

# Reduction of Otherwise Remarkably Stable Virus-specific Cytotoxic T Lymphocyte Memory by Heterologous Viral Infections

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## Summary

Experimental analyses of the acute cytotoxic T lymphocyte (CTL) response to viruses have focused on studying these infections in immunologically naive hosts. In the natural environment, however, viral CTL responses occur in hosts that are already immune to other infectious agents. To address which factors contribute to the maintenance and waning of immunological memory, the following study examined the frequencies of virus-specific CTL precursor cells (pCTL) not only using the usual experimental paradigm where mice undergo acute infections with a single virus, and in mice immune to a single virus, but also in immune mice after challenge with various heterologous viruses. As determined by limiting dilution assays, the pCTL frequency (p/f) per CD8<sup>+</sup> T cell specific for lymphocytic choriomeningitis virus (LCMV), Pichinde virus (PV), or vaccinia virus (VV) increased during the acute infections, peaking at days 7–8 with frequencies as high as 1/27–1/74. Acute viral infections such as these elicit major expansions in the CD8<sup>+</sup> T cell number, which has been reported to undergo apoptosis and decline after most of the viral antigen has been cleared. Although the decline in the total number of virus-specific pCTL after their peak in the acute infection was substantial, for all three viruses the virus-specific p/f per CD8<sup>+</sup> T cell decreased only two- to fourfold and remained at these high levels with little fluctuation for well over a year. The ratios of the three immunodominant peptide-specific to total LCMV-specific clones remained unchanged between days 7 and 8 of acute infection and long-term memory, suggesting that the apoptotic events did not discriminate on the basis of T cell receptor specificity, but instead nonspecifically eliminated a large proportion of the activated T cells. However, when one to five heterologous viruses (LCMV, PV, VV, murine cytomegalovirus, and vesicular stomatitis virus) were sequentially introduced into this otherwise stable memory pool, the stability of the memory pool was disrupted. With each successive infection, after the immune system had returned to homeostasis, the memory p/f specific to viruses from earlier infections declined. Reductions in memory p/f were observed in all tested immunological compartments (spleen, peripheral blood, lymph nodes, and peritoneal cavity), and on average in the spleen revealed a  $3 \pm 0.4$ -fold decrease in p/f after one additional viral infection and an  $8.4 \pm 3$ -fold decrease after two additional viral infections. Thus, subsequent challenges with heterologous antigens, which themselves induce memory CTL, may contribute to the waning of CTL memory pool to earlier viruses as the immune system accommodates ever-increasing numbers of new memory cells within a limited lymphoid population. This demonstrates that virus infections do not occur in immunological isolation, and that CD8<sup>+</sup> T cell responses are continually being modulated by other infectious agents.

Memory CD8<sup>+</sup> precursor CTL (pCTL)<sup>1</sup> specific for viruses proliferate and can be maintained for prolonged time periods *in vivo* in the absence of detectable viral antigens (1–5), but factors contributing to the maintenance

and modulation of CTL memory have remained undefined. Acute infections of mice with lymphocytic choriomeningitis virus (LCMV), Pichinde virus (PV), vaccinia virus (VV), and murine cytomegalovirus (MCMV) can lead to 5–20-

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<sup>1</sup>Abbreviations used in this paper: GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; LDA, limiting dilution assay; MCMV, murine cytomegalovirus; NP, nucleoprotein; pCTL, precursor CTL; PEC, perito-

neal exudate cell; p/f, pCTL frequency; PV, Pichinde virus; VSV, vesicular stomatitis virus; VV, vaccinia virus.

fold increases in the number of CD8<sup>+</sup> T cells in the spleen between days 6 and 9 after infection (6, 7, and our unpublished data). Thereafter, the T cell number declines, bringing about homeostasis in lymphocyte numbers (7–13) after most of the viral antigen has been cleared (7, 12, 13). In the case of LCMV, this decline in lymphocyte number is associated with enhanced susceptibility of isolated T cells to apoptosis *in vitro* (7, 8, 12, 13) and with high levels of apoptosis in the spleen T cell populations *in vivo* (11). The memory T cell population must therefore consist of pCTL that survive these apoptotic events. In the present study we examine, by limiting dilution assays (LDAs), the virus-specific pCTL frequency (p/f) to LCMV, PV, and VV before and after these apoptotic events and find that, even though there are large reductions in the total number of pCTL, the CTL p/f per CD8<sup>+</sup> T cell remains remarkably high after the apoptotic events have occurred and after detectable antigen has been cleared. The CTL p/f specific to LCMV, PV, or VV and to three LCMV-immunodominant peptides remain unchanged for well over a year, illustrating the remarkable stability in T cell memory.

Our previous studies, however, have shown that immune responses to viral infections are not mounted in immunological isolation, as the immune response to one virus may condition a host to make an altered response to a second unrelated or heterologous virus by reactivation of the memory CTL specific to the first virus (14, 15). This reactivation of memory CTL to an earlier virus is due at least in part to the ability of memory CTL to recognize in a cross-reactive manner heterologous viruses. There appears to be sufficient diversity within the memory pCTL repertoire specific for one virus to render some clones susceptible to activation by apparently unrelated heterologous viruses. Enhanced expression of adhesion molecules (6) and IL-2R (5, 16) on memory cells might make them particularly sensitive to stimulation by a low affinity, cross-reactive T cell antigen. This reactivation of memory CTL by heterologous viruses can prime the CTL response to challenge virus and play a role in either immunity (17) or immunopathology (18).

Given the nature of the activation of memory cells by heterologous viruses, we questioned whether such heterologous virus infections would impart a more lasting or retrospective effect on the memory CTL response to previous infectious agents. Here we demonstrate that heterologous viral infections disrupt the homeostasis of stable long-term CTL memory by causing reductions in the CTL p/f specific for viruses to which the host has previously been exposed.

## Materials and Methods

**Mice.** C57BL/6 (H-2<sup>b</sup>) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 2–24 mo of age.

**Viruses.** The LCMV Armstrong strain was propagated in BHK21 baby hamster kidney cells (19). The WR strain of VV (14) and the AN3739 strain of PV, an arenavirus only distantly related to LCMV, were propagated in L929 cells (14, 20). MCMV, strain Smith, was obtained from salivary glands of *in vivo*-infected BALB/c mice (21). Vesicular stomatitis virus (VSV), strain Indi-

ana, was propagated in BHK21 cells. For acute virus infections, mice were injected intraperitoneally with  $4 \times 10^4$  PFU of LCMV,  $8 \times 10^6$  or  $1.5 \times 10^7$  PFU of VV,  $10^6$  PFU of PV,  $10^5$  PFU of MCMV, or  $9 \times 10^8$  PFU of VSV in 0.1–0.3-ml volume.

