High Frequency of Cross-reactive Cytotoxic T Lymphocytes Elicited during the Virus-induced Polyclonal Cytotoxic T Lymphocyte Response

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Summary

Polyclonal stimulation of CD8+ cytotoxic T lymphocytes (CTL) occurs during infection with many viruses including those not known to transform CTL or encode superantigens. This polyclonal CTL response includes the generation of high levels of allospecific CTL directed against many class I haplotypes. In this report we investigated whether the allospecific CTL generated during an acute lymphocytic choriomeningitis virus (LCMV) infection of C57BL/6 mice were stimulated specifically by antigen recognition or nonspecifically by polyclonal mechanisms possibly involving lymphokines or superantigens. An examination of the ability of different strains of mice to induce high levels of CTL specific for a given alloantigen showed that most, but not all, strains generated high levels of allospecific CTL, and that their abilities to generate them mapped genetically to the major histocompatibility complex locus, exclusive of the class II region. This indicated that the virus-induced allospecific CTL generation was independent of the class II allotype, and mice depleted of CD4+ cells generated allospecific CTL, indicating independence of class II-CD4+ cell interactions and resulting CD4+ cell-secreted lymphokines. FACS® staining with a variety of Vß-binding antibodies did not show a superantigen-like depletion or enrichment of any tested Vß+ subset during infection. Several experiments provided evidence in support of direct stimulation of CD8+ cells via the T cell receptor: (a) both virus- and allo-specific killing were enriched within a given Vß subpopulation; (b) relative CTL precursor frequencies against different class I alloantigens changed during the course of virus infection; (c) the relative levels of virus-induced, allospecific CTL-mediated lysis at day 8 after infection did not parallel the CTL precursor frequencies before infection; and (d) limiting dilution analyses of day 8 LCMV-infected spleen cells stimulated by virus-infected syngeneic peritoneal exudate cells (PEC) revealed not only the expected virus-specific CTL clones, but also a high frequency of clones that were cross-reactive with allogeneic and virus-infected syngeneic targets. In addition to the virus cross-reactive allospecific CTL clones, virus-infected PEC also stimulated the generation of some allospecific clones that did not lyse virus-infected fibroblasts. Surprisingly, LCMV-infected PEC were much more efficient at stimulating allospecific CTL clones from day 8 LCMV-infected splenocytes than were allogeneic stimulators. These results indicate that at least part of the polyclonal allospecific CTL response elicited by acute virus infection is a consequence of the selective expansion of many clones of allospecific CTL which cross-react with virus-infected cells. These CTL may contribute to the early control of virus infection, when the viral burden is great, and before significant numbers of high affinity virus-specific CTL are generated.

Massive proliferative lymphocyte responses to virus infections have been shown with transforming viruses such as EBV in B cells (1) and with superantigen-encoding viruses, such as mouse mammary tumor virus (MMTV)1 in CD4+ T cells (2), but a profound polyclonal stimulation of CD8+ T cells appears to be also a natural consequence of infections with viruses not known to transform cells or to encode superantigen (3). In addition to the stimulation of virus-specific CTL, acute virus infections stimulate the generation of high levels of allospecific CTL and the reactivation of memory CTL specific for viruses from prior infections (4). This induced allospecific CTL response has been shown during infections with lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV), Pichinde virus (PV), and murine cyto-

1 Abbreviations used in this paper: c-RPMI, complete RPMI; FBS, fetal bovine serum; i, intercept, LCMV, lymphocytic choriomeningitis virus; LDA, limiting dilution assay; LU, lytic unit; MCMV, murine cytomegalovirus; MMTV, mouse mammary tumor virus; MOI, multiplicity of infection; PEC, peritoneal exudate cell; pf, precursor frequency.
megaviruses (MCMV) in the mouse (4), and with EBV in humans (5, 6). Acute virus infections such as mouse hepatitis virus (strain JHM) (7) and Coxackie B3 virus (CVB3) (8, 9) in the mouse and Kilham rat virus in the rat (10) have been reported to stimulate autoimmune T cells. The allospecific CTL induced in mice by LCMV or in humans by EBV infection have been shown to recognize the class I antigens on a broad range of allogeneic targets, indicating that a large spectrum of TCR specificities are elicited (6). This would suggest that virus infection causes many of the CD8+ T cells to proliferate and differentiate, and indeed, during the first 8 d of an acute LCMV infection, the number of CD8+ cells in the spleen increases 15–20-fold, whereas the total number of spleen cells increases two–four-fold over that of the uninfected spleen. Many of these virus-induced CD8+ T cells express activation markers. Approximately 75% express CD44, ~50% express CD11b (11), and ~25% express the large granular lymphocyte phenotype of cytotoxic cells (12).

The consequences of this polyclonal T cell response are as yet undetermined, but these CTL have the potential to contribute to autoimmunity and graft rejection. In addition, if the allospecific CTL represent selective expansions of virus-specific CTL which are cross-reactive with allogeneic targets, they may provide an early, important, and rapidly mobilized immune response to control virus infection and spread in the host.

The question we address in this report is whether the profound level of CD8+ cell proliferation and the induction of allospecific CTL is stimulated by an antigen-driven mechanism involving occupation of the TCR by viral or virus-induced antigens, or whether they are a consequence of a TCR-independent mechanism induced by the complex array of lymphokines produced during an acute virus infection. We have previously reported that the killing of allogeneic targets was not impaired by coincubation with unlabelled virus-infected syngeneic targets (4). This argued that the allospecific CTL response was not cross-reactive with the virus-specific response, and this result, along with the observation that targets expressing all tested allogeneic class I allogeneic (H-2\textsuperscript{d,d,k,p,r,t}) were lysed by CTL from C57BL/6 mice (H-2\textsuperscript{b}), led us to speculate that this may be a cytokine-driven polyclonal response independent of TCR stimulation. However, we also discussed that affinity differences of the CTL for the targets in cold target competition assays could account for lack of competition for T cells that recognize both targets (4).

