxic activity against uninfected L cells in the experiment shown in Fig. 1, we chose to assay bulk-cultured PP lymphocytes against additional targets (Fig. 3). While cytotoxicity to uninfected L cells was minimal in this experiment, significant cytotoxicity to both reovirus 1/L- and 3/D-infected L cells was observed. In addition, intermediate levels of killing of the natural killer cell (NK) target, Yac-1 were detected. However, both allogeneic and syngeneic

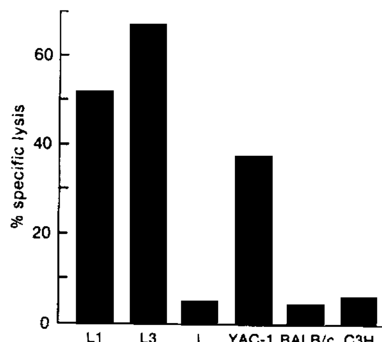

FIGURE 3. Cytotoxic activity of reovirus 1/L-primed PP lymphocytes. PP lymphocytes obtained from intraduodenally immunized mice were stimulated in vitro and effectors were tested at a 50:1 E/T ratio for cytotoxic activity to the NK target Yac-1, syngeneic (C3H) or allogeneic (BALB/c) LPS blasts, uninfected L cells, and reovirus 1/L- or 3/D-infected L cell targets.

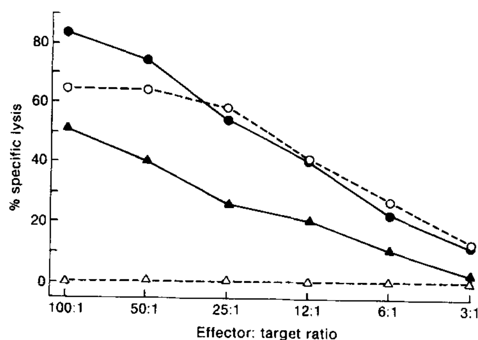

FIGURE 4. Cytotoxic activity of reovirus 1/L-primed splenocytes. Splenocytes obtained from intraperitoneally immunized C3H mice were stimulated in vitro and effectors were tested for cytotoxic activity to the following targets: uninfected L cells (△), reovirus 1/L-infected L cells (○), reovirus 3/D-infected L cells (●), Yac-1 cells (▲).

LPS blasts were not significantly lysed. Age-matched control mice gave PP cells that exhibited comparable levels of cytolysis to the NK target Yac-1 when cultured in the presence of conditioned medium (data not shown), indicating that the ability to lyse Yac-1 target cells is not a result of viral priming.

We analyzed splenocytes obtained from intraperitoneally immunized mice to determine whether they also generated cytotoxic lymphocytes that were not serotype-specific, as well as cytotoxicity to the NK target Yac-1. We found that primed splenocytes restimulated in bulk culture also efficiently lyse both reovirus 1/L- and 3/D-infected targets, and exhibit significant cytotoxicity to Yac-1 targets (Fig. 4). This demonstrates that in vitro-cultured lymphocytes, derived from either PP or spleen, can generate both reovirus-specific cytotoxic cells that do not distinguish reovirus serotype as well as cells that kill NK targets.

To examine the surface phenotypic markers of the reovirus-specific effectors generated in in vitro cultures obtained from primed PP, monoclonal antibodies to T cell determinants plus complement were used to eliminate subpopulations of effector cells. Substantial reduction of cytotoxicity against reovirus-infected L cells (reovirus 1/L or 3/D) was obtained by treatment of effector cells with either

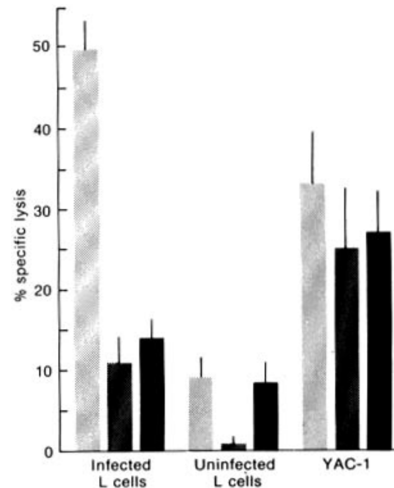

FIGURE 5. The surface phenotype of cytotoxic effector cells. PP lymphocytes obtained from intraduodenally immunized C3H mice were stimulated in vitro for 6 d. Effectors were harvested and pretreated with complement alone (*light bars*) anti-Thy-1 + complement (*dark bars*) or anti-Lyt-2 + complement (*black bars*) and tested for cytotoxic activity against the indicated targets. Results are the mean ± SE of three experiments. Reovirus 1/L–infected L cells were used as targets in all experiments, and reovirus 3/D–infected L cells were used in two of the three experiments.