**Cell Lines.** KO (H-2<sup>b</sup>), an SV40-transformed kidney cell line derived from a C57BL/6 mouse (22) and provided to us by Dr. Satvir Tevethia (Pennsylvania State Medical Center, Hershey, PA) was propagated in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 2 mM L-glutamine,  $5 \times 10^{-5}$  M 2-ME, 10 mM Hepes, and 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO). L929 (H-2<sup>b</sup>), a continuous liver cell line derived from C3H mice, was propagated in Eagle's MEM (GIBCO BRL) supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 2 mM L-glutamine, and 10% heat-inactivated FBS. KO cells were infected with LCMV or PV at a multiplicity of infection (MOI) of 0.1–0.2 PFU/cell and incubated for 2 d at 37°C. KO cells were infected with VV at a MOI of 4 for 3–4 h at 37°C.

**Responder Cells.** Adult C57BL/6 mice were inoculated intraperitoneally with the indicated viruses, and at the indicated times after infection spleens, LNs, peritoneal exudate cells (PECs), and peripheral blood leukocytes were harvested. The LN cells were derived from a pool of the bilateral axillary and inguinal LNs in each mouse. The LCMV-, PV-, and VV-specific p/f per CD8<sup>+</sup> were quantified by LDAs of unsorted cells (15, 23). The percentage of CD8<sup>+</sup> T cells was determined by fluorescent antibody staining and analyzed on either a FACS<sup>®</sup> 440 (Becton Dickinson, San Jose, CA) or FACStar<sup>®</sup> Plus (Becton Dickinson), as previously described (15, 23).

**Infection Protocol with Heterologous Viruses.** Mice were immunized with a sublethal dose of one virus. After the rise and fall of the acute T cell response and when the immune system had returned to homeostasis (usually 5 wk or longer), the mouse was inoculated with another virus. After the T cell response to the second virus returned to homeostasis, the animals were tested for pCTL specific for each virus. Subsequent infections with additional viruses were also tested. To strengthen the significance of the observations, the experimental design was set up to examine in one mouse the p/f simultaneously to two or three viruses within different memory cell compartments. For example, the LCMV-, PV-, and VV-specific p/f in the spleen, LNs, and/or PECs were assessed at the same time in the same mouse that had received LCMV+PV+VV+MCMV and compared with appropriate controls to determine the effect of subsequent virus infections on CTL responses to all three viruses.

The interassay variability for p/f may vary 2–10-fold and is dependent on prevailing culture conditions, such as the lot of FBS or cytokine supernatant that is used. Generally, we had very reproducible results between assays, as can be seen in Fig. 1; these memory p/f were done in separate experiments but all within a close time period with very similar culture conditions. Except for Fig. 1, all the data presented comparing changes in p/f were generated within the same LDA experiments. With our LDA protocol, six separate titrations of the spleen cells from the same LCMV-immune mouse within one LDA set up had a mean p/f per CD8<sup>+</sup> T cell of  $1/28 \pm 2.2$  (SEM; range: 1/21–1/36). No technically acceptable assays examining the deletion of spleen p/f by subsequent virus infections were deleted from these tables, i.e., there was no author-imposed selectivity in the presentation of these data.

**LDA for Virus-specific pCTL.** The assays used the procedure of Moskopidhis et al. (24) with modifications as previously described

(15, 23). Briefly, for LCMV and PV assays, splenic lymphocytes from infected mice (one mouse or two pooled) were harvested and titrated in U-96-well plates with 24 replicates at each titration. They were stimulated with virus-infected PECs ( $3-4 \times 10^4$ /well), supplemented with irradiated splenic feeders ( $1-2 \times 10^5$ /well) and growth factors provided by using a 16% culture supernatant from IL-2-secreting, gibbon lymphoma tumor cell line MLA.144 (American Type Culture Collection, Rockville, MD) (25). After 4 d, the cultures were fed with  $10^4$  irradiated, virus-infected PECs.

The highly lytic nature of VV *in vitro* required the use of a slightly altered method. PECs infected with VV ( $3-4 \times 10^4$  cells/well) *in vivo* were used as stimulators, and neutralizing polyclonal rabbit antiserum to VV was added at a 1/100 dilution final concentration (15, 26).

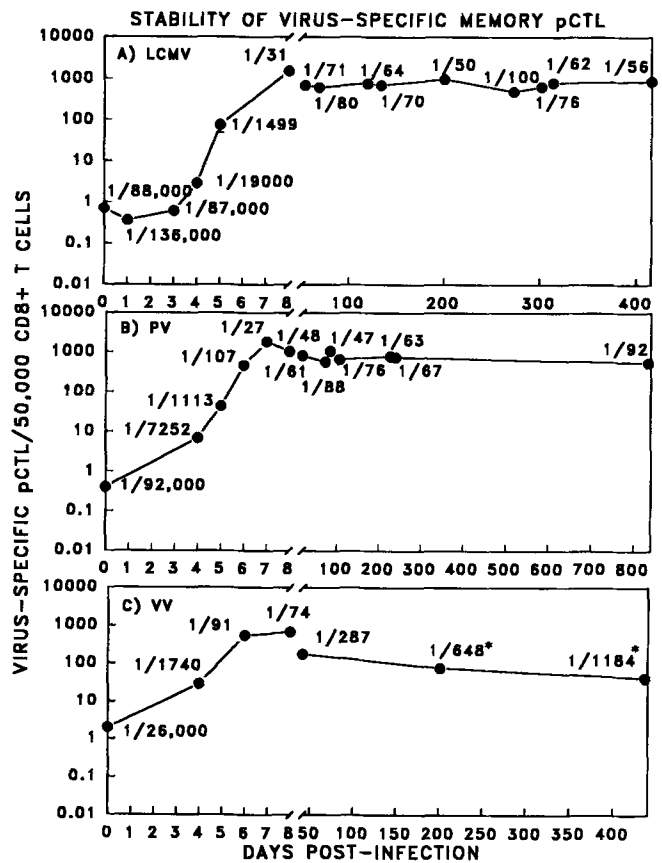
On days 7-8 of culture, individual wells were split twofold and assayed for cytolytic function on infected or peptide-coated targets and uninfected syngeneic target cells (KO) using a modified  $^{51}\text{Cr}$ -release assay.  $^{51}\text{Cr}$ -labeled targets ( $5 \times 10^3$ ) were added to all wells. The plates were incubated 8-10 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator, centrifuged for 5 min at 130 g (CRU-5000 centrifuge, International Equipment Co., Needham Heights, MA), and the supernatant was harvested. Radioactivity released into the supernatant was counted on a gamma counter (Clinigamma model 1272; LKB, Gaithersburg, MD). Positive wells were defined as those wells whose  $^{51}\text{Cr}$ -release exceeded the mean spontaneous release by  $>3$  SD. All wells that lysed uninfected syngeneic targets were eliminated from the analysis. Frequencies were calculated using chi squared analysis according to Taswell (27) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI).