The approach we have taken here to address these issues is to look for preferential selection of particular allospecificities over others during virus infection, and to determine if the virus-induced allospecific CTL are cross-reactive with viral antigen at the clonal level. In addition, we map genetically the generation of virus-induced allospecific CTL to MHC regions external to the class II genes and demonstrate that CD4+ cells do not play a prominent role in this induction process. We report that there is an active selection of allospecific CTL subsets during acute virus infection and that this can be attributed, at least in part, to cross-reactivity of virus-specific CTL clones for alloantigen.

**Materials and Methods**

**Mice.** Most strains of mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These included the H-2\textsuperscript{b} strains C57BL/6J, C57BL/10ScCr, and C57SW/SnJ; H-2\textsuperscript{d} strains BALB/cByJ, DBA/2J, and B10.D2SnJ; H-2\textsuperscript{a} strains C3H/HeN/SnJ and B10.BR/SgSnJ; and strains of other haplotypes such as B10.M/Sn (H-2\textsuperscript{b}), C3H.10B1/MpJ (H-2\textsuperscript{b}), SWR/J (H-2\textsuperscript{a}), B10.RII-7NS/J (H-2\textsuperscript{b}), and A.SW/SN (H-2\textsuperscript{b}). The H-2\textsuperscript{b} strain B10.S was purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The recombinant strains A.TBR1 (H-2\textsuperscript{b}+) and A.TBR2 (H-2\textsuperscript{b}−) (13) were kindly provided by Dr. Howard Passmore (Rutgers University, New Brunswick, NJ). Mice were used at 1–6 mo of age.

**Viruses.** The Armstrong strain of LCMV was used in all experiments. The virus was propagated on baby hamster kidney cells as reported elsewhere (14). Mice were injected intraperitoneally with 4 × 10\textsuperscript{6} PFU of LCMV for 7 or 8 d unless otherwise indicated. LCMV-immune mice were given secondary LCMV infection by injection with 4 × 10\textsuperscript{6} PFU of LCMV at least 4 wk after the primary LCMV infection.

**Target Cells.** L929 (H-2\textsuperscript{d}), a continuous liver cell line derived from C3H mice, and MC57G (H-2\textsuperscript{d}), a methylcholanthrene-induced fibroblast cell line from C57BL/6 mice were propagated in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 10% heat inactivated (56°C, 30 min) fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO). P815 (H-2\textsuperscript{a}), a methylcholanthrene-induced mastocytoma from DBA/2 mice, was maintained in RPMI media (Sigma Chemical Co.) supplemented as described for complete MEM (c-RPMI). KO cells (H-2\textsuperscript{b}−), a SV40-transformed cell line derived from a C57BL/6 mouse (15), and provided to us by Dr. Satvir Tevethia (Pennsylvania State Medical Center, Hershey, PA) were propagated in DMEM (Gibco Laboratories) supplemented as described for MEM, but with the addition of 5 × 10\textsuperscript{5} M 2-mercaptoethanol and 10 mM Hepes. MC57G, KO, and L929 cells were infected with LCMV at a multiplicity of infection (MOI) of 0.1-0.2 PFU/cell and incubated for 2 d at 37°C.

**Depletion of NK Cells In Vivo.** Mice were injected intraperitoneally with 5 μl of antiserum to asialo GM1 (Wako Chemical, Dallas, TX) in a volume of 0.1 ml on days 0 and 4 after infection. This greatly reduced NK cell activity for at least an 8-d time period.

**Effector (Responder) Cell Preparation.** Spleens were isolated and ground between the frosted ends of two glass microscope slides. The cell suspension was then passed through a fine nylon mesh to obtain a single cell suspension. Erythrocytes were lysed by briefly resuspending the spleen cell pellet in 0.84% NH\textsubscript{4}Cl solution before rinsing. For CD8+ effector cells, 10\textsuperscript{6} spleen cells were stained with 50 μl of directly FITC-conjugated, purified anti-CD8 antibody (Becton Dickinson & Co., Mountain View, CA) for 45 min on ice and rinsed three times before sorting. The stained cells were compared with unstained cells for background fluorescence and separated into CD8+ and CD8− subpopulations at a flow rate of 2,000–2,500 cells/s using a cell sorter (FACS® 440; Becton Dickinson & Co.). For TCR Vß staining of effector cells, tissue culture supernatants from hybridomas (Vß6 [JR 4-7]; Vß8.1 and 8.0 [KJ 16.133.18]; Vß11 [RRJ-3-15]; Vß9 [MR 10-2]; Vß5.1 and 5.2 [MR 9.4]; and Vß17 [KJ 23.588.1]), kindly provided by Dr. Ed Palmer (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) were used. The cells were incubated for 45 min on ice, rinsed three times, and treated with a mouse FITC-conjugated F(ab')\textsubscript{2} anti–rat IgG F(ab')\textsubscript{2} (Jackson Immunore-
and washed three times. The stained cells were sorted as described above. Rinsed three times. Streptavidin (Becton Dickinson & Co., San Jose, CA) was added to the biotin-treated samples for 30 rain on ice and bottomed microtiter wells in quadruplicate (Costar Corp., Cambridge, MA). For the CD8 inhibition studies, 0.05 ml of anti-Lyt anti-mouse Ig (Zymed, San Francisco, CA) for 30 rain on ice and was added to flat-bottomed microtiter wells in quadruplicate (Costar Corp., Cambridge, MA). For the CD8 inhibition studies, 0.05 ml of anti-Lyt 2 antibody (dilution of M12:17.2 ascites, kindly provided by Dr. Eric Martz (University of Massachusetts, MA) (17) was added to the plates for the duration of the 51Cr-release assay. Varying numbers of effector leukocytes were added in 0.1 ml of medium to achieve the desired E/T ratios. For a spontaneous 51Cr-release control, 0.1 ml of c-RPMI was added to the labeled target cells in place of effector cells. For the maximum 51Cr-release control, 0.1 ml of 1% NP-40 was added to target cells. After 6-8 h at 37°C, 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., Palo Alto, CA). Data were expressed as percent specific 51Cr-release = 100 × [(experimental cpm – spontaneous release cpm)/maximum release cpm – spontaneous release cpm]. Lytic units were calculated using the exponential fit and von Krogh methods (18) provided by software (Proteins International, Rochester Hills, MI). One lytic unit was defined as the number of effector cells required to lyse a given percentage of a population of 10^4 targets in an 8-h CTL assay.