anti-Thy-1 or anti-Lyt-2 monoclonal antibodies plus complement (Fig. 5), indicating that the virus-specific cytotoxicity was generated by effectors bearing the surface phenotype of CTLs. Cytotoxicity to the NK target, Yac-1, was little affected by treatment with anti-Thy-1 or anti-Lyt-2 monoclonal antibodies plus complement. These findings indicate that a separate population of effector cells mediate cytotoxic activity against L cells virally infected with either reovirus 1/L or 3/D, as opposed to that against Yac-1 targets.

The MHC restriction of PP-derived cytotoxic effector cells was analyzed in the experiment shown in Fig. 6. Reovirus-specific cytotoxic cells were generated from C3H (H-2^k) and DBA/2 (H-2^d) PP that were stimulated by intraduodenal application of reovirus 1/L 6 d previously. These primed effector cells, restimulated in vitro, were then assayed for virus-specific cytotoxicity against a panel of H-2^k or H-2^d cell lines infected either with reovirus 1/L or 3/D. As shown in Fig. 6*A*, C3H effectors efficiently lyse both reovirus 1/L- and 3/D-infected L cell targets. However, these same effectors were unable to lyse the H-2^d line, KD2SV, when infected with either reovirus 1/L or reovirus 3/D (Fig. 6*B*). In contrast, DBA/2J (H-2^d) effectors, which do not lyse reovirus 1/L- or 3/D-infected L cells (Fig. 6*A*), are capable of lysing reovirus 1/L- or 3/D-infected KD2SV targets (Fig. 6*B*). Further, both these DBA/2J effectors as well as C3H effectors are capable of lysing L cells that have been transfected with H-2^d class 1 genes if they are infected with either serotype of reovirus (Fig. 6*C*). The ability of DBA/2J effectors to lyse reovirus-infected L cells that simultaneously express syngeneic class 1 molecules clearly demonstrates that class 1 MHC molecules serve as the restriction element for PP-derived cytotoxic effector cells.

PP lymphocytes, stimulated in vivo and in vitro with reovirus serotype 1/L,

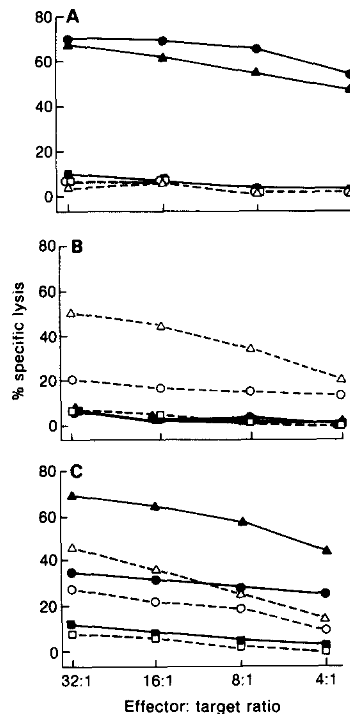

FIGURE 6. PP CTLs are H-2 restricted. PP lymphocytes, obtained from intraduodenally primed C3H (H-2^k; *closed symbols, solid lines*) or DBA/2J (H-2^d; *open symbols, dashed lines*) mice, stimulated in vitro and effectors were tested for cytotoxic activity to uninfected targets (□), reovirus 1/L-infected targets (○), or reovirus 3/D-infected targets (△). In *A*, the targets are L cells (H-2^k), in *B* the targets are KD2SV cells (H-2^d), and in *C* the targets are a 1:1 mixture of the H-2^d-transfected L cells DM-1 and LM-1.

were assayed for their ability to lyse either normal, reovirus-infected or vaccinia virus-infected L cell targets (Fig. 7*A*). We found that these effectors were cytotoxic for reovirus-infected, but not vaccinia virus-infected L cells. In contrast, splenocytes primed in vivo and in vitro with vaccinia virus were cytotoxic for vaccinia virus-infected but not reovirus-infected L cells (Fig. 7*B*). These observations demonstrate that reovirus-specific CTLs are not crossreactive with unrelated viral antigens.