**Peptide-coated Targets.** KO cells were incubated overnight with 100-200  $\mu\text{M}$  of indicated peptide. The LCMV H-2<sup>b</sup>-restricted immunodominant peptides, nucleoprotein (NP)397 (QPQNGQ-FIHFY), glycoproteins (GP)34 (AVYNFATCG) and GP278 (VEN-PGGYCL), were synthesized by Dr. Robert Carraway (University of Massachusetts Medical Center). These peptides were purified to  $>95\%$  homogeneity by reverse-phase HPLC. The high concentrations of peptides used were selected after peptide titration studies and were chosen to enhance the efficiency in detecting pCTL in LDAs, where the low numbers of effectors present in each well are further split into two wells for the cytotoxicity assays. Furthermore, KO target cells were found to express relatively low levels of class I MHC antigens. These assays remained quite specific, as PV-induced CTL did not lyse KO cells coated with any of the LCMV peptides.

**Statistical Analyses.** Spearman's correlation coefficient and the one-sample *t* test were used for data analysis where appropriate.

## Results

**Stability of Long-term CTL Memory after Acute Viral Infections.** During the early stages of infection, the virus-specific CTL p/f per splenic  $\text{CD8}^+$  T cell increased, peaking at 1/31, 1/27, and 1/74 for LCMV, PV, and VV, respectively, on days 7-8 (Fig. 1, A-C). Under these conditions, infectious virus was cleared from the spleen by day 7, with no evidence of long-term persistence of these viruses (1, 18, 28). Analysis of p/f subsequent to the peak of the T cell response and the ensuing apoptosis events surprisingly revealed that, although freshly isolated T cells were not directly cytotoxic to virus-infected targets, the p/f per  $\text{CD8}^+$



**Figure 1.** Stability of long-term CTL memory after acute viral infections. Adult C57BL/6 (H-2<sup>b</sup>) mice were inoculated intraperitoneally with LCMV, PV, or VV. At indicated times after infection, the LCMV- (A), PV- (B), and VV-specific (C) p/f per  $\text{CD8}^+$  was quantified by LDAs of unsorted splenocytes. The virus-specific p/f per  $\text{CD8}^+$  cell are displayed above the pCTL/50,000  $\text{CD8}^+$  splenocytes plotted. For LCMV, the data from days 0 to 8 are averages of two to four experiments at each time point. For PV and VV, the data from days 0 to 8 represent one single experiment with all time points tested in the same LDA, including one immune mouse time point. The pCTL from immune mice ( $>6$  wk) for all three viruses were derived from separate experiments. (\*) The same stock and input titers of virus were used for all of the LCMV and PV experiments. The 202- and 437-d samples in the VV experiment were from mice receiving  $8 \times 10^6$  PFU of one virus stock and the rest of the time points were from mice receiving  $1.5 \times 10^7$  PFU of a second stock.

splenic T cell was only two- to fourfold less in immune mice than it was at the peak of the acute CTL response (Fig. 1, A-C). Thereafter the p/f remained remarkably stable for LCMV and PV, even out to 14-27 mo (Fig. 1, A and B). The p/f also appeared very stable for VV, though the data for 202 and 437 d after infection shown in Fig. 1 C were generated from a different virus input stock receiving half the virus dose of that in the rest of the figure.

This rather small drop in p/f per  $\text{CD8}^+$  T cell between the acute and memory T cell response has not been appreciated before (1, 6, 7, 13, 29), but it is consistent with previously reported data for LCMV showing a greater drop in p/f per splenocyte if it were corrected for  $\text{CD8}^+$  T cell number (1). The apparent greater drop per splenocyte is a consequence of a significant drop in the percentage of

CD8<sup>+</sup> T cells per spleen ( LCMV day 8 CD8: 38% ± 4 vs. LCMV-immune CD8: 13% ± 0.6, *n* = 3), as well as a major reduction in the total number of splenocytes (6, 7, 13). In three experiments there were on average  $1.5 \times 10^6$  pCTL/spleen on day 8 vs.  $2.2 \times 10^5$ /spleen in the immune state, or about a sevenfold reduction in total pCTL number.

**Stability of the LCMV-immunodominant, Peptide-specific CTL p/f.** Similar stabilities in p/f per CD8<sup>+</sup> T cell were found not only in the quantitative but also in the qualitative nature of the CTL response. The p/f per CD8<sup>+</sup> T cell for each of three H-2<sup>b</sup>-restricted, LCMV-immunodominant peptides was, like the total LCMV-specific pCTL, reduced about twofold between day 8 and immune, and the ratios of peptide-specific to total LCMV-specific clones were unchanged (Table 1). These unchanged specificities in the CD8<sup>+</sup> T cell populations suggest that after infectious virus has been cleared, the apoptotic downregulation in CD8<sup>+</sup> T cell number occurs across-the-board among the activated lymphocytes and is apparently not influenced by the specificity of the TCR. This argues that there is little selective elimination or protection of the virus-specific T cells during this phase of memory cell development.

