Cross-reactivities in Memory Cytotoxic T Lymphocyte Recognition of Heterologous Viruses

By Liisa K. Selin, Sharon R. Nahill, and Raymond M. Welsh

From the Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Summary

Analyses of the relationships between different viruses and viral proteins have focused on homologies between linear amino acid sequences, but cross-reactivities at the level of T cell recognition may not be dependent on a conserved linear sequence of several amino acids. The CTL response to Pichinde virus (PV) and vaccinia virus (VV) in C57BL/6 mice previously immunized with lymphocytic choriomeningitis virus (LCMV) included the reactivation of memory cytotoxic T lymphocyte (CTL) specific to LCMV. Limiting dilution assays (LDA) demonstrated that at least part of this reactivation of memory cells in LCMV-immune mice related to cross-reactivity at the clonal level, even though acute infections with these viruses in nonimmune mice elicited CTL responses that did not cross-react in conventional bulk CTL assays. Precursor CTL (pCTL) to LCMV were generated in splenic leukocytes from LCMV-immune mice acutely infected with PV or VV when stimulated in vitro with only the second virus but not with uninfected peritoneal exudate cells (PECs). Cytotoxicity mediated by LCMV-specific CTL clones activated by PV infection was greatly inhibited by anti-CD8 antibody, suggesting that these memory CTL clones recognizing LCMV-infected targets were of low affinity. LCMV-immune splenocytes stimulated in vitro with PV or VV demonstrated a low but significant precursor frequency (p/f) to the heterologous viruses, and splenocytes from PV- or VV-immune mice when stimulated in vitro against LCMV generated a low but significant p/f to LCMV. Short-term CTL clones cross-reactive between LCMV and PV were derived from splenic leukocytes from LCMV-immune mice acutely infected with PV. To distinguish whether the cross-reactivity was directed against a viral peptide or a virus-induced endogenous cellular neoantigen, we demonstrated that a pCTL frequency to PV about 1/4–1/7 that of the frequency to LCMV could be generated from LCMV-immune splenic leukocytes stimulated with the immunodominant LCMV NP peptide. A partially homologous PV peptide generated from the equivalent site to the LCMV NP peptide did not sensitize targets to lysis by either LCMV- or PV-specific CTLs, suggesting that the cross-reactivity in killing was not due to evolutionarily conserved equivalent sequences. Experiments also indicated that prior immunity to one virus could modulate future primary immune responses to a second virus. Elevated pCTL frequencies to PV were seen after acute PV infection of LCMV-immune mice, and elevated pCTL frequencies to LCMV were seen after acute LCMV infection of PV- and VV-immune mice. The implication of these analyses is that, at the T cell level, there may be a great deal of immunological cross-reactivity between heterologous viruses, and by virtue of this proposed remote T cell cross-reactivity, the host's response to a virus infection may be modified by its previous experience with other putatively unrelated pathogens.

Analyses of the relationships between viruses have focused on homologies in nucleotide or amino acid sequences. Sharing of a linear sequence of amino acids between different viral proteins can result in serological cross-reactivities, leading to cross-protective immunity. Selective pressure at the level of viral neutralization has led to high variation of virion surface proteins between virus species, and it is this lack of cross-reactivity at the level of neutralizing antibodies that is used as a major criterion for identifying distinct virus species. Cross-reactivities at the level of T cell recognition may be altogether different and much less dependent on a conserved linear sequence of several amino acids. Some studies analyzing immunodominant epitopes of infectious agents have found that apparently unrelated peptides from the same or a heterologous virus can sensitize a target cell to the same CTL line or clone (1–4). Analyses of the amino acid sequences of these peptides have shown relatively little amino acid homology, with the exception that the peptides needed to comply with

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the sequence motif for the presenting MHC molecule and therefore sometimes shared identical amino acids in two or three positions of the peptide nonamer (5). The implication of these analyses is that, at the T cell level, there may be a great deal of immunological cross-reactivity between serologically distinct viruses.

We have been analyzing the specificities and cross-reactivities of CTL induced in mice by LCMV, which elicits a profound polyclonal $CD8^+$ CTL response associated with a 10–40-fold increase in total number of activated proliferating $CD8^+$ T cells/spleen (6). The vast majority of these cells display activation markers such as the IL-2R (CD25) (7), the memory cell marker Pgp-1 (CD44) (8) and the adhesion molecule and memory marker Mac-1 (CD11b) (9), and at least 25% of the cells have the large granular lymphocyte morphology of cytotoxic effectors (10). Part of the CTL response consists of allospecific CTL detectable in primary bulk assays and specific for cells expressing a wide variety of alloantigens (6, 11, 12). Limiting dilution assay (LDA)[1] analyses demonstrated many allospecific CTL clones cross-reactive with virus-infected syngeneic targets. This induction of allospecific CTL also occurred in mouse infections with vaccinia virus (VV), Pinchinde virus (PV), and murine cytomegalovirus (MCMV) (11, 12), and in the human during acute EBV infection (13, 14).

A second observation was that in lymphocytic choriomenigitis virus (LCMV)-hyperimmune mice, i.e., mice challenged with LCMV one to two times after original infection, a portion of the CTL response elicited during acute infection with a second virus (PV, VV, or MCMV) consisted of memory CTLs specific for the first virus, LCMV (12). The present study demonstrates that these LCMV-specific CTLs are easily detectable in mice having received a single LCMV infection and later challenged with a heterologous viral infection (PV, VV). Analogous to the allospecific CTL at the clonal level, an unexpectedly large portion of these LCMV-specific CTLs were cross-reactive with MHC-syngeneic target cells infected with the second virus. In addition, a history of LCMV infection caused a more vigorous CTL response to either PV or VV.

This study demonstrates that there may be much greater than anticipated immunological cross-reactivity at the T cell level between viruses that are serologically unrelated and that such cross-reactivity may be of significance in one's "natural" resistance to subsequent infections with unrelated viruses.

Materials and Methods

Mice. The C57BL/6 ($H\text{-}2^b$) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were used at 1–6 mo of age.

Viruses. The LCMV Armstrong strain was propagated in BHK21 baby hamster kidney cells (15). The WR strain of VV and the AN3739 strain of PV, an arenavirus only distantly related to LCMV, were propagated in L929 cells (16). Mice were injected intraperitoneally with 4×10^4 PFU of LCMV, 7×10^6 PFU of VV, or 10^6 PFU of PV in 0.1 ml vol/mouse for acute virus infection.