Virus-specific Cytolytic Activity Is More Pronounced in PP than in Peripheral Lymph Nodes After Intraduodenal Stimulation. We have demonstrated that virus-specific CTLs can be generated from PP 6 d after intraduodenal application of virus. We examined the level of virus-specific cytotoxicity obtained from cultures of PP, mesenteric lymph nodes (MLN), spleen, and PLN 6 d (Fig. 8*A*) or 2 d (Fig. 8*B*) after intraduodenal application of reovirus 1/L. In both cases, substantially higher levels of cytotoxic activity to reovirus-infected L cells were generated from PP as compared with PLN and spleen. MLN contained substantial cytotoxic activity at 2 d, and intermediate levels of cytotoxicity at 6 d.

Limiting-dilution analysis was performed to determine whether the difference in responsiveness of various lymphoid tissues observed in nonlimiting cultures was related to the frequency of precursor CTLs. While we did not detect virus-

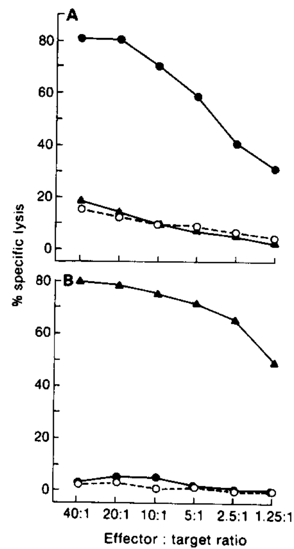

FIGURE 7. Reovirus-reactive CTLs are not crossreactive with unrelated viral antigens. PP lymphocytes obtained from C3H mice stimulated intraduodenally with reovirus 1/L (*A*) or splenocytes obtained from CBA mice stimulated intravenously with vaccinia virus (*B*) were cultured in vitro with the immunizing virus, and effectors were tested for cytotoxic activity to uninfected L cells (○), reovirus 3/D-infected L cells (●), and vaccinia virus-infected L cells (▲).

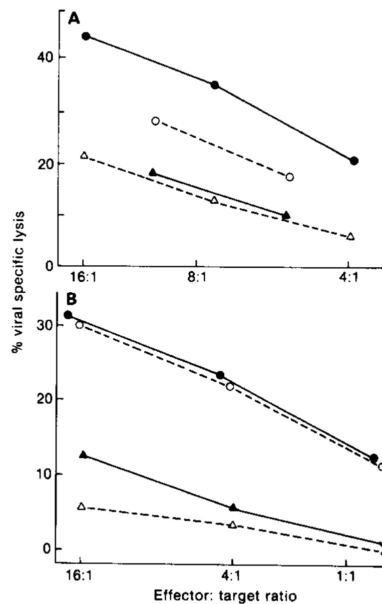

FIGURE 8. Tissue distribution of reovirus-specific cytotoxic activity. PP lymphocytes (●), MLN lymphocytes (○), PLN lymphocytes (△), or splenocytes (▲) were tested 6 d (*A*) or 2 d (*B*) after intraduodenal immunization with reovirus 1/L for virus-specific cytotoxic activity.