In Vivo Depletion of CD4+ Cells. CD4+ cells were depleted following the procedure of Buller et al. (19). Briefly, 0.1 ml of GK 1.5 hybridoma ascites propagated in BALB/c mice in our facility, control ascites from BALB/c, or PBS was injected intraperitoneally on -4, -2, and +3 d after infection to deplete CD4+ cells. On the day of the assay, the mice were bled to determine IgG levels in the serum. The spleens were removed and used in cell-mediated cytotoxicity assays and FACS® analyses to determine the presence or absence of CD4+ cells using a mouse FITC-conjugated F(ab')2 anti-rat IgG F(ab')2 second antibody (Jackson Immunoresearch Laboratories). IgG levels were determined by cytoplasmic staining of fixed LCMV-infected MC57G cells using serum dilutions of control and CD4+ cell-depleted sera. The second antibody was a FITC-labeled goat anti-mouse IgG (Zymed).

Limiting Dilution Assay (LDA) for Virus-Specific CTL Precursors. The assays used the procedure of Moskophides et al. (20) with the following modifications. Stimulators were prepared from peritoneal exudate cells (PEC), isolated from C57BL/6 mice having received 1 ml of thioglycolate broth (Difco Laboratories Inc., Detroit, MI) intraperitoneally before plating. The PEC were infected with an MOI of 0.1 PFU of LCMV and plated in 96-well, U-bottomed microtiter dishes (Costar Corp.) at 5 x 10^4 per/well in 0.1 ml culture media. After 2 d in culture, the 96-well plates received 2,000 rad of 131Iesium gamma irradiation from an irradiator (Gammacell 40; Atomic Energy of Canada, Ltd., Ottawa, Canada). Culture medium was removed (50 μl) from each well and replaced with 50 μl of media containing 10^8 irradiated (2,000 rad), syngeneic spleen cells. Varying numbers of responder cells from C57BL/6 spleens were added in 0.1 ml of media to 16-32 well replicates, and the plates were cultured at 37°C in a humidified CO2 (5%) incubator. After 4 d, the cultures were fed by removing 0.1 ml of media from each well and adding 5 x 10^4 irradiated, virus-infected PEC in 0.1 ml of media. The culture medium throughout the assay was either c-RPMI (Sigma Chemical Co.) supplemented with 0.1 mM nonessential amino acids (Gibco Laboratories), 0.1 μM sodium pyruvate (Gibco Laboratories), 1% 1 M Hepes, and 5 x 10^-3 M 2-ME (MLC-RPMI) or complete AIM V medium (Gibco Laboratories) supplemented with 10% heat-inactivated FCS. Growth factors were provided by the addition of 25% culture supernatant from the IL-2-secreting, gibbon lymphoma tumor cell line MLA.144 (American Type Culture Collection, Rockville, MD) (21). On days 5-8 of culture, individual wells were split three- or fourfold and assayed for cytolytic function on infected or uninfected syngeneic target cells (MC57G or KO) and on allogeneic target cells (L929 and P815) using a modified 51Cr-release assay. 51Cr-labeled targets (5 x 10^5) were added to all wells to allow a final volume of 200 μl/well. The plates were incubated 8 h at 37°C, centrifuged for 5 min at 130 g (IEC CRU-5000 centrifuge), and 0.1 ml of supernatant was harvested. Radioactivity released into the supernatant was counted on a gamma counter (Beckman Instruments, Inc.). Positive wells were defined as those wells whose 51Cr-release exceeded the mean spontaneous release by >3 SD. All wells that lysed uninfected syngeneic targets or lysed all targets tested were eliminated from the analysis. Frequencies were calculated using χ^2 analysis according to Tawse (22) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI).

LDA to Determine Precursor Frequencies of Allogeneric CTL. Allogenetic stimulator cells were prepared from the splenies of allogeneic mice irradiated (2,000 rad) 1 h before killing. Varying numbers of responder spleen cells (from infected or control mice) were cultured in 96-well flat-bottomed microtiter plates (Costar Corp.) in the presence of 10^6 irradiated allogeneic spleen cells in 0.2 ml supernatant-supplemented MLC-RPMI (as described above). The cultures were incubated at 37°C in a humidified 5% CO2 atmosphere for 4 d, at which time they were fed by replacing 0.1 ml of the culture medium in each well with fresh supplemented MLC-RPMI. After 4 d more of culture, a modified 51Cr-release assay and precursor analyses were performed as described above.