Cell Lines. KO cells ($H\text{-}2^b$), a SV40-transformed kidney cell line derived from a C57BL/6 mouse (17) and provided to us by Dr. Satvir Tevethia (Pennsylvania State Medical Center, Hershey, PA), were propagated in DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 10 mM Hepes, and 10% heat inactivated (56°C, 30 min) FBS (Sigma Chemical Co., St. Louis, MO). L929 ($H\text{-}2^k$), a continuous liver cell line derived from C3H mice, and MC57G ($H\text{-}2^b$), a methylcholanthrene-induced fibroblast cell line from C57BL/6 mice, were propagated in Eagle's MEM (Gibco Laboratories) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 10% heat inactivated FBS. KO and MC57G 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.

Depletion of NK Cells In Vivo. Antiserum to asialo GM_1 (5 μl; Wako Chemical, Dallas, TX) was injected intraperitoneally in a volume of 0.1 ml, on days 0 and 4 of acute infection. In immune mice not receiving a second viral infection, the antibody was given 2 d before the harvesting of spleens. As the asialo GM_1 antigen can be expressed on some T cells (18, 19), we carefully titrated every lot to obtain a concentration of antiserum that selectively reduces NK cell activity without affecting CTL activity. This regimen of antibody nearly completely depleted NK cell activity for at least an 8-d time period.

Cytotoxicity Assays. Cell-mediated cytotoxicity was determined using a standard microcytotoxicity (CTL) assay (16). KO cells ($1\text{–}3 \times 10^6$) were pelleted, resuspended in 0.2 ml of $Na\text{-}^{51}Cr$ (Amersham Corp., Arlington Heights, IL), and incubated for 1 h at 37°C in a humidified 5% CO_2 incubator. They were rinsed three times and resuspended to 5×10^4/ml, and 0.1 ml was added to round-bottomed microtiter wells (Falcon Labware, Oxnard, CA). Varying numbers of effector leukocytes were added in triplicate in 0.1 ml of medium to achieve the desired E/T ratio. For spontaneous ^{51}Cr release controls, 0.1 ml of media was added to the labeled target cells in place of effectors. For maximum ^{51}Cr-release control, 0.1 ml of 1% NP-40 (United States Biochemical Co., Cleveland, OH) was added to the labeled target cells. After 6–8 h at 37°C in a 5% CO_2 incubator, the microtiter plates were centrifuged at 200 *g* for 5 min. Supernatant was removed (0.1 ml) from each well and counted on a gamma counter (model 5000; Beckman Instruments, Inc., Fullerton, CA). Data are expressed as percent specific ^{51}Cr release = 100 × [(experimental cpm − spontaneous cpm)/(maximum release cpm − spontaneous release cpm)].

LDA for Virus-specific CTL Precursors. The assays used the procedure of Moskophidis et al. (20) with modifications as previously described (6). Briefly, splenic lymphocytes were harvested, titrated in U-96-well plates with 24 replicates at each tritration. They were stimulated with virus infected peritoneal exudate cells (PECs) (3×10^4/well), supplemented with irradiated splenic feeders ($1\text{–}2 \times 10^5$/well) and growth factors provided by using a 16% culture supernatant from the IL-2–secreting, gibbon lymphoma tumor cell line MLA.144 (American Type Culture Collection, Rockville, MD) (21). These studies were routinely done with splenocytes isolated from mice depleted of NK cells by antiserum to asialo GM_1 in order to minimize NK cell outgrowth in the LDA. This treatment did not significantly alter the frequency of the LCMV-specific CTL precursors. In our earlier report we showed that the LCMV-specific CTL precursors were $CD8^+$ cells (6).

In the case of VV, which was a highly lytic virus in vitro, an

[1] *Abbreviations used in this paper:* LCMV, lymphocytic choriomeningitis virus; LDA, limiting dilution assay; MCMV, murine cytomegalovirus; NP, nucleoprotein; pCTL, precursor CTL; PEC, peritoneal exudate cell; p/f, precursor frequency; PV, Pichinde virus; VV, vaccinia virus.

alternate method was used. The thioglycollate-injected mice were also injected with 7×10^6 PFU of VV in 0.1 ml i.p. 12 h before plating. The isolated PEC were then plated at 3×10^4 cells/well in 50 μl of media, and neutralizing polyclonal rabbit antiserum to VV was added at 1/50 dilution final concentration (22). On days 5–8 of culture, individual wells were split threefold and assayed for cytolytic function on infected and uninfected syngeneic target cells (KO) using a modified ^{51}Cr release assay. ^{51}Cr-labeled targets (5×10^3) were added to all wells to a final volume of 200 μl/well. The plates were incubated 9–10 h at 37°C, in a 5% CO_2 incubator, centrifuged for 5 min at 130 *g* (IEC CRU-5000 centrifuge; International Equipment Co., Needham Heights, MA), and 0.1 ml of supernatant was harvested. Positive wells were defined as those wells whose ^{51}Cr release exceeded the mean spontaneous release by >3 SD. All wells that lysed uninfected syngeneic targets tested were eliminated from the analysis. Frequencies were calculated using χ^2 analysis according to Taswell (23) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI).

Anti-CD8 Blocking and Complement Depletion of CTL. Anti-CD8 produced by hybridoma M12/7.2, well-documented to block CTL-mediated lysis, was kindly provided by Dr. Eric Martz (University of Massachusetts, Amherst, MA) (6). A 1:160 final dilution of an NH_4SO_4 cut of the hybridoma culture fluid was added to the LDA effector cells for 1 h before adding the targets on the day of the cytotoxicity assay. Splenocytes were depleted of CD8$^+$ cells with antibody and rabbit complement as previously described (12).

NP-peptide Stimulation in LDAs. LCMV H-2^b restricted immunodominant nucleoprotein (NP) peptide (QPQNGQFIHFY) (5) was synthesized by Dr. Robert Carraway (University of Massachusetts Medical Center). These peptides were purified to >95% homogeneity by reverse-phase HPLC. LDAs were set up as described above with the following exceptions: PECs were pulsed for 0.5 h with the peptide at 100 μM concentration and then placed in the LDA (100 μl/well). All media used in the LDA were the same as above except that they contained IL-7 (Genzyme Corp., Cambridge, MA) at 15 U/ml.