**Stability of LCMV-induced, Allospecific CTL Repertoire.** We have previously shown that LCMV induces allospecific as well as virus-specific CTL activity; clonal analyses have shown that individual clones propagated on LCMV-infected syngeneic APCs may lyse both a virus-infected syngeneic target and an uninfected H-2<sup>k</sup> allogeneic target, or, surprisingly, lyse the allogeneic target but not the virus-infected target (23). We examined an additional indicator of the sta-

**Table 1.** Stability of the LCMV-specific and Viral Peptide-specific p/f after Ds 7-8 of LCMV Infection

pCTL per CD8 <sup>+</sup> cells*	Ds 7-8 (pCTL/50,000 CD8 <sup>+</sup> cells)	LCMV immune <sup>‡</sup>
LCMV-specific	2,133 ± 305 (5) <sup>§</sup> (1/25) <sup>  </sup>	1,230 ± 264 (7) (1/51)
GP278-specific <sup>¶</sup>	628 ± 69 (3) (1/82)	424 ± 107 (3) (1/142)
GP278/LCMV ratio	0.31	0.35
GP34-specific <sup>¶</sup>	1,054 ± 83 (3) (1/48)	662 ± 170 (3) (1/91)
GP34/LCMV ratio	0.53	0.55
NP-specific <sup>¶</sup>	349 ± 141 (2) (1/171)	196 ± 42 (4) (1/294)
NP/LCMV ratio	0.15	0.17

\* LCMV- and peptide-specific p/f per CD8<sup>+</sup> cell were quantified as described in Materials and Methods.

<sup>‡</sup> LCMV-immune refers to mice >6 wk after infection.

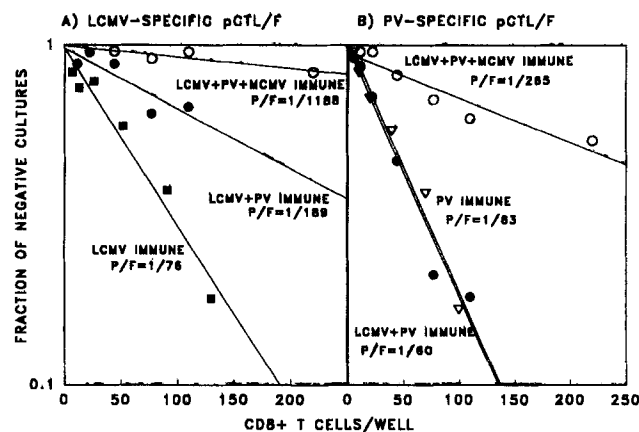
<sup>§</sup> Number of experiments.

<sup>||</sup> p/f per CD8<sup>+</sup> cell.

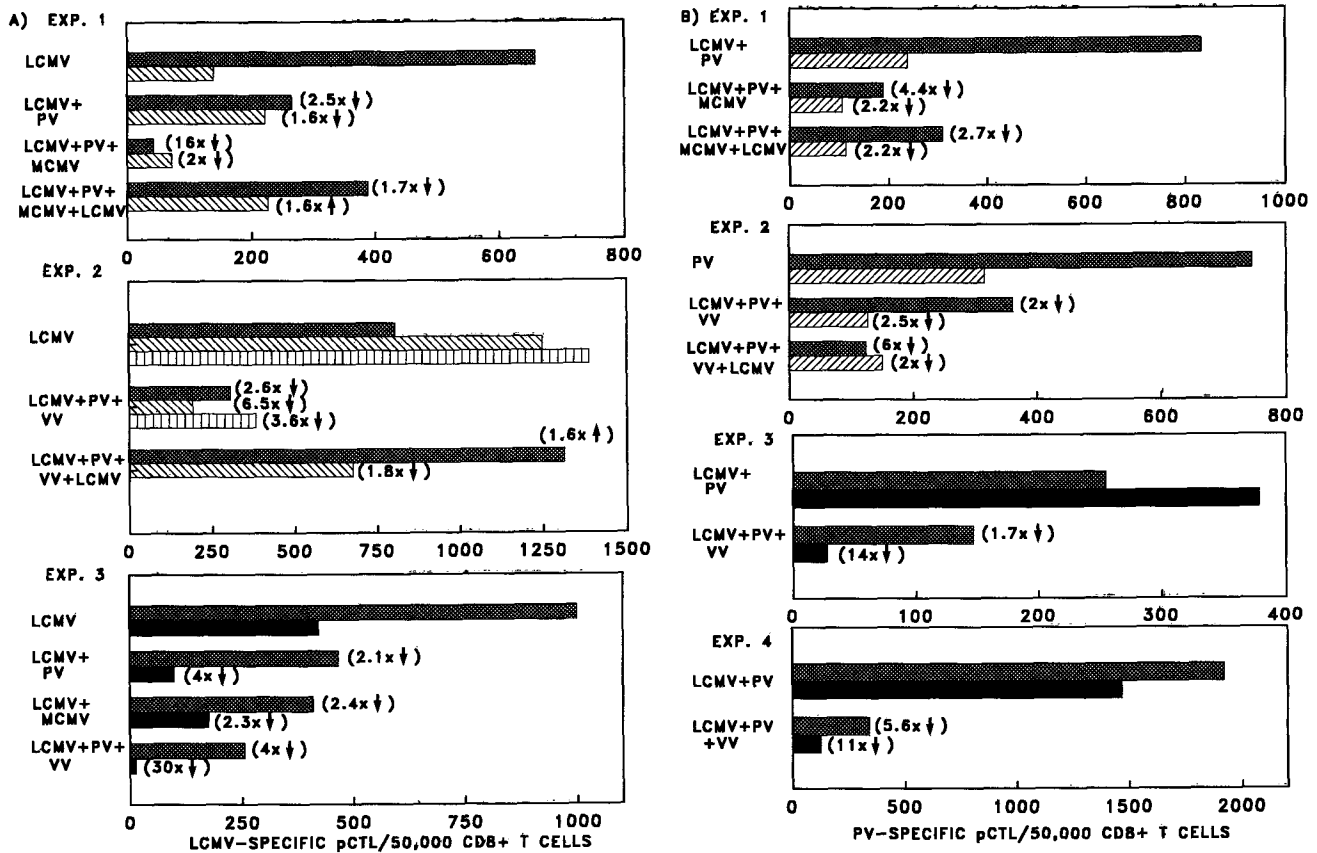
<sup>¶</sup> LCMV H-2<sup>b</sup>-restricted immunodominant peptides are NP397, GP34, and GP278.

bility of the specificity of the CTL repertoire during the conversion of the acute response to the memory response, the p/f of H-2<sup>k</sup>-specific CTL propagated on LCMV-infected APCs. A study of 114 LCMV-specific CTL clones derived at day 8 after infection, and of 114 CTL clones from LCMV-immune mice, revealed an identical 10% (11/114) that cross-reacted with H-2<sup>k</sup>, as demonstrated by the lysis of L-929 cells. An additional 11% (14/128) of day 8 clones and 14% (18/132) of immune clones propagated on LCMV-infected syngeneic APCs lysed allogeneic (L-929) targets without lysing virus-infected syngeneic targets. These observations add further support to the hypothesis that the relative specificity of the LCMV-induced T cell repertoire does not change between day 8 and long-term memory.

