Minimal Bystander Activation of CD8 T Cells during the Virus-induced Polyclonal T Cell Response

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Summary

Acute infections with viruses such as lymphocytic choriomeningitis virus (LCMV) are associated with a massive polyclonal T cell response, but the specificities of only a small percentage of these activated T cells are known. To determine if bystander stimulation of T cells not specific to the virus plays a role in this T cell response, we examined two different systems, HY-specific T cell receptor (TCR)-transgenic mice, which have a restricted TCR repertoire, and LCMV-carrier mice, which are tolerant to LCMV. LCMV infection of HY-transgenic C57BL/6 mice induced antiviral CTLs that lysed target cells coated with two of the three immunodominant epitopes previously defined for LCMV (glycoprotein 33 and nucleoprotein 397). Although LCMV-induced cytotoxic T lymphocytes (CTLs) from C57BL/6 mice could lyse uninfected H-2k and H-2d allogeneic targets, LCMV-induced CTLs from HY mice lysed only the H-2k-expressing cells. The HY mice generated both anti-H-2k and anti-H-2d CTL in mixed leukocyte reactions, providing evidence that the generation of allospecific CTLs during acute LCMV infection is antigen specific. During the LCMV infection there was blastogenesis of the CD8+ T cell population, but the HY-specific T cells (as determined by expression of the TCR-a chain) remained small in size. To examine the potential for bystander stimulation under conditions of a very strong CTL response, T cell chimeras were made between normal and HY mice. Even in the context of a normal virus-induced CTL response, no stimulation of HY-specific T cells was observed, and HY-specific cells were diluted in number by day 9 after infection. In LCMV-carrier mice in which donor and host T cells could be distinguished by Thy1 allotypic markers, adoptive transfer of LCMV-immune T cells into LCMV-carrier mice, whose T cells were tolerant to LCMV, resulted in activation and proliferation of donor CD8 cells, but little or no activation of host CD8 cells. These results support the hypothesis that the massive polyclonal CTL response to LCMV infection is virus-specific and that bystander activation of non–virus-specific T cells is not a significant component of this response.

During the course of a viral infection there are profound physiological changes that take place as the immunocompetent host mounts an immune response to the invading pathogen. These responses include activation of the cellular and humoral arms of the immune system. A useful model system for studying the activation and function of CD8+ T lymphocytes has been lymphocytic choriomeningitis virus (LCMV) infection. Upon infection of C57BL/6 mice with LCMV, there is a massive 5–20-fold expansion of the number of CD8+ lymphocytes in the spleen (1–3). The majority of these cells express activation markers such as CD44 (4), IL-2R (CD25; 5), and the adhesion molecule Mac-1 (6). By day 6 after infection many CD8+ cells are blast sized (7), and by day 8, 25% of the splenic T cells contain clearly defined azurophilic cytoplasmic granules containing perforin and serine esterases, indicative of a highly activated T cell population (8, 9).

This highly activated CD8+ T cell population not only contains CTLs capable of lysing virus-infected targets, but also contains T cells that will lyse various uninfected allogeneic targets (3). Many T cell clones isolated during the acute LCMV infection and propagated on virus-infected APCs lyse uninfected allogeneic targets, and some of these lyse the allogeneic target much better than the virus-infected syngeneic target (1). This suggests that the T cell response to viral infections consists of T cells with a wide array of affinities and specificities. Infection of LCMV-immune mice with heterologous viruses such as vaccinia virus (VV), Pichinde virus (PV), and murine cytomegalovirus (MCMV)
reactivated LCMV-specific memory CTL (3, 10), as shown by direct bulk CTL assays ex vivo. Surprisingly, clonal CTL assays showed that these heterologous virus infections could reactivate memory CTLs cross-reactive between the two viruses. This indicates that memory cell reactivation could also contribute to the virus-induced polyclonal T cell activation.

Using bulk CTL assays and limiting dilution analyses (LDAs), however, we have only been able to account for the specificities of between 5–10% of these activated CD8+ lymphocytes. LCMV encodes no known superantigen, and the explanation for this profound expansion in the numbers of CD8+ T cells remains unresolved. One mechanism could be that many of these T cells are not virus-specific, but are activated via bystander stimulation due to the large concentration of cytokines that are present during the acute phase of an LCMV infection. Another possibility is that these cells are activated through their TCRs, which react with LCMV-peptide–MHC complexes on the surface of virus-infected cells. Many of these T cells, however, would probably be of low affinity, and would not be detected in bulk or even in clonal cytotoxicity assays, but would be activated in vivo due to the plethora of antigens and cytokines present during acute infection. To determine whether viral antigen-independent bystander stimulation of non-cross-reactive T cells was occurring, we examined the CD8+ T cell response to LCMV infection in the HY-specific TCR-transgenic mouse (11) and in persistently infected normal mice whose T cells are tolerant to LCMV.