Results

Viral Infections Activate CTL Specific for Viruses from Previous Infections. C57BL/6 mice were inoculated with LCMV to generate a pool of LCMV-specific memory T cells and used in experiments 1–4 mo later. These are referred to as LCMV-immune mice. As can be seen in Figs. 1 (day 5 post infection) and 2 (day 7 post acute infection), spleen leukocytes from uninfected control or from LCMV-immune mice failed to lyse significantly any of the targets tested, indicating that LCMV-specific CTLs are not detectable in the LCMV-immune mice in bulk CTL assays with freshly isolated lymphocytes. Acute infection of C57BL/6 mice (depleted of NK cells) with LCMV, VV, or PV resulted in the generation of cytotoxicity specific for the infecting virus. The level of cytotoxic activity was low at day 5 for LCMV and PV and reached high levels at day 7, whereas the level of cytotoxic activity for VV peaked earlier at day 5. The CTLs induced by each virus were virus-specific and lysed cells infected with different viruses only at very low levels and comparable to uninfected targets. However, infection of LCMV-immune mice with VV or PV stimulated both challenge virus-specific and LCMV-specific killing. In previous experiments, we showed that the phenotype of the virus-specific killer cells activated by VV or PV infection of LCMV-hyperimmune mice were CD8$^+$, Thy1.2$^+$, asialo GM_1^- T cells (12). This indicated that memory CTL reactive against viral antigens from a previous heterologous viral infection were stimulated. These LCMV-specific CTLs appeared by day 5 (Fig. 1) post infection and persisted at day 7 (Fig. 2).

Limiting Dilution Analysis of Spleen Leukocytes from LCMV-immune Mice Infected with a Second Virus. LDAs were performed in order to determine at the clonal level if this reactivation of memory CTLs to the first viral infection could relate to possible remote cross-reactivities between the viruses. At day 7 after infection with PV, a splenic leukocyte precursor frequency (p/f) of 1/329 was derived against PV-infected target cells (Fig. 3 *A*). This value is in the same range as those obtained for LCMV precursor (p)CTL at day 7 post LCMV infection (6). During an acute PV infection pCTL against LCMV-infected targets were not detected (p/f <1/100,000) when leukocytes were stimulated in vitro against PV (Fig. 3 *A*). However, in an LCMV-immune mouse acutely infected with PV for 7 d, splenic leukocytes stimulated with PV in vitro generated a p/f not only for PV-infected targets (1/212) but also for LCMV-infected targets (1/7636) (Fig. 3 *B*).

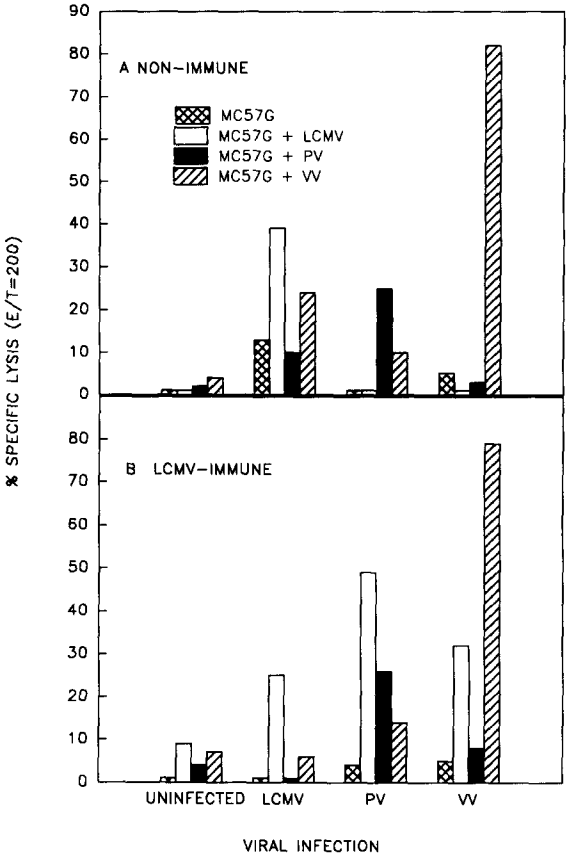

Figure 1. Reactivation of LCMV-specific memory CTL after acute (day 5) infection with heterologous viruses. (*A*) C57BL/6 mice were injected intraperitoneally with various viruses, and CTL activity was assessed at day 5 of acute infection. (*B*) C57BL/6 mice were inoculated intraperitoneally with LCMV and injected 2–4 mo later with other viruses. CTL activity was then assessed.

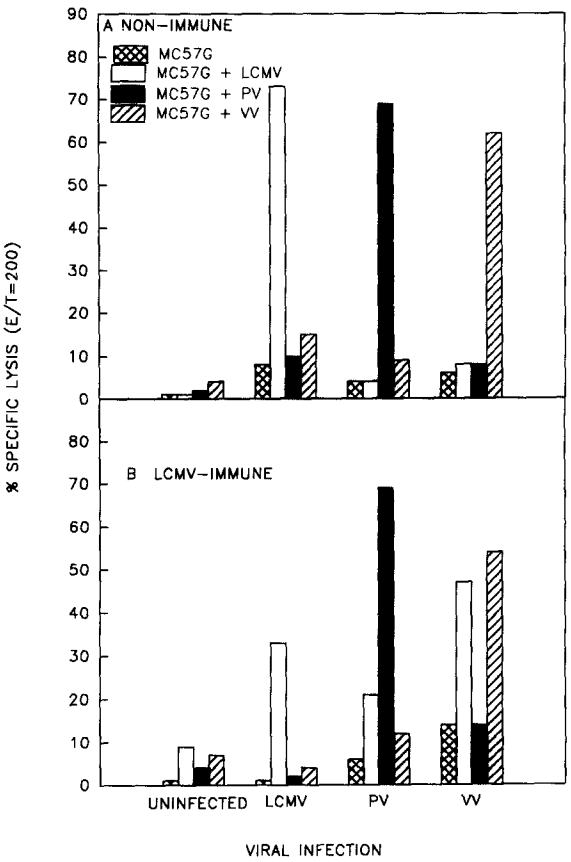

Figure 2. Reactivation of LCMV-specific memory CTL after acute (day 7) infection with heterologous viruses. (*A*) C57BL/6 mice were injected intraperitoneally with various viruses, and CTL activity was assessed. (*B*) C57BL/6 mice were inoculated with LCMV and inoculated 2–4 mo later with other viruses. CTL activity was then assessed.