MLC. For micro MLC, 2 x 10^6 irradiated, allogeneic stimulator cells were plated into flat-bottomed microtiter wells (Costar Corp.) Doses ranging from 25 to 200 x 10^6 responder cells in 0.1 ml volume of MLC-RPMI were added to the stimulators in replicas of four wells. The cultures were incubated in a 37°C, humidified, 5% CO2 incubator for 4 d. 51Cr-labeled target cells (10^4, P815, L929, and MC57G) in 50 μl of c-RPMI were added to each culture well. For spontaneous release of 51Cr, targets were added to wells containing only medium. For maximum release of 51Cr, targets were added to wells that contained 0.1 ml of 1% NP-40 in PBS. The plates were incubated for 6 h at 37°C in a humidified incubator, centrifuged at 130 g, and 0.1 ml of supernatant was removed from each well and counted in a gamma counter (Beckman Instruments, Inc.). The percent specific 51Cr-release was calculated as described above. Standard MLC were prepared by incubating 2 x 10^6 effectors in the presence of 8 x 10^6 irradiated, allogeneic stimulator cells in 25 ml of MLC-RPMI in a 25 cm² flask (Costar Corp.). These were incubated in the upright position, 37°C in a humidified 5% CO2 incubator for 4 d. The viable cells were collected and used as effectors in the standard cytotoxicity assay described above.
Results

Induction of Allospecific CTL and Their Sensitivity to Treatment with Anti-CD8 mAb. Table 1 (Expts. 1, 2, and 3) shows that at 8 d after primary infection, high levels of cytotoxicity develop against LCMV-infected syngeneic targets, and moderate levels of cytotoxicity develop against the uninfected allogeneic H-2^k and H-2^d targets. Virtually no cytotoxicity was observed against uninfected syngeneic targets in these mice, which had been depleted of NK cells. We have previously shown that this cytotoxic activity is mediated by CD8^+ CTL (3). The CD8 molecule on the surface of CTL is responsible for strengthening low affinity interactions between the CTL and the target cell by binding to the α3 domain of the class I MHC molecule (23). Antibodies directed against CD8 are thought to inhibit the interactions of low affinity CTL more than those of high affinity (24). We questioned whether there were differences in the CD8 requirements between the virus- and allospecific CTL-mediated lysis. Table 1 shows the effects of anti-CD8 antibody on the lysis of LCMV-infected syngeneic targets (H-2^b + LCMV) and allogeneic targets (H-2^k and H-2^d) by day 8 LCMV-infected spleen cells in a ^{51}Cr-release assay. With excess anti-CD8 antibody, there was significant inhibition in the lysis of both allogeneic and virus-infected syngeneic targets, though a small component of the virus-specific killing was not inhibited. This contrasts with the high affinity, uninhibitable interactions seen with allo- or virus-specific CTL, respectively, from secondary MLC (Expts. 2 and 3) or from secondary in vivo virus infection (Expt. 4).

The Induction of Virus-Induced Allospecific CTL in Congenic and Recombinant Strains of Mice. The levels of LCMV-induced allospecific CTL generation varied with the strain of mice (Fig. 1). H-2^k and ^i strains generated relatively low levels of lysis against the targets L929 (H-2^k) and P815 (H-2^d); H-2^b, ^f, ^p, and ^s strains generated high levels. We questioned what genetic factors contributed to this strain variation and tested the ability of various congenic mouse strains to generate anti-H-2^d CTL after LCMV infection. The allospecific CTL induction against H-2^d targets mapped within the MHC. Congenic strains that presented the H-2^d (C57BL/6, C57BL/6 mice were infected with LCMV for 8 d before the assay. The 2^o MLC effectors were C57BL/6 spleen cells primed in vivo with allogeneic tumor cells (P815 or L929) for 1-2 mo before 2^o stimulation in vitro. The 2^o LCMV-induced effectors were C57BL/6 spleen cells that were infected with LCMV in vivo 1-2 mo before secondary LCMV infection for 6 d in vivo. The anti-Lyt 2 (anti-CD8) antibody was titrated for maximum inhibition of virus-specific lysis and was used in the ^{51}Cr-release assay at 1:300-600 dilution. Target cells: H-2^b, MC57G; H-2^d, P815; and H-2^k, L929.

Table 1. Inhibition of Virus- and Allospecific Lysis by Anti-CD8 Antibody

| Expt. | Effectors | Targets |
|-------|-----------|--------|
|       | H-2^b + LCMV | H-2^k | H-2^d | H-2^f | H-2^s |
| 1.    | LCMV-infected H-2^b | 44-50 | 22-25 | 12 | 50 | 10-12 | 3 | 50 | 10-12 | 3 | 50 | 10-12 | 3 |
|       | LCMV + anti-CD8 | 60 | 16 | 5 | 3 | 8 | 2 | -2 | -4 |
| 2.    | LCMV-infected H-2^b | 36 | 9 | 6 | 4 | 6 | 2 | 4 | 3 |
|       | LCMV + anti-CD8 | 67 | 38 | 27 | 10 | 27 | 10 | 9 | 7 |
|       | 2^o MLC (H-2^b anti-H-2^d) + anti-CD8 | 31 | 14 | 30 | 9 | 30 | 11 | 1 | 1 |
|       | 2^o MLC (H-2^b anti-H-2^d) + anti-CD8 | 22 | 8 |
| 3.    | LCMV-infected H-2^b | 36 | 19 | 17 | 4 | 17 | 5 | 3 | 2 |
|       | LCMV-infected H-2^b + anti-CD8 | 20 | 10 | 1 | 1 | 8 | 2 | 3 | -4 |
|       | 2^o MLC (H-2^b anti-H-2^d) + anti-CD8 | 67 | 28 | 7 | 1 |
|       | 2^o MLC (H-2^b anti-H-2^d) + anti-CD8 | 56 | 25 | 5 | 2 |
| 4.    | 2^o LCMV-infected H-2^b | 29 | 17 |
|       | 2^o LCMV-infected H-2^b + anti-CD8 | 21 | 14 |