There are two useful attributes of the T cell compartment of the HY mouse. The first trait is that all T cells in the HY mouse express a single, transgenic Vβ8.2-containing TCR chain. As a result, these animals have a restricted T cell repertoire in which all immune responses must be orchestrated in the context of a single TCR–β chain. The second feature is that between one-third and one-half of the CD8+ T cells also express the transgenic Vα3 chain. Dual expression of these two transgenic TCRs imparted upon the T cell specificity for the male-specific HY antigen in the context of H-2Db. These HY-specific T cells can be distinguished from the rest of the CD8+ T cells by the monoclonal antibody T3.70, which detects a clonotypic determinant on the transgenic α chain (12). Using these particular characteristics of the HY transgenic mouse, we analyzed the kinetics and specificities of the antiviral T cell response and examined the fate of the non-LCMV-reactive HY-specific T cells during virus infection.

To look for the bystander activation of a more conventional nontransgenic T cell population containing both naïve and memory T cells, we made use of LCMV-carrier mice. This was a system in which we could distinguish memory and naive host cells from memory donor cells that were responding to the antigen in the LCMV-carrier mice. Using these congenic mice, we observed that despite a vigorous immune response carried out by the donor cells, the host CD8 T cells did not increase in size or number and did not change expression of activation markers. Together these systems indicate that bystander activation of non-

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**Materials and Methods**

Mice. Conventionally housed male and female C57BL/6 (H-2b: Thy1.2), B6.PL Thyl1/Cy (H-2b: Thy1.1), and LCMV-carrying C57BL/6 mice were either purchased from Jackson Labs, (Bar Harbor, ME) or bred in the Department of Animal Medicine at the University of Massachusetts Medical Center (Worcester, MA). C57BL/6 HY-transgenic mice, whose transgenic TCR is specific for the male antigen HY in the context of H-2Db, were provided by B.J. Fowlkes (National Institutes of Health, Bethesda, MD). HY-transgenic and (C57BL/6 × bm12)F1 mice were bred and maintained in the Department of Animal Medicine at the University of Massachusetts Medical Center under microisolator conditions. Transgenic mice were typed using anti-CD8-PE (GIBCO BRL, Gaithersburg, MD) and anti-Vβ8.1/8.2 FITC (PharMingen, San Diego, CA) to stain PBLs obtained via blood drawn from the retro-orbital sinus.

Cells and virus. MC57G (H-2b), a methylcholanthrene-induced fibroblast cell line from C57BL/6 mice, and vero cells were propagated in MEM (GIBCO BRL) supplemented with 100 U/ml of penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, 10 mM Hepes (United States Biochemical Corp., Cleveland, OH), and 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO). The TAP-2-deficient cell line RMA-S was from Hans-Gustaf Ljunggren (Karolinska Institute, Stockholm, Sweden) and was grown in RPMI-1640, supplemented as above. The LCMV Armstrong strain was propagated in baby hamster kidney BHK21 cells. Mice were injected intraperitoneally with 4 × 105 PFU of virus unless otherwise indicated. PV was propagated as described by Y ang et al. (3). Splenocytes from infected mice were homogenized and titrated for virus by plaque assay on vero cell monolayers.