This was a consistent observation both at days 5 and 7 during an acute PV infection in an LCMV-immune mouse (Fig. 4, *A* and *B*). As expected at day 5 of an acute PV infection, before the peak in PV-specific bulk CTL activity, the p/f for PV-infected targets was lower (1/1509) than at day 7 (1/280), which is near the peak of CTL activity. This is the first time to our knowledge that p/f's for PV infection have been described, and although the mouse is not a normal host for PV it did generate p/f's very similar to those previously observed for LCMV (6). The ratio of the p/f between PV and LCMV (stimulated against PV) was 1:3 at day 5 and 1:14 at day 7, suggesting a selection for the non-cross-reactive PV-specific CTL as the PV infection progressed.

LCMV and PV are both arenaviruses and share some amino acid homology, but they can be considered heterologous viruses because their immunological cross-reactivity is quite remote (12, 24–26). Reactivation of LCMV-specific memory cells by the completely unrelated virus, VV, was also assessed in an LDA. When an LCMV-immune C57BL/6 mouse was acutely infected with VV, and at day 5 of infection splenic leukocytes were stimulated in vitro with VV-infected PECs, p/f's both for VV- (1/2,402) and LCMV- (1/18,000) infected targets were derived (Fig. 5). These LDA results suggest that some form of cross-reactivity may exist between these serologically unrelated viruses.

Anti-CD8 Blocking of Cytotoxicity by Cross-reactive pCTL. To determine whether the phenotype of the cross-reactive killer cells was that of $CD8^+$ CTL and not, for example, NK cells, splenocytes from LCMV-immune mice infected for 6 d with PV were treated with anti-CD8 plus complement before inclusion in an LDA stimulated against PV-infected PEC. In the control group, complement alone, the p/f for PV kill was 1/950 and for LCMV kill was 1/12,276, whereas in the CD8-depleted group there was a significant decrease in p/f's to 1/20,755 and 1/60,653, respectively. This indicates that both types of killing were mediated by $CD8^+$ T cells. To determine whether CD8 was needed in the killing process, anti-CD8 antibody was added directly to the cytotoxicity assay of a similar LDA set up with untreated splenic leukocytes. The anti-CD8 antibody reduced the frequency of wells killing PV-infected targets by ~4-fold and almost completely blocked lysis of the LCMV-infected targets (120-fold) (Fig. 6).

Specificities of Short-term, PV-induced pCTL Clones Derived from PV-infected LCMV-immune Mice. To further confirm

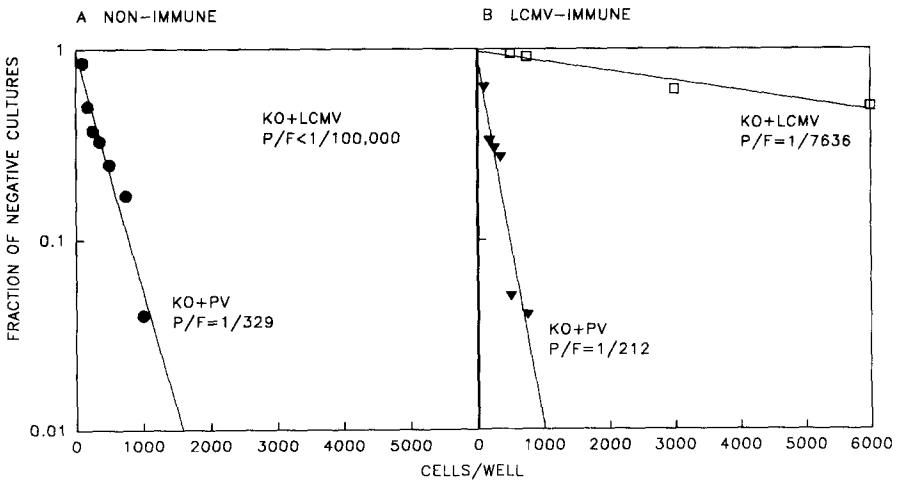

Figure 3. LCMV-immune mice acutely infected with PV (day 7) generate in an LDA pCTL to LCMV when stimulated in vitro with PV-infected PEC. (*A*) Nonimmune mice. C57BL/6 mice were inoculated with PV intraperitoneally, and LDA were set up at day 7 of infection. (*B*) LCMV-immune mice. C57BL/6 mice were inoculated intraperitoneally with LCMV, and 1–4 mo later with PV. LDA were set up day 7 of acute PV infection.

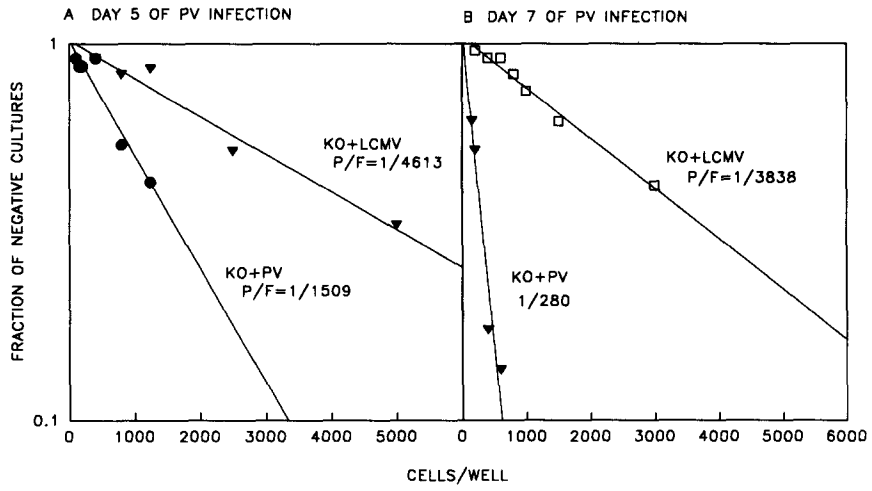

Figure 4. LCMV-immune mice acutely infected with PV generate in an LDA pCTL to LCMV when stimulated in vitro with PV-infected PECs. (*A*) Day 5 of acute PV infection. (*B*) Day 7 of acute PV infection.