**Reduction in Virus-specific CTL p/f after Heterologous Virus Infections.** Although the frequency of virus-specific memory CTL remained remarkably stable over a period of many months (Fig. 1), infections with other viruses disrupted this homeostasis. Below are shown a series of experiments designed to determine whether heterologous viral infections would positively or negatively influence immunological memory to earlier viruses. The strategy was to immunize mice with a sublethal acute infection of one virus, allow for the rise and fall of the T cell response with a return to homeostasis, and then inoculate with another virus. After the T cell response to that second infection returned to homeostasis, the animals were tested for pCTL specific for each virus. Subsequent infections with additional viruses were also tested. Fig. 2 A is a representative experiment from a single LDA demonstrating a 2.5-fold decrease in LCMV-specific splenic p/f per CD8<sup>+</sup> T cell after a subsequent PV infection and a 16-fold decrease after a third virus, MCMV (Fig. 3 A). Fig. 2 B, done at the same time as Fig. 2 A, demonstrates in the same LCMV+PV immune mouse



**Figure 2.** Reduction of LCMV- (A) and PV-specific (B) memory CTL p/f per CD8<sup>+</sup> spleen cells after subsequent heterologous virus infections. Quantification by LDA was made of LCMV- (A) and PV-specific (B) splenic CTL p/f per CD8<sup>+</sup> T cell in C57BL/6 mice. These mice were immunized with a sublethal acute infection of LCMV, the T cell response was allowed to rise and return to homeostasis (>6 wk after infection), and then PV was given, followed by MCMV, allowing each time for a return to homeostasis before giving the next virus or testing for CTL p/f.



**Figure 3.** Deletion of LCMV- (A) and PV-specific (B) memory CTL in different memory compartments by heterologous viral infections and boosting of LCMV-specific memory CTL by reinfection of these mice with LCMV. LCMV- or PV-specific splenic (hatched bar, A and B: Exp. 1-4), LN (right diagonal striped bar, A and B: Exp. 1 and 2), peripheral blood (vertical striped bar) (A, Exp. 2), and PEC (solid bar, A and B: Exp. 3 and 4) pCTL/50,000 CD8<sup>+</sup> cells were quantified by LDA as previously described. The same individual mouse was used to derive the data for the different memory compartments within each experiment. The same mouse used to derive the PV-specific p/f in Exp. 1-3 was used to derive the LCMV-specific p/f. These experiments were carried out in clean, pathogen-free environments. However, for more strict pathogen-free conditions, some experiments were done in mice housed in microisolator cages (Fig. 3 B, Exp. 4). The bracketed numbers over each bar represent the fold change in pCTL/50,000 CD8<sup>+</sup> T cells in mice after receiving the indicated heterologous virus infections in comparison to the control, which did not receive any more subsequent infections.

that, as the decrease in LCMV-specific p/f is occurring after a subsequent MCMV infection, there is a simultaneous fourfold decrease in PV-specific p/f after a subsequent MCMV infection. Fig. 2 B also shows that a prior infection with LCMV does not significantly alter the PV-specific p/f. Not only was there a reduction in the pCTL/CD8 cell caused by subsequent infections, but the total number of pCTL also decreased. The total numbers of LCMV-specific pCTL/spleen in Fig. 2 A were the following: LCMV—274,914; LCMV+PV—58,173; and LCMV+PV+MCMV—12,030. The total numbers of PV-specific pCTL/spleen were the following: PV—173,502; LCMV+PV—184,843; and LCMV+PV+MCMV—54,032.

Tables 2-4 are a composite of eight experiments demonstrating 27 comparisons in which mice receiving various sequential infections with LCMV, PV, VV, MCMV, and VSV were tested for CTL specific to LCMV, PV, or VV, respectively. All paired observations were done at the same time in the same LDA, and each experiment number throughout the three tables represents LDAs run on a single

day as parts of the same experiment. Table 2 i demonstrates the expected result that, when an LCMV-immune mouse was reinfected with LCMV instead of a heterologous virus, there was a dramatic increase in p/f per CD8<sup>+</sup> spleen cell or PEC. In virtually every case, however, subsequent infections of immune mice with heterologous viruses reduced the frequency of memory pCTL specific to viruses from earlier infections. In mice initially infected with LCMV there were means of 2- ( $\pm 0.3$ , SEM;  $n = 3$ ), 3- ( $\pm 0.5$ ;  $n = 3$ ), and 3.9-fold ( $\pm 0.3$ ;  $n = 2$ ) decreases in p/f per CD8<sup>+</sup> spleen cell after infections with PV, PV+VV, and PV+VV+MCMV, respectively (Table 2, ii, iii, and iv). This gradual decrease in LCMV-specific p/f with increasing numbers of subsequent heterologous infections (LCMV+VV+VV+MCMV) was significant, as determined by Spearman's correlation coefficient ( $P = 0.016$ ). In one experiment in which MCMV was given as the third virus instead of VV, a more profound 16-fold decrease was observed (Table 2 iii, Fig. 2 A). Some restoration of LCMV-specific pCTL was noted after rechallenge with LCMV (Table 2 v).