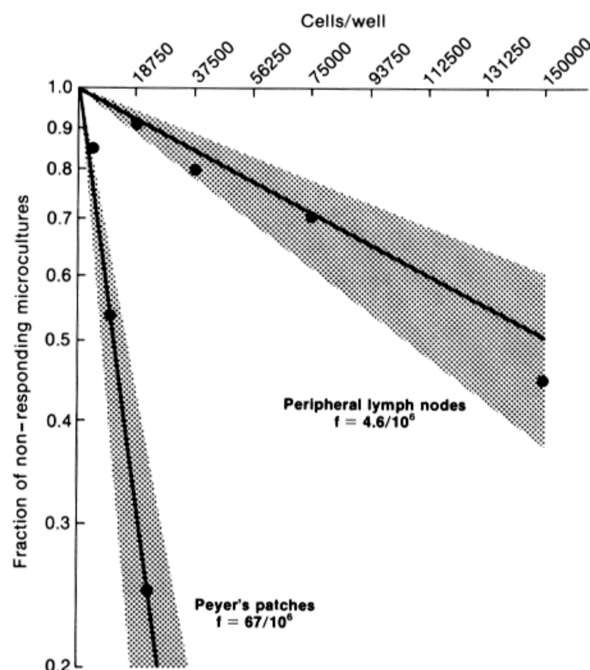

FIGURE 9. Limting-dilution analysis of pCTL frequencies. 6 d after intraduodenal immunization, the frequency of pCTLs present in PP and PLN was analyzed by limiting-dilution analysis. The shaded areas represent the 95% confidence limits of the maximum likelihood estimates.

specific pCTLs from any lymphoid tissue of control animals (data not shown), PP obtained from mice intraduodenally primed with reovirus 1/L 6 d previously showed a precursor frequency of $67/10^6$ PP lymphocytes (Fig. 9 and Table II). The frequency of reovirus 1/L–specific pCTLs obtained from PLN was 14.6-fold lower ($4.6/10^6$) than that occurring in PP (Fig. 9 and Table II). We have also examined the presence of virus–specific pCTLs in PP and PLN 6 mo after intraduodenal immunization with reovirus 1/L. We found that pCTLs were present in both tissues, with a 2.2-fold higher frequency detectable in PP ($50/10^6$) as compared to PLN ($23/10^6$) (Table II).

Discussion

In this report we have examined the ability of intraduodenally administered reovirus 1/L to perturb, in an antigen-specific manner, both T and B cell subsets that occur in PP. Because reovirus 1/L selectively enters PP via M (microfold) cells (17, 18), we postulated that it would be an efficacious mucosal immunogen capable of expanding the numbers of virus-specific T and B cells in this location. The finding of high frequencies of IgA-committed memory cells in PP and spleen after intraduodenal but not intraperitoneal application of this virus confirmed its effectiveness and implicated the PP as the site of priming. We have previously reported that this process of IgA priming occurs naturally for certain antigenic determinants associated with normal gut-commensal organisms, such as phosphocholine and inulin (35, 36), and can be duplicated when germ-free animals,

TABLE II
Frequencies of Reovirus-specific pCTLs Present after Intraduodenal Immunization

Tissue	Frequency (per 10^6 cells)*	p*	Frequencies normalized to:	
			Thy-1^+ ‡	Lyt-2^+ §
PP‖	67	0.3345	270	1,675
PLN‖	4.6	0.6301	6	17
PP¶	50	0.0931	200	1,235
PLN¶	23	0.7480	28	85

* Frequency and p values were calculated by the maximum likelihood method (31).
‡ Absolute frequencies were normalized to the proportion of Thy-1^+ cells present in PP (25%) and PLN (75%) as determined by flow-cytometric analysis.
§ Absolute frequencies were normalized to the proportion of Lyt-2^+ cells present in PP (6%) and PLN (25%) as determined by flow-cytometric analysis.
‖ Donor mice were primed by the intraduodenal injection of 3×10^7 PFU of reovirus 1/L 6 d before sacrifice.
¶ Donor mice were primed by the intraduodenal injection of 3×10^7 PFU of reovirus 1/L 6 mo before sacrifice.

which have low frequencies of B cells specific for these determinants, are colonized with normal gut-commensal organisms (36, 37). This process of IgA priming can also occur when nonenvironmental antigens are applied via the mucosal route. To date, cholera toxin has been found to be the most efficacious nonenvironmental antigen for generating an IgA-committed B cell response in PP after mucosal application (6). While the ability of cholera toxin to generate an IgA-committed response may be related to its pharmacological properties (38), it has also been hypothesized that its ability to avidly bind to gut mucosal surfaces through ganglioside GM1 (39) and to be selectively absorbed by the dome epithelium (40) may be a critical determinant in allowing entry into the patch, with subsequent generation of a B cell response dominated by IgA-committed clones. Our current finding that reovirus 1/L effectively stimulates a virus-specific IgA response further demonstrates that, for antigens that do not chronically persist in the gut, binding to the dome epithelium overlying PP may be crucial for the generation of IgA memory B cells. Whether the effectiveness of enterically-applied reovirus 1/L at priming for an IgA response is related to a specific interaction between viral surface proteins and receptors on M cells is currently under investigation.