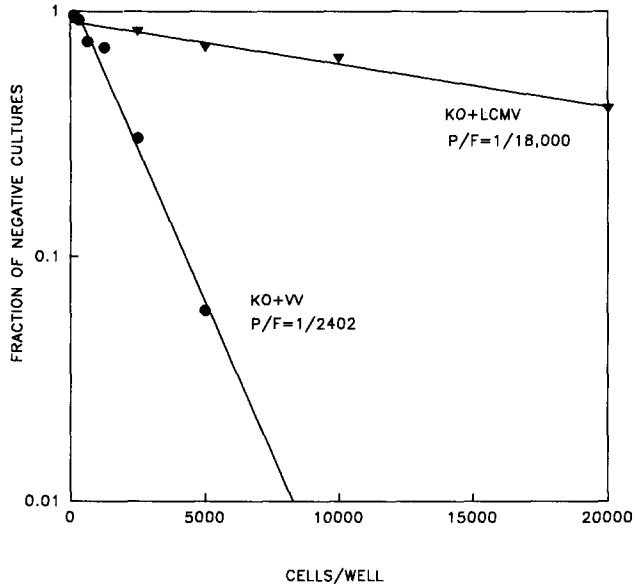

Figure 5. LCMV-immune mice acutely infected with VV generate in an LDA pCTL to LCMV when stimulated in vitro with VV-infected PEC at day 5 of acute VV infection.

whether the same clone of T cells was recognizing targets infected with either of two viruses, LDAs were set up to produce a large number of wells statistically containing <0.5 pCTL/well for either LCMV- or PV-infected targets (i.e., monoclonal wells). In these studies, as in Figs. 3 and 4, LCMV-immune mice were acutely infected with PV, and CTL clones were generated in LDA from spleen leukocytes stimulated against PV-infected PEC. Thus, the arising LCMV-specific clones should be cross-reactive with PV, as they were cultivated with a PV stimulus. Statistical analyses by 2-tail Fisher's Exact Test in five different experiments demonstrated that the wells containing effector cells lytic to targets infected with each virus were not due to random associations of distinct PV-specific and LCMV-specific clones in the same well (Table 1). This indicates that the same CTL clone lysed both targets. At day 5 of the acute PV infection, ~23% (range 15–31%) of the wells containing PV-specific pCTL clones were cross-reactive with LCMV at the level of cytotoxicity, lysing both PV- and LCMV-infected targets. Approximately 62% (range 38–83%) of the wells containing LCMV-specific pCTL clones stimulated with PV-infected PEC were cytolytically cross-reactive with PV, lysing both PV-infected and LCMV-infected

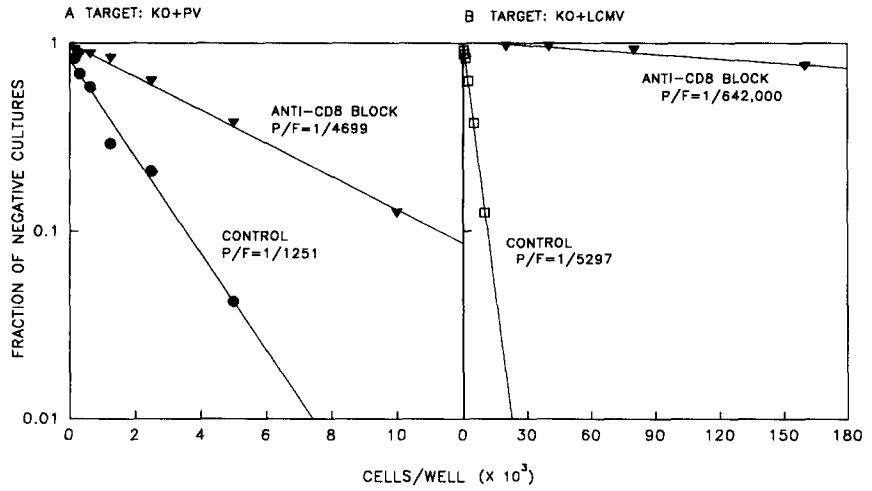

Figure 6. Anti-CD8 blocks lysis of (*A*) PV-infected targets and (*B*) LCMV-infected targets by pCTL from LCMV-immune mice acutely infected with PV (day 6) and stimulated in vitro with PV.

Table 1. *Specificities of Short-term, PV-induced CTL Clones Derived from PV-infected LCMV Immune Mice**

Expt. no.	Clone specificities‡ LCMV + PV +	LCMV + PV −	LCMV − PV +	LCMV − PV −	p value§
			No. of clones		
1	5	1	11	55	0.001
2	3	5	7	142	0.01
3	3	1	17	51	0.06
4	4	4	20	153	0.012
5	4	10	14	68	0.3

* C57BL mice were inoculated intraperitoneally with LCMV. 1–2 mo later these immune mice were inoculated with PV. At 5 (Expts. 1–4) to 7 (Expt. 5) d post infection spleen leukocytes from these mice were stimulated in vitro in LDA with PV-infected PECs, as described in Materials and Methods.

‡ These data represent LDA wells with <0.5 pCTL/well for both LCMV- and PV-infected targets when screened for CTL activity in a 10-h ^{51}Cr release assay.

§ Statistical analysis of the distribution of clones by 2-tail Fisher's Exact test demonstrated the probability that the cross-reactive wells were not a random association of separate PV-specific and LCMV-specific clones.