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levels were tested. As shown in Fig. 3, mice with low responder alleles at the I-A (A.TBR2), or I-A and I-E (A.TBR1) generated high levels of allospecific CTL. This result shows that the generation of allospecific CTL maps outside the class II region. In these and in a variety of other experiments, high responder class I alleles (b or s) overlapping either the K or D class I loci resulted in a high allospecific CTL phenotype. No congenic mice were available that expressed high responder alleles (b or s) at the class II loci. However, the data are consistent with the concept that relative levels of LCMV induction of allospecific CTL may involve the class I loci and are independent of the class II haplotype.

Generation of Virus-induced Allospecific CTL In Vivo in the Absence of CD4⁺ Cells. To determine if the allospecific CTL generated during acute virus infection were being stimulated by high levels of IL-2 or other growth factors secreted by activated CD4⁺ T cells, C57BL/6 mice were depleted of CD4⁺ cells by multiple intraperitoneal injections of anti-CD4 antibody (GK 1.5) or control ascites before and during the acute infection with LCMV. The effector cells were also depleted in vivo of NK cell activity with antibody to asialo GM₁ to eliminate background NK cell–mediated lysis. Fig. 4 shows that spleen cells from C57BL/6 (H-2b) mice treated with control ascites lysed allogeneic P815 and L929 cells and virus-infected but not uninfected, syngeneic MC57G cells. The depletion of CD4⁺ T cells did not affect the induction of allospecific CTL, nor, as shown previously by others, did it reduce virus-specific killing (25) (Fig. 4). Cytoplasmic staining of LCMV-infected MC57G cells with sera collected

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Figure 1. Levels of LCMV-induced allospecific CTL in different strains of mice. 100:1 E/T ratio. Data represent average lysis from six experiments using C57BL/6; four experiments using A.SW; three experiments using C3H.NB and C3H; two experiments using B10.RIII; and one experiment using B10.M and B10.S.

Figure 2. The generation of virus-induced allospecific CTL in MHC congenic mice. NK cell–depleted mice were infected with LCMV 7 d before CTL assay. Splenocytes were tested for cytotoxicity against the allogeneic target, P815 (H-2d), and against infected and uninfected syngeneic target cells. The syngeneic target for H-2b effectors was MC57G and for H-2k effectors, L929. 100:1 E/T ratio. The average of the virus-specific lysis between strains at an E/T ratio of 100:1 was 65 ± 10%.

Figure 3. Induction of allospecific CTL during LCMV infection of class I and II recombinant strains of mice. The analyses of LCMV-infected A.TBR1 and A.TBR2 strains were based on the average lysis by splenocytes from four individual spleens. Representative data from two C3H mice were included. Other C3H data are presented in Figs. 1 and 2. The LCMV-infected syngeneic target for A.TBR1 and A.TBR2 strains was MC57G, and for C3H strain, L929.
from the antibody- or sham-treated mice showed that the production of antiviral IgG, a CD4+ T cell-dependent event, was reduced by ~1,000-fold in the anti-CD4 antibody-treated mice, and FACS® showed that the lymphocyte population was largely depleted of CD4+ cells (data not shown). These results indicate that the polyclonal stimulation of allospecific CTL during virus infection does not require high levels of the lymphokines secreted by the CD4+ T cell population.

**Lack of Evidence for a CD8+ T Cell Superantigen Response.**

"Superantigen"-encoding pathogens such as MMTV and Staphylococcus preferentially stimulate the expansion of peripheral CD4+ T cells expressing particular Vβ in their TCR (2, 26). To determine whether LCMV-induced allospecific CTL were the result of the expansion of CD8+ T cells activated by an LCMV-induced or -encoded superantigen, spleen cells from LCMV-infected or -encoded control C57BL/6 mice were stained with a panel of TCR V3 antibodies (V3 levels of the lymphokines secreted by the CD4+ T cell-specific CTL during virus infection does not require high numbers of allospecific CTL (H-2a vs. H-2k) should not be absolutely "polyclonal" but more oligoclonal, and the relative numbers of allospecific CTL (H-2k vs. H-2d) and their precursors before and after infection should change depending upon which allospecific CTL are responding to superantigen (data not shown).

**Selective Expansion of H-2k vs. H-2d Allospecificity in the LCMV-infected C57BL/6 Mouse.**

A possible mechanism for the induction of allospecific CTL during acute virus infection is that they are cross-reactive CTL selected to proliferate via TCR occupancy by a viral or endogenous antigen presented in the context of self MHC class I antigen. If so, the response should not be absolutely "polyclonal" but more oligoclonal, and the relative numbers of allospecific CTL (H-2k vs. H-2d) and their precursors before and after infection should change depending upon which allospecific CTL more closely resembles the conformation of self MHC and antigen that is driving the stimulation in vivo. LDA, MLC, and CTL assays were performed to determine the responsiveness of uninfected and day 8 LCMV-infected C57BL/6 (H-2b) spleen cells to the H-2k or H-2d alloantigen. MLC of uninfected C57BL/6 (H-2b) spleen cells cultured in the presence of irradiated H-2d (BALB/c or DBA/2) or H-2d (C3H or B10.BR) spleen cells show that the lytic units (LU) specific for H-2d targets (L929) were only half as great as those for H-2k targets (P815) (ratio, 0.47 ± 0.07 (Table 2). This ratio is reflected in the precursor frequency estimation of H-2k- or H-2d-specific CTL in the uninfected C57BL/6 spleen (1/6553 [H-2k] vs. 1/3412 [H-2d], respectively, in paired experiments). Yet, in spleen cells taken 8 d after LCMV infection, there were approximately equivalent CTL precursor frequencies (H-2k, 1/3,407 vs. H-2d, 1/3,739), as well as comparable amounts of killing in 51Cr-release assays against the two alloantigens (H-2k, 1.68 LU vs. H-2d = 1.29 LU) (Table 2). More often than not, the direct virus-induced allospecific CTL-mediated lysis was greater against the H-2d (L929) cell target than the H-2k (P815) cell target, even though these mice had twice as many H-2d-specific precursors before infection. These results demonstrate a greater selection of H-2k- than H-2d-specific CTL and suggest that