Our observation that PP are capable of generating a CTL response to nominal (virus encoded) antigens extends earlier observations that CTL activity directed against MHC differences can be generated in secondary MLR cultures of PP lymphocytes from either normal mice or mice that had been chronically fed allogeneic tumor cells (41, 42). No CTL response occurred in PP when mice were chronically fed tumor cells bearing minor histocompatibility differences (42). While these earlier studies demonstrated that a CTL response could occur in PP, the generation of allospecific CTLs, whose precursors are present at high

frequency among normal T cells (43), may not be representative of the potential of PP to generate CTLs specific for nominal antigens. More recently, it was inferred (44) that virus-specific CTLs are generated in rat PP after the enteric administration of vaccinia virus. Virus-specific helper T cells and CTLs were found in MLN, but not PLN, after enteric application of vaccinia virus. However, cytotoxic or helper activity was not detected in PP at any time after enteric immunization. It was inferred that the virus-specific T cells that were found in the MLN after enteric priming were derived from precursors locally primed in the patches. Because our data demonstrate that virus-specific helper T cells and CTLs can occur in PP, it is likely that a vaccinia virus–specific T cell response also occurred in this system. We feel that we were successful in detecting a CTL response to nominal antigens in PP, while others have failed, in part because we used a more sensitive assay system that depends on expansion of effector CTLs by in vitro restimulation after viral priming in vivo. The ability of reovirus to generate both a virus-specific helper and CTL response further demonstrates its efficacy as a mucosal immunogen.

In our characterization of PP-derived CTLs, we found that while the virus-specific cytotoxic cells were not selective for the immunizing serotype of reovirus, they were not crossreactive with unrelated viral antigens. This lack of serotype specificity is in contrast to previous studies, which reported that reovirus-specific CTLs derived from the spleen after intraperitoneal immunization predominantly recognized serotypically unique determinants expressed on the viral hemagglutinin (16, 24, 45). We found that the lack of serotype specificity was not unique to PP but was also displayed by intraperitoneally primed splenocytes. Thus, the discrepancy is not due to the tissue source of the pCTLs and remains unexplained.

We feel that the CTL activity observed in PP was locally generated. In nonlimiting cultures, significantly higher levels of virus-specific cytotoxicity were observed from PP as compared to PLN 2 or 6 d after intraduodenal immunization. Our observation that PP also contain virus-specific helper activity after intraduodenal priming demonstrates that they contain the cellular elements required for the generation of effector CTLs. The finding of virus-specific CTLs in the MLN after intraduodenal immunization is a likely result of dissemination, via lymphatic drainage from PP, of either activated CTLs or infectious virus. Because the finding of a minimal response in PLN after enteric priming implies that the route of immunization may be an important determinant in the generation of a mucosal vs. peripheral T cell response, it was necessary to determine whether the differences in responses seen in nonlimiting cultures were in fact related to the precursor frequencies present in these tissues. We found a 14-fold higher precursor frequency in PP as compared to PLN 6 d after enteric immunization. The divergence in frequencies present in PP vs. PLN becomes more pronounced when these are normalized to the proportion of T cells present (determined by flow-cytometric analysis), as PP contain fewer T cells (25% Thy-1^+, 6% Lyt-2^+) as compared to PLNs (75% Thy-1^+, 25% Lyt-2^+). When normalized to the proportion of Thy-1^+ cells, a 45-fold difference is observed, whereas the difference is 100-fold when normalized to Lyt-2^+ cells (Table II). Because we have demonstrated such a discrepancy in the frequency of pCTLs present 6 d after enteric immunization, we have examined these tissues for virus-specific

pCTLs 6 mo after the single intraduodenal application of 3×10^7 PFU of reovirus 1/L (Table II). We found a detectable CTL response in both PP and PLN at this time, with a twofold higher frequency in PP as compared to PLN. While this difference is not as large as that which occurred 6 d after priming, the divergence in frequencies becomes significant when normalized to the proportion of Thy-1^+ (sevenfold) or Lyt-2^+ (14.5-fold) cells. This experiment demonstrates that virus-specific pCTLs persist for many months in PP after viral challenge. We feel that this analysis is valid, as it is likely that the precursor cells that generate reovirus-specific CTLs in in vitro cultures are of the T cell lineage. The phenotype of these pCTLs is currently under investigation.