Table 2. *Cross-reactive pCTL Elicited from Immune Mice Infected with Only One Virus*

Immune status	In Vitro stimulus	Expt.		Targets KO + LCMV	KO + PV
LCMV	PV	1*	pCTL‡	50 (34–60)	6 (2–8)
			p/f	1/20,300	1/198,000
		2§	pCTL	8 (2–14)	12 (2–22)
			p/f	1/135,000	1/81,700
PV	LCMV	1	pCTL	2 (1–4)	2 (2–4)
			p/f	1/473,000	1/377,000
LCMV	LCMV	1	pCTL	972 (726–1,218)	76 (38–116)
			p/f	1/1,028	1/13,000
		2	pCTL	366 (226–508)	34 (19–56)
			p/f	1/2,726	1/30,187
PV	PV	1	pCTL	10 (4–22)	1,062 (690–1,434)
			p/f	1/104,000	1/942
LCMV	PEC‖	1	pCTL	<0.25	<0.25
			p/f	<1/4,000,000	<1/4,000,000
PV	PEC	1	pCTL	<0.5	<0.5
			p/f	<1/2,000,000	<1/2,000,000
Naive¶	PEC	1	pCTL	0.5	0.6
			p/f	1/2,700,000	1/7,000,000
Naive	LCMV	1	pCTL	1.7	0.8
			p/f	1/562,000	1/1,300,000
Naive	PV	1	pCTL	0.3	0.6
			p/f	1/3,500,000	1/1,800,000

* In all experiments designated Expt. 1 the LDA was harvested after 8 d in culture (stimulated twice with virus-infected PECs, at day 0 and day 4).

‡ pCTL means the number of pCTL/10^6 splenic leukocytes.

§ In all experiments designated Expt. 2 the LDA was harvested after 5 d in culture (stimulated only once with virus-infected PECs, at day 0).

‖ PEC are uninfected PECs used as antigen presenting cells.

¶ Uninfected mice.

targets. However, a significant number of clones (38%) in each of these experiments lysed only the LCMV-infected targets. This is an unexpected observation, since these clones were stimulated to grow by PV-infected PEC. This observation could be explained by affinity differences or if the cross-reactive stringency for induction of proliferation of the memory LCMV-specific pCTL is lower than that for target recognition and lysis. Alternatively, differences in antigen presentation (6, 27) or adhesion molecules (28, 29) between the stimulating PEC and the CTL assay targets (KO cells) might account for clones cytolytically specific only for LCMV.

Cross-reactive pCTL Elicited from Immune Mice Infected with Only One Virus. We questioned whether cross-reactive CTL could be generated from splenocytes of immune mice which had not been challenged in vivo with a second virus. Splenocytes from LCMV-immune mice not receiving acute PV infection when stimulated in vitro with heterologous virus (PV) generated p/f's to both LCMV- (1/135,000) and PV-infected (1/81,600) targets (Table 2). PV-immune splenocytes stimulated in vitro with the heterologous virus, LCMV, also generated p/f's to both targets (LCMV-infected targets, 1/473,000; PV-infected targets, 1/377,000) but at a lower level than observed with the LCMV-immune splenocytes. VV-immune splenocytes when stimulated with LCMV in vitro generated pCTL to both LCMV- and VV-infected targets, but the p/f curves were nonlinear (estimated p/f's: LCMV-infected targets, 1/341,000; VV-infected targets, 1/177,000) (not tabulated).

Splenocytes from an LCMV-immune mouse when stimulated in vitro with homologous LCMV generated a low but consistent p/f to PV-infected targets (1/13,000; 1/30,000) (Table 2). A similar observation of pCTL to LCMV-infected targets was made with PV-immune (1/104,000) and VV-immune (1/321,000) (not tabulated) animals, as they were able to generate a low p/f to LCMV-infected targets when stimulated in vitro with the initial infecting virus. It should be noted that the p/f for LCMV- and PV-immune mice stimulated with uninfected PECs or for uninfected naive mice stimulated with LCMV- or PV-infected PECs was $<1/10^6$.

pCTL in LCMV-immune Splenocytes Stimulated In Vitro with LCMV NP Peptide. To determine whether the cross-reactivity between virus-infected cells was due to recognition of viral peptides, as opposed to a common virus-induced cellular antigen, we examined the p/f to LCMV- and PV-infected targets using LCMV-immune splenocytes stimulated in vitro with LCMV NP peptide. These experiments were done in the presence of IL-7, as this cytokine has recently been demonstrated to enhance the stimulation of virus-specific CTL by peptide alone in naive uninfected mice (30, 31). Under these conditions, not only was an appropriate p/f to LCMV-infected targets (1/10,000; 1/24,000) generated but also a significant p/f to PV-infected targets (1/71,000; 1/100,000) (Fig. 7). NP-specific CTL compose ~20% of the pCTL to LCMV in an LCMV-immune C57BL/6 mouse. The usual LCMV p/f in an LCMV-immune C57BL/6 mouse is in our experimental system 1/1000–1/2000. Therefore, an LCMV-specific p/f of 1/10,000–1/20,000 would be appropriate in an LCMV-immune C57BL/6 mouse stimulated with NP peptide. Although 1/71,000 (1/100,000) is a low p/f, it is nonetheless significant, as stimulation of naive uninfected mice or LCMV-immune mice with uninfected PECs resulted in p/f's to either LCMV- or PV-infected targets of $<1/10^6$. These results support the concept that viral peptides are involved in the recognition process by cross-reactive T cells between different viruses.

Priming of CTL Response to Subsequent Acute Viral Infections by Previous Viral Infection. At both day 5 and day 7 of acute PV infection there was a reproducibly enhanced pCTL to challenge virus (PV) in LCMV-immune mice as compared