**Table 2. Preferential Stimulation of H-2k CTL during LCMV Infection**

| Assay | Effectors | Relative response H-2k/H-2d |
|-------|-----------|-----------------------------|
| MLC   | H-2k spleen cells | 0.48*                       |
| LDA   | H-2k spleen cells | 0.52†                      |
| CTL   | H-2k LCMV-infected spleen cells | 1.49§                     |
| LDA   | H-2k LCMV-infected spleen cells | 0.91‖                     |

* The relative response is based on LU of H-2k- and H-2d-specific lysis by C57BL/6 spleen cells in the micro MLC. Data presented are the average LU of two representative experiments: 0.48 ± 0.08 SD.
† The relative response is based on precursor frequencies (pf) of H-2k- and H-2d-specific CTL in the uninfected C57BL/6 spleen. Data represent the average precursor frequencies in three experiments in which both the H-2k- and H-2d-specific precursors were estimated from the same spleen cell population. For the H-2k precursor analyses: the average pf = 6,553 ± 435, p = 0.67 ± 0.07, and the intercept (i) = 0.99 ± 0.05. For the H-2d precursor analyses: average pf = 3,412 ± 1,669, p = 0.68 ± 0.47, and i = 0.97 ± 0.02.
§ The relative response is based on lysis (LU) of H-2k (L929) and H-2d (P815) targets by LCMV-infected C57BL/6 spleen cells in an 8-h 51Cr-release assay. Data represent the average LU in two experiments: 1.49 ± 0.28.
‖ The relative response is based on pf of H-2k- and H-2d-specific CTL in the LCMV-infected C57BL/6 spleen. Data represent the average of H-2k-specific pf in five experiments and H-2d-specific pf in four experiments. Average H-2k-specific pf = 3,739 ± 1,339, p = 0.68 ± 0.17, and i = 1.02 ± 0.08. Average H-2d-specific pf = 3,407 ± 1,157, p = 0.64 ± 0.15, and i = 1.09 ± 0.15.
Table 3. Virus-specific and Allospecific Lysis by Vβ8+ LCMV-infected Spleen Cells

| Expt. | Targets         | Lysis by effector cells | Stained unsorted | Vβ 8+ | Vβ 8- |
|-------|-----------------|-------------------------|------------------|-------|-------|
| 1     | H-2b + LCMV     | 9.3*                    | 47.7             | 10.8  |
|       | H-2b            | 2.2                     | 6.3              | 2.8   |
| 2     | H-2b + LCMV     | 16.1                    | 141.0            | 12.0  |
|       | H-2b            | 8.0                     | 79.3             | 4.9   |

C57BL/6 mice were infected with LCMV for 8 d, stained and sorted for Vβ 8 expression, and tested for lysis against L929 (H-2k) and LCMV-infected MC57G (H-2b + LCMV) targets in a standard 51Cr-release assay. Effector cells in expt. 1 were enriched for T cells by panning (31). Whole spleen leukocytes were used in expt. 2.

* Lysis is represented in LU/10^6 cells.

The virus-induced alloreactivity may be due to the selective expansion of discrete clones of allospecific CTL.

Representation of Both Virus- and H-2b-specific CTL in the Vβ8+ Population of Virus-infected Spleen Cells. To determine the relationship between the virus- and allospecific CTL, we questioned whether a population of T cells bearing a given Vβ and enriched for virus-specific CTL activity would also be enriched for allospecific CTL activity. The Vβ 8 TCR was selected because it is expressed on a greater number of T cells in the C57BL/6 mouse (27) than other Vβ TCR for which there are antibodies. When LCMV-infected spleen cells were sorted using flow cytometry for expression of Vβ 8+ TCR, virus- and allospecific lysis were both highly represented in the Vβ 8+ population (Table 3), suggesting that phenotypically similar cells are mediating both functions. This experiment also formerly proves that at least some of the allospecific CTL response is mediated by conventional CTL expressing TCR-β. Substantial virus- and allospecific CTL activity was also found in the Vβ 8− populations.