**Table 2.** Reduction in LCMV-specific pCTL/50,000 CD8<sup>+</sup> T Cells after Heterologous Virus Infections

Exp. No. <sup>‡</sup>	LCMV-specific pCTL/50,000 CD8 <sup>+</sup> T Cells*			
	Control		Test	Fold change
i) Rechallenge with Homologous Virus:				
1.	LCMV	1,000	LCMV+LCMV	4,545 4.5↑
2.	LCMV	500	LCMV+LCMV	3,125 6.2↑
1.	LCMV (PEC) <sup>§</sup>	424	LCMV+LCMV (PEC)	5,000 11.7↑
2.	LCMV (PEC)	105	LCMV+LCMV (PEC)	6,250 59.0↑
ii) Challenge with One Subsequent Heterologous Virus:				
1.	LCMV	1,000	LCMV+PV	467 2.0↓
3.	LCMV	658	LCMV+PV	265 2.5↓
4.	LCMV	185	LCMV+PV	126 1.5↓
1.	LCMV	1,000	LCMV+MCMV	410 2.4↓
1.	LCMV+PV	467	LCMV+PV+VV	256 1.8↓
4.	LCMV+PV	126	LCMV+PV+VV	78 1.6↓
3.	LCMV+PV	265	LCMV+PV+MCMV	42 6.3↓
1.	LCMV+PV+VV	256	LCMV+PV+VV+MCMV	242 1.1↓
4.	LCMV+PV+VV	78	LCMV+PV+VV+MCMV	52 1.5↓
iii) Challenge with Two Subsequent Heterologous Viruses:				
1.	LCMV	1,000	LCMV+PV+VV	256 4.0↓
5.	LCMV	806	LCMV+PV+VV	305 2.6↓
4.	LCMV	185	LCMV+PV+VV	78 2.4↓
3.	LCMV	658	LCMV+PV+MCMV	42 16.0↓
4.	LCMV+PV	126	LCMV+PV+VV+MCMV	52 2.4↓
1.	LCMV+PV	467	LCMV+PV+VV+MCMV	242 1.9↓
iv) Challenge with Three Subsequent Heterologous Viruses:				
1.	LCMV	1,000	LCMV+PV+VV+MCMV	242 4.1↓
4.	LCMV	185	LCMV+PV+VV+MCMV	52 3.6↓
v) Rechallenge with LCMV after Multiple Sequential Heterologous Viruses:				
3.	LCMV+PV+MCMV	42	LCMV+PV+MCMV+LCMV	388 8.8↑
5.	LCMV+PV+VV	305	LCMV+PV+VV+LCMV	1,315 4.3↑
1.	LCMV+PV+VV	256	LCMV+PV+VV+LCMV	273 1.1↑

\*Quantification by LDA was made of LCMV-specific, splenic pCTL/50,000 CD8<sup>+</sup> T cells in C57BL/6 mice. These mice were immunized with a sublethal acute infection of one virus; the T cell response was allowed to rise and return to homeostasis (>6 wk), and then a second heterologous virus was given, followed by a third, and fourth, allowing each time for a return to homeostasis before giving the next virus or testing for p/f. This p/f was compared with an age-matched control mouse that in that same time period had only received the initial virus or other prior viruses. Each pair of virus-specific pCTL/50,000 CD8<sup>+</sup> splenic T cells for the control versus test comparison sequence of homologous or heterologous virus(es) was set up with the same experiment in the same single LDA. The fold change quantifies either the fold increase or decrease in pCTL comparing the test to the control sequence.

<sup>‡</sup>Experiment number indicates the various sequences of viruses that were assessed during one single experimental LDA set up whether assessing LCMV- (Table 2), PV- (Table 3), or VV-specific (Table 4) pCTL.

<sup>§</sup>Groups followed by (PEC) represent data for pCTL/50,000 CD8<sup>+</sup> T cells present in peritoneal exudate instead of splenic T cells.

Similar but more pronounced reductions in p/f were seen when PV-specific CTL were examined (Table 3). Because the design of the experiments with PV frequently used mice previously infected with LCMV, we first determined whether a prior LCMV infection significantly altered the p/f to PV. The PV-specific p/f was similar whether or not mice had previously been infected with LCMV (Fig. 2 B;

Table 3 i). In most of these experiments the control for assessing PV-specific p/f was a mouse that had in the past received LCMV (i.e., LCMV+PV). The same individual mouse was used to derive the PV- and LCMV-, as well as the VV-specific pCTL in multiple compartments. We feel this experimental strategy strengthens the significance of the observation, as we were able to demonstrate within one

**Table 3.** Reduction in PV-specific pCTL/50,000 CD8<sup>+</sup> T Cells after Heterologous Virus Infections

Exp. <sup>‡</sup>	PV-specific pCTL/50,000 CD8 <sup>+</sup> T cells*				Fold change
	Control		Test		
(i) Prior infection with LCMV:					
1.	PV	187	LCMV+PV	255	1.4↑
3.	PV	794	LCMV+PV	833	1.05↑
6.	PV	83	LCMV+PV	119	1.4↑
(ii) Challenge with One Subsequent Heterologous Virus:					
1.	LCMV+PV	255	LCMV+PV+VV	147	1.7↓
4.	PV <sup>§</sup>	816	LCMV+PV+VV	108	8↓
7.	LCMV+PV	1,923	LCMV+PV+VV	342	5.6↓
1.	LCMV+PV	255	LCMV+PV+MCMV	39	6.5↓
3.	LCMV+PV	833	LCMV+PV+MCMV	189	4.4↓
4.	LCMV+PV+VV	108	LCMV+PV+VV+MCMV	34	3.2↓
(iii) Challenge with Two Subsequent Heterologous Viruses:					
4.	LCMV+PV	816	LCMV+PV+VV+MCMV	34	24↓

\*Quantification by LDA was made of PV-specific, splenic pCTL/50,000 CD8<sup>+</sup> T cells in C57BL/6 mice. These mice were immunized with a sublethal acute infection of one virus, the T cell response was allowed to rise and return to homeostasis (>6 wk), and then a second heterologous virus was given, followed by a third, and a fourth, allowing each time for a return to homeostasis before giving the next virus or testing for p/f. This p/f was compared with an age-matched control mouse that in that same time period had only received the initial virus or other prior viruses. Each pair of virus-specific pCTL/50,000 CD8<sup>+</sup> splenic T cells for the control versus test comparison sequence of homologous or heterologous viruses(es) was set up within the same experiment in the same single LDA. The fold change quantifies either the fold increase or decrease in pCTL, comparing the test to the control sequence.

<sup>‡</sup>Experiment number indicates the various sequences of viruses that were assessed during one single experimental LDA set up whether assessing LCMV- (Table 2), PV- (Table 3), or VV-specific (Table 4) pCTL.