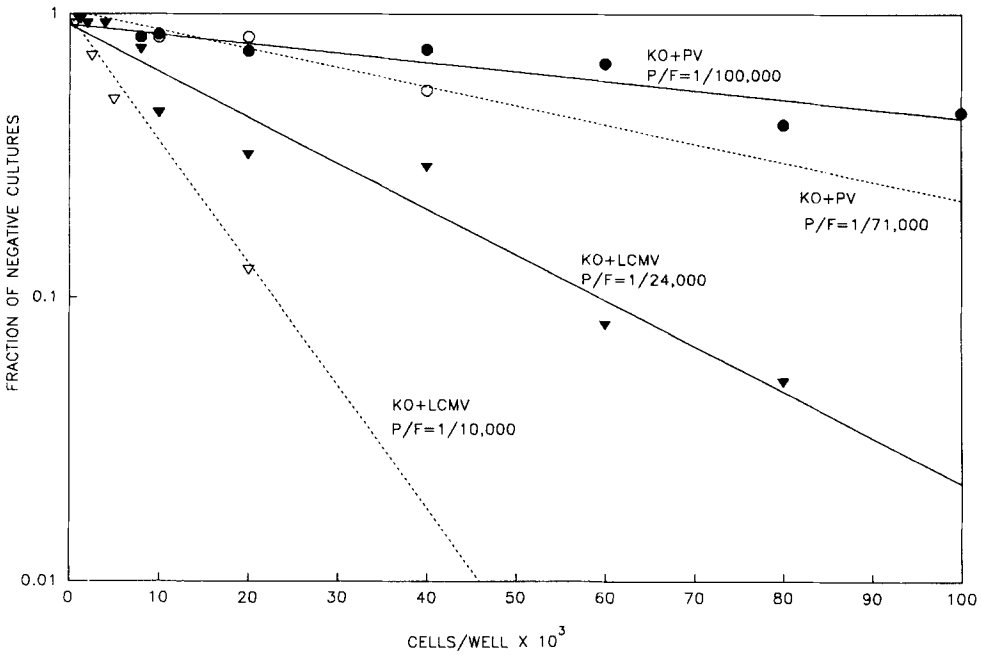

Figure 7. LCMV-immune leukocytes stimulated in vitro with the immunodominant LCMV NP peptide in the presence of IL-7 in an LDA. C57BL/6 mice were inoculated with LCMV, and 1–4 mo later their spleens were harvested. Splenic leukocytes were then set up in LDA stimulated in vitro with LCMV NP peptide-sensitized PECs, in the presence of 15 U/ml of IL-7. ^{51}Cr release assays were done against both LCMV- and PV-infected targets. *Dotted line* represents the p/f's curves of Expt. 1 and *solid line* of Expt. 2. In Expt. 1 only a two-way split-well analysis was done, whereas in Expt. 2 a three-way split-well analysis was done. This may help to explain why the p/f in Expt. 1 is slightly higher than in Expt. 2.

Table 3. *Increased pCTL to Heterologous Virus after Infection of Immune Mice*

Expt.	Day*		Viral infections	
			Acute PV‡	LCMV-immune + acute PV§
1	5	pCTL‖	288 (220–354)	502 (324–682)
		p/f	1/4,377	1/1,990
2	7	pCTL	3,040 (2,400–3,650)	4,716 (3,676–5,748)
		p/f	1/329	1/212
3	7	pCTL	1,139 (752–1,527)	1,901 (1,366–2,439)
		p/f	1/878	1/526
4	7	pCTL	532 (283–781)	1,309 (845–1,773)
		p/f	1/1,880	1/764
5	5	pCTL	637 (410–864)	2,967 (2,066–3,861)
		p/f	1/1,571	1/337
			Acute LCMV¶	PV-immune + acute LCMV**
6	7	pCTL	3,816 (2,688–4,950)	6,666 (4,546–8,772)
		p/f	1/262	1/150
			Acute LCMV	VV-immune + acute LCMV‡‡
7	7	pCTL	3,816 (2,688–4,950)	6,896 (4,310–9,524)
		p/f	1/262	1/145

* Day of acute infection with second virus.
‡ C57BL mice acutely infected with PV.
§ C57BL mice were inoculated with LCMV and 1–4 mo later acutely infected with PV.
‖ pCTL are the number of precursor CTL/10^6 splenic leukocytes.
¶ C57BL mice were acutely infected with LCMV.
** C57BL mice were inoculated with PV and 1–4 mo later acutely infected with LCMV.
‡‡ C57BL mice were inoculated with VV and 1–4 mo later acutely infected with LCMV.

with nonimmune mice when splenic leukocytes were stimulated in vitro with PV (Table 3). Similarly, in individual experiments higher pCTL to LCMV were generated after LCMV infection of PV-immune or VV-immune mice than after infection of nonimmune mice (Table 3). These results indicate that a prior viral infection can prime a CTL response to a heterologous virus in a subsequent infection.

Discussion

This study shows that memory CTL specific to one virus contribute to the primary polyclonal CTL response during infection with a second heterologous virus. This reactivation of memory CTL by heterologous viruses could be due to either (*a*) a nonspecific stimulation of memory cells, particularly the blast-sized IL-2R–bearing cells, with lymphokines generated during the T cell response to the heterologous virus infection; or (*b*) a specific stimulation of memory cells due to the fact that they cross-reacted, albeit possibly at a remote level, with MHC-presented heterologous viral or virus-induced cellular peptides. Enhanced expression of adhesion molecules and IL-2R by memory cells might make them particularly sensitive to stimulation by a low affinity T cell antigen. Our data have not ruled out the possibility of substantial levels of nonspecific stimulation, but they do convincingly support the hypothesis of cross-reactivity. This type of cross-reactivity was demonstrated in a number of ways at the clonal level using LDA analyses. (*a*) Splenic leukocytes from LCMV-immune mice acutely infected with PV or VV generated pCTL to LCMV when stimulated in vitro with PEC's infected only with the second virus but not with uninfected PEC's. (*b*) LCMV-immune splenocytes stimulated in vitro with PV or VV demonstrated a low but significant CTL p/f to the heterologous viruses, and splenocytes from PV- or VV-immune mice when stimulated in vitro against LCMV generated a low but significant p/f to LCMV. (*c*) LCMV-immune splenocytes stimulated in vitro with an immunodominant LCMV peptide generated a p/f to PV. (*d*) In short-term LDA cultures using split-well analyses, CTL clones cross-reactive between LCMV and PV were observed in splenic leukocytes

from LCMV-immune mice acutely infected with PV. It should be noted that VV and PV were propagated in different cells (L-929) than was LCMV (BHK21), and the fact that the NP-peptide preparation was able to stimulate cross-reactive pCTL in LCMV-immune mice makes it unlikely that a cellular contaminant was the source of the cross-reactivity. In addition, plaque purified stocks of PV and LCMV were tested and found to show the same type of cross-reactivity.