Cross-reactivity of Short-Term LCMV-specific CTL Clones Generated in LDA. An explanation for the preferential selection of certain allospecific CTL specificities could be that the abundance of viral antigen present during acute virus infection stimulates the proliferation of any CTL with the capacity to bind or cross-react with the viral peptide-self-MHC class I complex. Some of these CTL might be cross-reactive with alloantigen. To determine if the selection of allospecific CTL was due to cross-reactivity with virus-infected cells, short-term (4–8 d in culture) virus-specific CTL clones were generated in LDA and tested in split-well analyses for cross-reactivity against LCMV-infected and uninfected syngeneic (H-2b) and uninfected H-2b or H-2d allogeneic targets. Fig. 5 shows the LDA of CD8+ splenic T cells taken from mice at days 5, 7, 8, and 9 after infection. The precursor frequency of virus-specific CD8+ T cells derived from C57BL/6 mice peaked at 7 d of LCMV infection, but was estimated to be only 1 in 61 CD8+ spleen cells (Fig. 5), despite the fact that most of the CD8+ cells at that time point have an activated phenotype (11). This demonstrates how only a very small subpopulation of this activated CD8+ T cell population grows out in these LDA. The precursor frequency was determined for each experiment, and all the culture wells estimated to contain <0.5 virus-specific CTL/well were exposed to 51Cr-labeled allogeneic targets to determine if any of the

![Figure 5](image_url)

Figure 5. LDA of LCMV-specific CTL precursors per CD8+ cell during LCMV infection of the C57BL/6 mouse. Four representative precursor analyses from different days after infection are shown. The effectors for days 7 and 8 after infection were CD8+ sorted cells. The precursor analyses for days 5 and 9 after infection used unsorted spleen cells, and the CD8+ CTL precursor frequency was estimated based on the percentage of CD8+ cells in the starting population. Intersection with the horizontal line represents the point at which 37% of the wells were negative and the precursor frequency was estimated. The p value and intercept for each regression plot were as follows: day 5, p = 0.99, i = 1.07; day 7, p = 0.93, i = 0.84; day 8, p = 0.99, i = 1.00; and day 9, p = 0.95, i = 1.1. The LCMV-infected target was KO and lysis of uninfected KO cells was below 3 SD of their spontaneous 51Cr-release.
virus-specific clones also lysed allogeneic targets. As can be seen in Table 4, experiments 1–5 generated high levels of virus-specific CTL cross-reactive with H-2^k or H-2^d (27/78 or 35%) and used a commercially available, supplemented RPMI medium. Experiments 6–11 used an alternative medium, AIM V, which generated similar virus-specific CTL precursor frequencies but with greater rates of cell proliferation in the 8-d culture period. Lower, but still significant levels of CTL cross-reactive with H-2^k were stimulated with this proprietary medium (11/114 or 10%) (Table 4). This lower level of cross-reactivity in the AIM V media suggests that the cross-reactive allospecific CTL may have different growth requirements than some of the virus-specific CTL.

The generation of the high levels of allospecific CTL clones required virus-infected PEC for their stimulation, suggesting that the clones must cross-react with virus-infected PEC. It could be argued, however, that a strong virus-specific T cell reaction might provide factors for a bystander stimulation of allospecific CTL without recognition of the virus-infected PEC by the allospecific CTL. Analyses indicated that the cross-reactive CTL were, indeed, clones and not mixtures of two CTL clones, each with a different specificity. There was a linear single-hit distribution of allospecific killing at limiting concentrations, indicating that the allospecific CTL were not dependent on a second (i.e., virus specific) T cell type for their generation. The precursor frequencies for the virus-specific CTL were not of a sufficient magnitude to contaminate all the wells of allospecific CTL precursors and to allow for linear allospecific kinetics in an environment of excess virus-specific “helper cells.” Statistical analysis (χ^2, goodness of fit) of the distribution of clones at <0.5 spleen cells/well showed that the cross-reactive clones were not merely due to the chance appearance of an alloreactive clone in the same well as a virus-specific clone, because many more wells showed lysis of both allogeneic and virus-infected targets than would have if the cross-reactivity were attributable to a random association of two clones (two-tail χ^2 p value <0.001; Fisher value <0.001%). In summary, these analyses of short-term, virus-specific CTL clones show that a significant amount of the cross-reactivity seen during LCMV infection can be attributed to virus-specific CTL that cross-react with allogeneic H-2^d or MHC antigens (38/192 or 20%) (Table 4) and that the outgrowth of these CTL is sensitive to indeterminant factors present in media.

The Generation of Allospecific CTL Clones not Lytic for Virus-infected Syngeneic Fibroblasts. The virus-specific, allospecific CTL clones generated in LDA did not account for all of the allospecific CTL clones. Split-well analyses also revealed allospecific clones that were stimulated to proliferate in vitro with virus-infected, syngeneic PECs, but were unable to lyse the infected, syngeneic fibroblast cell line, MC57G. Under the limiting conditions that generated 192 H-2^k plus virus-specific CTL clones (where the effector cell density was <0.5 virus-specific CTL/well), 35 clones were found to lyse only the allogeneic target, L929 (H-2^k) or P815 (H-2^d) or both, but not the virus-infected or uninfected syngeneic targets, MC57G or KO cells (Table 3). The generation of allospecific CTL occurred in the LDA cultures stimulated with virus-infected PEC but not with uninfected PEC (data not shown). The allospecific clones appeared in wells seeded with fewer than 250 spleen cells, which was a remarkably high frequency approaching that of virus-specific CTL. These results suggest that these allospecific CTL recognized (a) a viral antigen presented by LCMV-infected PEC, but not by virus-infected MC57G or KO cells; (b) virus-induced cellular antigens presented on the PEC; or (c) presented viral antigens with affinities sufficient for stimulation of proliferation and differentiation, but not sufficient for cytotoxicity against virus-infected targets.