Reynolds and coworkers (14) have suggested that two populations of T lymphocytes can be distinguished based on their preferential recirculation pathways through PLN vs. the gut mucosa. We should be able to determine whether gut immunization generates a population of antigen-reactive T cells that preferentially repopulate mucosal tissues by adoptive transfer experiments aimed at determining which tissue source and which route of priming is most efficient at populating mucosal vs. nonmucosal tissues with virus-specific T cells. That such preferential population of PP vs. PLN occurs after enteric immunization is suggested by the minimal response observed in PLN in bulk and limiting cultures 6 d after priming. We will expand our studies to include the lamina propria and intraepithelial compartments for analysis, as these compartments have been described as containing cytotoxic effectors against a variety of targets (46, 47). Enriching for populations of lymphocytes bearing high or low levels of the MEL-14 antigen, which is associated with binding to PLN but not PP venule high endothelium (48), should enable us to specifically probe for subpopulations of lymphocytes that may display preferential tissue lodging after being primed by antigenic stimuli at different locations.

In this report, we have found that PP lymphocytes are capable of generating cytotoxic activity to the NK cell target Yac-1 upon in vitro culture with conditioned medium. This cytotoxicity to Yac-1 targets is generated from reovirus 1/L–primed as well as control PP. While we do not claim these cultured effectors to be classic NK cells, their presence was unexpected because PP have been described as poor sources for NK cytotoxicity (49). The finding of this type of activity from in vitro–cultured PP indicates that precursor populations for these nonspecific cytotoxic cells occur in PP. It is possible that these precursor populations, when activated in the patch, migrate and develop into functionally competent lytic cells in other tissues. If this is the case, it would be similar to the observation that PP lack terminally differentiated B cells (plasma cells) even though they are capable of generating antibody secreting cells in in vitro cultures (50).

The ability of enterically applied reovirus 1/L to generate a CTL response in PP that can persist for many months demonstrates that a CTL response exists among the repertoire of immune responses that can occur in PP. For viruses that initially impinge on the wet epithelium, the presence of virus-specific CTLs and natural effector (i.e., NK) cells at this location could result in the local containment of the infectious agent before its dissemination. The ability to

mount a mucosal CTL response may be advantageous to the host, because local containment would prevent sequelae associated with infection at distal sites.

Summary

In this report we have shown that reovirus 1/L is an effective mucosal immunogen capable of generating a cytotoxic T cell (CTL) and associated helper T cell response to the nominal antigens associated with reovirus 1/L. The effectors that mediate reovirus-specific cytotoxicity are Thy-1^+, Lyt-2^+, and major histocompatibility complex (MHC)-restricted in their recognition of reovirus antigens, and can therefore be classified as CTLs. Frequency analysis of precursor CTLs occurring in Peyer's patches (PP) and peripheral lymph nodes (PLN) 6 d and 6 mo after intraduodenal stimulation have demonstrated that a persistent gradient of precursors is established, with higher frequencies present in PP. The generation of a CTL response in PP may be important in preferentially repopulating mucosal tissues with effector CTLs that could result in the local containment of infections in the gut.

We also found that reovirus 1/L generates a virus-specific B cell response that is dominated by IgA memory cells after intraduodenal immunization. We hypothesize that the efficacy of reovirus 1/L at stimulating T and B cells in the gut mucosa is related to its ability to selectively enter PP via microfold (M) cells after enteric application.

In this study we have also demonstrated that PP cells, upon in vitro culture and unrelated to prior reovirus priming, can generate natural killer–like (NK) cytotoxic activity. This may be an in vitro correlate of the in vivo generation of effectors that may populate mucosal tissues (i.e., the intestinal epithelium) with NK-like effector cells.

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