<sup>§</sup>As PV and LCMV+PV controls have similar p/f and an age-matched LCMV+PV infected mouse was not available for this experiment, a mouse infected with PV alone was used.

mouse simultaneously a reduction in memory pCTL to two or three different viruses within different organs. There was a 5.2-fold ( $\pm 1.7$ ;  $n = 3$ ) drop in PV-specific pCTL after subsequent VV infections and a 5.5-fold ( $\pm 1$ ;  $n = 2$ ) drop after subsequent MCMV infections (Table 3 ii). In one experiment after infection with two subsequent viruses (VV+MCMV) there was a 24-fold drop (Table 3 iii). Similarly, infection with heterologous viruses caused a reduction in VV-specific p/f (Table 4). Examination of all the virus combinations tested showed that one subsequent heterologous virus infection decreased spleen p/f 3.0  $\pm$  0.4-fold ( $n = 16$ ), and two subsequent infections decreased it 8.4  $\pm$  3-fold ( $n = 8$ ) (Tables 2–4). The percent reduction by either one or two virus infections was significant ( $P < 0.0005$ ) as analyzed by the one-sample *t* test.

**Simultaneous Decrease of Memory pCTL in Multiple T Cell Compartments.** The above data from Tables 2–4 focussed on pCTL in the spleen, but this deletion of LCMV- (Fig. 3 A) and PV-specific (Fig. 3 B) memory pCTL after heterologous virus infections was a global event occurring in all lymphoid compartments tested, including the spleen (Fig. 3), LNs (Fig. 3, A and B, Exp. 1 and 2), peritoneal cells (Fig. 3, A and B, Exp. 3 and 4), and peripheral blood (Fig. 3 A, Exp. 2). This suggests that the memory cells were not traf-

ficking to a location where they were protected from deletion, though it remains possible that they became sequestered in a tissue that was not examined.

**Homeostasis of the Lymphoid System.** By 6 wk after each virus infection, homeostasis in lymphoid number occurred. In the experiments described here, the numbers of spleen leukocytes after two or more infections ( $1.2 \times 10^8 \pm 0.04$  SEM;  $n = 25$ ) were no greater than after one infection ( $1.2 \times 10^8 \pm 0.08$ ;  $n = 14$ ). The same was true of the numbers of LN cells ( $2.2 \times 10^6 \pm 0.22$ ;  $n = 12$  vs.  $2.1 \times 10^6 \pm 0.19$ ;  $n = 10$ ), and PECs ( $1.6 \times 10^6 \pm 0.17$ ;  $n = 13$  vs.  $1.3 \times 10^6 \pm 0.15$ ;  $n = 5$ ).

The percentage of CD8<sup>+</sup> spleen cells did not vary significantly between experiments, nor did it significantly change over time with increasing numbers of viral infections: LCMV immune (CD8<sup>+</sup> cells, 13%  $\pm$  0.6,  $n = 3$ ); PV immune (10%  $\pm$  0.6,  $n = 3$ ); LCMV+PV immune (11%  $\pm$  0.3,  $n = 3$ ); and LCMV+PV+VV immune (15%  $\pm$  0.7,  $n = 3$ ); and LCMV+PV+VV+MCMV (14%,  $n = 1$ ). The percentage of LN CD8<sup>+</sup> cells did not significantly vary between individual mice and was independent of virus treatment (32%  $\pm$  1.0,  $n = 8$ ). Because there was no significant change in the total number in CD8<sup>+</sup> splenocytes and lymph node cells, the reductions in p/f per CD8 cell in the

**Table 4.** Reduction in VV-specific pCTL/50,000 CD8<sup>+</sup> T Cells after Heterologous Virus Infections

Exp. No.‡	VV-specific pCTL/50,000 CD8 <sup>+</sup> T cells*			Fold change	
	Control		Test		
i) Prior Infection with LCMV and PV:					
5.	VV	77	LCMV+PV+VV	44	1.8↓
ii) Challenge with One Subsequent Heterologous Virus:					
8.	VV	42	VV+MCMV	15	2.8↓
iii) Challenge with One Subsequent Heterologous Virus and Rechallenge with LCMV:					
5.	LCMV+PV+VV	44	LCMV+PV+VV+MCMV+LCMV	17	2.6↓
iv) Challenge with Two Subsequent Heterologous Viruses:					
8.§	VV	42	LCMV+PV+VV+MCMV+VSV	3	14↓

\*Quantification by LDA was made of VV-specific, splenic pCTL/50,000 CD8<sup>+</sup> T cells in C57BL/6 mice. These mice were immunized with a sublethal acute infection of one virus, the T cell response was allowed to rise and return to homeostasis (>6 wk), and then a second heterologous virus was given, followed by a third, fourth, and fifth, allowing each time for a return to homeostasis before giving the next virus or testing for p/f. This p/f was compared with an age-matched control mouse that in that same time period had only received the initial virus or other prior viruses. Each pair of virus-specific pCTL/50,000 CD8<sup>+</sup> splenic T cells for the control versus test comparison sequence of homologous or heterologous virus(es) was set up within the same experiment in the same single LDA. The fold change quantifies either the fold increase or decrease in pCTL comparing the test to the control sequence.

‡Experiment number indicates the various sequences of viruses that were assessed during one single experimental LDA set up whether assessing LCMV- (Table 2), PV- (Table 3), or VV-specific (Table 4) pCTL.

§As VV and LCMV+PV+VV controls have p/f within twofold of each other and an age-matched LCMV+PV+VV infected mouse was not available for this experiment, a mouse infected with VV alone was used.

immune mice directly correlate with the total number of pCTL. However, the percentage of CD8<sup>+</sup> PECs, which represent only a small number of cells in comparison to splenocytes or LN cells, was significantly different ( $P = 0.017$ , Student's *t* test) between mice that had received only one virus ( $18\% \pm 3$ ,  $n = 5$ ) versus those that received two or more ( $30\% \pm 3$ ,  $n = 11$ ).

## Discussion

Our recent studies have indicated that immune responses to viruses are not mounted in isolation by exclusively naive cells of the immune system (12–15, 17). Instead, the immune response to one virus can predispose a host to make an altered immune response when encountering a second unrelated virus. This altered immune response consists, at least in part, of stimulated memory T cells cross-reactive between two heterologous viruses. This is not surprising because T cells recognize short peptides, and *in vitro* studies with defined T cell clones have shown that the same clone may recognize distinct peptides with very different amino acid sequences (30–33). Given the polyclonal nature of the CTL response to viral infections, there appears to be sufficient diversity within the memory pCTL repertoire specific for one virus to render some clones susceptible to reactivation by apparently unrelated heterologous viruses. We have shown that this reactivation of memory cells can prospectively prime the immune response to the second virus (15), and it may contribute either to enhanced protective immunity (17, and our unpublished data) or to en-

hanced immunopathology (18, and our unpublished data). In the present study we show that the immune response to one virus not only has prospective but also has retrospective effects on the memory responses specific for viruses to which the host had been previously exposed. These retrospective effects in the systems studied invariably involved a significant reduction in pCTL specific for pathogens from previous infections.