### Table 4. Specificities of Short-Term, LCMV-induced CTL Clones (H-2^k)

| Clone specifics | Expt. 1–2 (RPMI) | Expt. 3–5 (RPMI) | Expt. 6–11 (AIM V) |
|----------------|-----------------|-----------------|-------------------|
| 1. LCMV + H-2^b | 36              | 42              | 114               |
| 2. H-2^k        | 24              | 19              | 25                |
| 3. H-2^d        | 5               | ND              | ND                |
| 4. LCMV + H-2^b and H-2^k | 15   | 9               | 11                |
| 5. LCMV + H-2^b and H-2^d | 3    | ND              | ND                |
| 6. H-2^k alone  | 7               | 10              | 14                |
| 7. H-2^k alone  | 2               | ND              | ND                |
| 8. H-2^k and d  | 2               | ND              | ND                |

Lines 1–3 represent the specificities of the total numbers of clones generated in expts. 1–11. Lines 4–8 represent the fine specificities of the clones listed in rows 1–3. The media used for each experiment is represented in parentheses.
reactivity or be lost. This did not occur in six clones that were maintained for 4 wk in culture. The first clone maintained specificity for L929 (H-2k), the second was cross-reactive for L929 (H-2k) and virus antigen, the third was specific for P815 (H-2b) and L929 (H-2k), and the remaining three were specific for virus-modified self (i.e., H-2k + LCMV) but not H-2b or H-2k (Fig. 6).

Discussion

These results show that infection with a nontransforming virus elicits a profound polyclonal CTL response in the absence of any detectable Vβ-binding superantigens, as significant alterations in the numbers of cells expressing TCR Vβ genes are not observed during infection. Although not all Vβ gene products were examined, the expansion of an untested Vβ subset should still lead to a reduction in other subsets, which did not occur. A corollary of this polyclonal CTL response was the generation of low affinity, allospecific CTL, independent of CD4 + T cells or the allotype of the class II antigens. The target of the allospecific CTL was shown previously to be class I MHC antigens (4), and the ability of various strains of mice to generate high levels of CTL to a given allotype mapped to the MHC locus outside of the class II region, and may in fact be a product of the T cell repertoire educated by host class I antigens. At least a portion of the allospecific CTL response generated during acute virus infection is due to a selective stimulation of many, though discrete, clones bearing cross-reactivity with syngeneic cells expressing viral antigen. Many of the allospecific CTL generated in vivo and detected directly in cytotoxicity assays probably have a low affinity for virus-modified self because unlabeled virus-infected targets did not interfere with the lysis of radiolabeled allogeneic targets in bulk cytotoxicity assays (3). Additionally, however, the allospecific CTL are themselves probably of low affinity, as their ability to lyse allogeneic targets is highly inhibitable by anti-CD8 antibody (Table 1). The large range of allogeneic haplotypes recognized (4) implies that the numbers of these allospecific CTL must be great. This may provide an explanation for why virus-specific precursor frequencies are only 1 in 61 CD8 + cells in the spleen (Fig. 5), yet 1 in 4 of the CD8 + cells in the day 7 LCMV-infected spleen have the large granular lymphocyte morphology characteristic of activated cytotoxic cells (12). The majority of the CTL present in the acutely infected spleen may not proliferate in our LDA because their affinity for virus is too low, yet the allospecific CTL probably proliferate in vivo, as we have shown previously that both the allospecific and virus-specific cells are found in the blast-sized cell fractions (4).

"Affinity," as it is used here, is a loosely defined term which in the case of the CTL probably includes not only the physical affinity of the TCR for MHC plus peptide (which has been shown to be a very weak interaction) (28), but also the interactions of accessory molecules and their receptors (CD2-LFA 3; CD8-MHC; CD11b-intercellular adhesion molecule (ICAM)-1 or C3bi; and LFA-1-ICAM-1 or 2), as has been suggested by Williams et al. (29). In contrast with the acute infection, the precursor frequencies of virus-specific CTL in the peritoneal cavity of mice 2 mo after infection correlate closely with the numbers of CD8 + CTL expressing the memory CD11b + phenotype in vivo (data not shown) and are quite high (>1 in 10), indicating that there is no inherent poor efficiency in generating precursors in the LDA and suggest that the LDA culture conditions may be favoring the proliferation of memory cells. The high frequency of cross-reactive clones generated in short-term LDA from acutely infected splenocytes contrasts with reports on the specificities of a number of LCMV-specific clones generated in long-term, bulk cultures. These highly selected clones show no cross-reactivity with H-2d or H-2k alloantigens (30).

It is surprising that we found many CTL clones whose proliferation depended upon virus-infected PEC, but which were able to lyse only allogeneic cells but not virus-infected fibroblast targets. These CTL may have been cross-reactive for self PEC antigens induced by virus infection or for viral antigens presented by the virus-infected PEC used to stimulate their growth, but not presented on the virus-infected fibroblasts used for assaying their cytotoxicity. This distinction will be addressed in future experiments. Recently there have been reports of virus infections inducing autoimmune T cells in the mouse (7-9) and the rat (10). Some of the allospecific CTL observed in our studies may be stimulated by a virus-induced, tissue-specific neo-antigen to which the host is not tolerized.

PV, MCMV, VV, and LCMV induce allospecific CTL, suggesting that the allospecific CTL induction is a normal part of the immune response to virus infection (4). We propose that these cross-reactive CTL may play an important role in viral clearance at early times after infection when the viral
burden is great, before high affinity CTL appear in large numbers. These cells may be directly cytotoxic to virus-infected targets in vivo or secrete cytokines amplifying other clearance mechanisms and possibly the proliferation of CD8+ cells. The fact that so many CD8+ cells respond to infection may provide an explanation as to why many virus-specific CTL responses do not require help from CD4+ T cells (25). It has been suggested (Hagerty, D. T., and E.M. Allen, manuscript submitted for publication) that high concentrations of antigen select a broad range of TCR affinities whereas low concentrations of antigen select high affinity T cells. LCMV grows to very high titers in the spleens of the mouse during the first few days of infection, and this would allow efficient antigen presentation to CTL with a broad range of affinities for virus plus MHC class I. Presumably, high affinity CTL would be selected as the virus is cleared, and antigen becomes rare.

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