Analyses of the relationships between different viruses and viral proteins have focused on homologies between linear amino acid sequences, but cross-reactivities at the level of T cell recognition may not be dependent on a conserved linear sequence of several amino acids. Cross-reactivity in the recognition of unrelated viral peptides by T cell lines and clones has been previously reported (1–4). Anderson et al. (1) described an influenza A NP-specific CTL line which could lyse not only NP peptide-sensitized targets but also targets sensitized with two different influenza A basic polymerase 2 (PB2) peptides. Shimojo et al. (2) described cross-reactive recognition of a human rotavirus VP4 peptide by an HLA-A2.1–restricted CTL line induced by a very dissimilar influenza A matrix peptide. Kuwano et al. (3) demonstrated recognition of two distinct influenza peptides from HA and NS1 by an H-2K^d–restricted influenza-specific CTL clone. Recently Kulkarni et al. (4) demonstrated that immunization of mice with a VV recombinant of the M2 protein of respiratory syncytial virus induced CTL that lysed in a cross-reactive manner targets sensitized with either of two peptides derived from different nonoverlapping regions of the same M2 protein. Analyses of the amino acid sequences of the peptides used in these experiments have showed relatively little homology, and the cross-reactive epitope was often of lower affinity, as defined by the fact that it required much higher concentrations of the peptides to sensitize the target.

Many of the cross-reactive CTL between heterologous viruses in our experiments may be of low affinity to at least one of the viruses. This is supported by the observation that anti-CD8 was more efficient at blocking lysis of the LCMV-infected target rather than the PV-infected target in LCMV-immune mice acutely infected with PV. It has been reported that blocking with anti-CD8 is one method to differentiate high affinity and low affinity CTL clones (32). These putatively low affinity cross-reactive interactions may be best observed once a large memory pool has been defined and during an acute infection when activating lymphokines have increased the CTL expression of ancillary adhesion molecules that may enhance the ability of a low affinity interaction to stimulate a T cell expansion.

It is not surprising that processed peptides from different proteins could cross-react, as the presented peptides must comply with the sequence motif for the presenting MHC molecule and therefore may share similar amino acids in two or three critical positions of the peptide nonamer. Numerous virus-specific clones recognizing noninfected cells expressing different alleles of class I molecules have also been identified. We have shown high frequencies of LCMV-specific short-term clones in H-2^b mice that cross-reacted with various different class I MHC alleles, including H-2^k or H-2^d (6). HSV- (33) and Moloney-specific (34) H-2K^b–restricted CTL generated after secondary in vitro stimulation recognize uninfected cells expressing K^{bm} molecules.

LCMV and PV are both arenaviruses, and they do share considerable amino acid homology, but their serological cross-reactivity is nevertheless quite remote. Antisera do not cross-neutralize, although there is a report of some cross-reactive complement-fixing antibodies to the viral nucleoprotein (24) and one monoclonal antibody directed against a common arenavirus glycoprotein (GP-C) sequence (25). They share no 9 amino acid sequence overlapping any of the defined LCMV T cell immunodominant peptides, and, in fact, acute PV infection does not generate CTL that can in bulk assays lyse LCMV infected targets, and vice versa (Figs. 1 and 2; reference 12). Our results, however, suggest that some form of CTL cross-reactivity may exist between these two heterologous viruses.

Screening of the published PV NP amino acid sequence revealed at the site of homology with the LCMV NP peptide a nonamer with five amino acids in common with the LCMV H-2^b–restricted immunodominant NP LCMV peptide (LCMV = FQPQNGNFI (5); PV = YQPDTGNYI). However, the PV sequence does not have the major fifth position asparagine anchoring amino acid required for binding to the H-2D^b motif (5), and we have not been able to show that the synthesized PV peptide can sensitize targets to CTL induced by either PV or LCMV. This supports the concept that, although LCMV and PV have some amino acid homology, they are heterologous viruses in regards to CTL recognition and do not have similar immunodominant epitopes. The immunodominant epitopes for PV are as yet undefined, and in fact, a search for CTL epitopes on the PV nucleoprotein was unsuccessful (35). These results also indicate that it may be difficult to predict which viral peptides will be cross-reactive at least until the TCR binding motifs for various viral peptides can be identified. Nevertheless, our data clearly argue that at the level of CTL there is definitely cross-reactivity between LCMV and PV and probably cross-reactivity between LCMV and VV, both unexpected findings, as cross-reactivities between these viruses are not seen in conventional bulk assays using CTL from mice infected with a single virus.

The implication of these analyses is that, at the T cell level, there may be a great deal of immunological cross-reactivity between serologically unrelated infectious agents, and by virtue of this proposed remote T cell cross-reactivity, memory T cells from a previous unrelated infection may be reactivated and contribute to the primary response of a putatively unrelated infectious agent. Jones et al. have demonstrated that an MHC-restricted T cell proliferative response to *Plasmodium falciparum* malaria antigens could occur in donors not immune to malaria. However, this response required the presence of memory T cells (UCHL1$^+$ cells), suggesting that this cross-reactive response was due to epitopes shared between *P. falciparum* and some other common immunogens that induced a memory T cell pool (36). Cross-reactive memory T cells may account for the high frequency of T cells expressing mRNA for activating lymphokines (IFN-γ, IL-2) at day 3 early in viral infection long before the primary

virus-specific pCTL could have expanded to detectable levels from naive mice (37). There is evidence in the alloantigen system that naive and memory T cell subsets differ in their ability to synthesize and respond to a variety of cytokines and that each subset can produce cytokines that amplify the response of the other subset (7). Both subsets appear to be activated to the same extent by alloantigen, and it has been suggested that they synergize in initiating potent responses against transplanted allografts.

These data together suggest that prior immunity to one virus might provide some level of protective natural immunity to an unrelated infectious agent. Consistent with this concept is our previous work showing that VV replication in LCMV-immune mice was reduced by ~10-fold (38), and we have recently found a similar 10-fold reduction of PV titers in LCMV-immune mice, and splenocytes from LCMV-immune mice when transferred to nonimmune recipients (one splenic equivalent/mouse) conferred a similar degree of resistance to PV (data not shown). The mechanism for this resistance to heterologous virus infection has not been clarified, but may involve cross-reactive $CD8^+$ cells, as $CD8^+$ CTL are major regulators of infection for each of these viruses (26, 39). It is equally feasible that cross-reactivity exists at the CD4 level, and these cells could provide resistance to infection via the secretion of cytokines. These results do, however, suggest that prior immunity to one virus enhances the immune system's ability to handle future viral infections, thereby contributing to natural resistance.

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Address correspondence to Dr. Raymond M. Welsh, Department of Pathology, University of Massachusetts Medical Center, Worcester, MA 01655.

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