In the first part of this study detailed analyses of the frequencies and specificities of the CTL responses during the progression of the acute to the memory response led to several surprising results. Examination of the virus-specific p/f per CD8<sup>+</sup> T cell showed that, even though the total splenocyte and total CD8<sup>+</sup> T cell number declined dramatically at the conclusion of the acute infection, there was only a two- to fourfold decrease in p/f per CD8<sup>+</sup> T cell between the acutely infected and immune mice for the three viruses tested (LCMV, PV, and VV). The p/f per CD8<sup>+</sup> T cells, about 1:50 for LCMV, thus remained at very high levels for the lifetime of the animals. Considering that not all LCMV-specific CD8<sup>+</sup> T cells would be cytotoxic precursors and that the efficiency of the LDA must be <100%, these results would indicate that a history of a viral infection must significantly alter the immunological repertoire available to combat other infections. The finding that mice given a second LCMV infection will maintain a p/f of ~1:11 (Table 2) further illustrates the point that a significant population of the T cells in a host can be specific to a pathogen long after the acute infection has resolved. Reports of more dramatic declines in virus-specific p/f at the



conclusion of acute virus infections can be attributed to either (a) reporting the frequencies as per spleen or per splenocyte, rather than per CD8<sup>+</sup> T cell (1); (b) the use of very high doses of LCMV strains (clone 13 or Docile) that can disseminate and cause an apoptotic clonal exhaustion of CTL (34); or (c) suboptimal LDA techniques inadequate to efficiently stimulate memory cells, which are at a lower activation state than the CTL present during the acute infection.

The immunodominant peptide specificities relative to the total LCMV-specific CTL response also did not change between day 8 of the acute infection and the immune state. This indicates that when the apoptotic events causing a reduction of the total CD8<sup>+</sup> T cell number at the end of the acute infection occurred, there was no further selection of T cells based on their target specificity. This would be consistent with the observation that most LCMV antigen is gone by day 8. In contrast, in models of high dose infection with disseminating LCMV infection, where high antigen load is present at day 8, there is a selective elimination of virus-specific CTL and pCTL (34). We believe that these events demonstrate two contrasting mechanisms of apoptosis in these infections, one which is antigen dependent and occurs in the presence of excess antigen, and the other, described by us here and elsewhere (7, 8, 11, 12), that represents an antigen-independent across-the-board decline in CD8<sup>+</sup> T cell number after most of the antigen has cleared and without a selective exhaustion of antigen-specific T cells. Selection for immunodominant peptide specificity in the LCMV system must therefore take place in earlier stages of infection, before day 8 and the ensuing apoptotic events.

The impressive homeostasis in the memory CTL pool is disrupted when heterologous viruses are introduced, as these heterologous infections first activate (14, 15, 35) and then, as shown here, induce a reduction in the memory pCTL pool to earlier viruses. The mechanism for this decline in CTL p/f is as yet not known. A dilution of pCTL number to an earlier virus may occur after a selective expansion of pCTL specific to the second virus and the ensuing apoptotic downregulation and return to homeostasis thereafter. The explanation for this decline in pCTL may, however, be more complex, as suggested by our previous observation that heterologous viruses can stimulate expansion of memory T cells cross-reactive with earlier viruses (15). The consequences of this could be that there would be not only quantitative but also qualitative changes in CTL memory. Preliminary data supporting this supposition demonstrate that the ratio of the three immunodominant peptides to LCMV-specific pCTL, which is normally unchanged between days 7 and 8 and long-term memory, is altered by heterologous viral infections (Selin, L.K. and R.M. Welsh, unpublished data). In addition, within the same mice im-

mune to both LCMV and PV, subsequent infections reduced PV-specific p/f more efficiently than LCMV-specific p/f, arguing for some selectivity in pCTL deletion (Tables 2 and 3). A change in the qualitative specificity of the T cell repertoire reactive with viruses from previous infections would be consistent with the hypothesis that cross-reactive T cell responses are important determinants modifying this memory cell loss. A decrease in pCTL memory may also be influenced by viral replication within and subsequent lysis of memory lymphocytes activated in response to a heterologous virus infection. All of the viruses tested are capable of some, though usually limited, replication in lymphocyte populations, and activated lymphocytes are better hosts for viral replication than are resting lymphocytes (36).

Factors contributing to the maintenance of memory CTL remain unclear, and opinions differ regarding the need for persisting antigen to sustain this highly stable memory response when there is no interference with other infections. We did not address the question of antigen persistence maintaining memory, but none of these viruses establish detectable persistent infections under these conditions (1, 18, 28), suggesting that replicating viral antigen may not be needed to drive memory. Our present data do not, however, negate the theory that cross-reactivity could play a role in maintaining memory cells. It is possible that either self or even foreign antigens presented during perhaps a different type of immunological milieu (e.g., a lower intensity immune response with an altered cytokine bath, in contrast to that in these potent virus infections) can activate memory cells in a cross-reactive manner but not result in their deletion.

Because the natural host would have many viral infections throughout a lifetime, and because p/f can be very high after each new infection (as high as 1/11 CD8<sup>+</sup> T cells after two infections with LCMV; Table 2 v), it would not be possible to maintain lymphoid homeostasis if pCTL specific for previous infections did not decline as new infections occurred. It is not known to what level the CTL p/f has to fall before memory responses are compromised to biologically significant levels, but a meaningful depletion in T cell memory would seem likely after a lifetime of infections, unless the host is continually reexposed to the original immunizing antigen or to other antigens that stimulate cross-reactive T cells (1-4, 15, 37-39). The results presented here and elsewhere thus indicate that viral infections do not occur in immunological isolation, that the immune response to each infection is influenced by prior exposures to other antigens, and that each new infection affects the previous T cell memory pool. This type of continual modulation of CD8<sup>+</sup> T cell responses may have a significant positive or negative impact on an individual's ability to respond to an infectious agent.

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