Spleen cell preparation and cytotoxicity assays. Spleen cell suspensions were depleted of erythrocytes by briefly suspending the cell pellet in a 0.84% NH4Cl solution before rinsing. Cell-mediated cytotoxicity was determined using a standard microcytotoxicity assay (13). In brief, target cells were pelleted and resuspended in 100 μl of NaCl (Amerham Corp., Arlington Heights, IL) per 106 cells and incubated at 37°C in a humidified 5% CO2 incubator for 1 h. Varying numbers of effector cells were added in 0.1-ml volumes to achieve the desired E:T ratios. For a spontaneous 51Cr-release control, 0.1 ml of complete MEM was substituted for effector cells. Maximal release was determined by adding 0.1 ml of 1% Naodid P-40 to the well. After 6 h at 37°C, the plates were centrifuged at 200 g for 5 min, and 0.1 ml of supernatant was removed from each well and counted on a gamma counter (model 5000; Beckman Instruments, Inc., Palo Alto, CA). Data were presented as percent specific 51Cr-release = 100 × (experimental cpm – spontaneous cpm) / (maximum release cpm – spontaneous cpm)). Limiting dilution assays to determine the precursor frequencies for LCMV-specific CTL were performed using the procedure of Selin et al. (10), and frequencies were calculated using χ² analysis according to Tawsewell (14) on a computer program provided by R. Miller (University of Michigan, Ann Arbor, MI). For mixed lymphocyte cultures, varying numbers of splenocytes from female C57BL/6 mice or female HY-transgenic mice were co-cultured with 2 × 10⁶ female irradiated (3,000 R ad) allogeneic splenocytes in the presence of 5 Cetus units of recombinant human IL-2/ml. After 4 d the cultures were tested for
cytotoxic activity against syngeneic and allogeneic targets in a standard CTL assay.

Detection of Transgenic T Cells. Spleen cells were prepared as stated above, and 10^6 cells were incubated with normal mouse serum for 20 min at 4°C. The cells were then incubated with the biotin-labeled monoclonal antibody T3.70 (a gift from H. Teh, University of British Columbia, Vancouver, Canada) for 30 min on ice. The cells were then washed twice and blocked again with normal mouse serum at 4°C for 20 min. Next, the cells were stained with anti-CD8-PE (GIBCO BRL) and streptavidin-FITC (Becton Dickinson, San Jose, CA) for 25 min at 4°C. The cells were then washed twice and fixed with 2% paraformaldehyde. After staining, cells were analyzed or sorted by flow cytometry using a FACSTAR®. Data analysis was performed using the program PC-LYsis (Becton Dickinson).

A adoptive Transfer of Immune Spleen Cells into LCMV-carrier Mice. A total of 2–4 × 10^7 spleen cells from LCMV-immune B6.PL Thy1^a/Cy mice (Thy1.1^) were injected intravenously via the retro-orbital sinus into C57BL/6 LCMV-carrier mice (Thy1.2^). Spleens were harvested at various days after transfer and evaluated by flow cytometry.

Results

Antiviral Response in HY-transgenic Animals. To study the specificity and magnitude of the antiviral CD8^+ T cell response in the HY-transgenic mice, we first questioned whether the restrictions on the T cell repertoire would prevent a virus-specific T cell response from taking place. We therefore chose first to examine if the HY-transgenic animals were capable of clearing infectious virus from the spleen and how this compared to viral clearance in wild-type animals. As shown in Table 1, HY-transgenic and normal female C57BL/6 mice had similar viral titers 4 d after infection, but thereafter, HY-transgenic animals cleared LCMV with delayed kinetics when compared to normal mice. These observations are complementary to those of Ewing et al. (15) who showed that transgenic CBA/Ca mice expressing only a single randomly selected TCR^b chain locus of BALB/c mice resulted in LCMV infection than did wild-type mice (16) and those of Woodland et al., where it was observed that a large deletion in the TCR^b chain locus of BALB/c mice resulted in a reduction in the response to many common antigens (17).

The higher levels of background killing observed with lymphocytes from the transgenic mice against uninfected syngeneic M(57G (H-2^b)) targets were probably due to NK cells, which would likely undergo a period of prolonged activation in these animals due to the fact that the virus persisted in the spleen for a longer duration than in the wild-type C57BL/6 mice (Table 1). Consistent with this hypothesis, an anti-CD8 antibody that inhibited CTL-mediated killing failed to inhibit the lysis of uninfected M(57G cells, and in vivo administration of antibodies to the NK cell marker asialo GM1 reduced this killing (data not shown).

Presursor CTL Frequencies in LCMV-immune HY-transgenic Mice. To determine whether the relatively modest CTL response during acute infection of HY mice was also subsequently reflected in the memory response, we performed LDAs to determine the number of precursor CTLs (pCTLs) present in LCMV-immune mice. It has been shown that the number of LCMV-specific pCTL remains fairly constant for over a year after infection and that this memory

| Table 1. | Kinetics of Viral Clearance from Normal and Transgenic Mice |
| Days after infection | Log_{10} PFU/spleen |
| Days after infection | C57BL/6 mice | HY mice |
| 4 | 5.1 ± 0.3 | 5.4 ± 0.2 |
| 7 | <2.0† | 3.1 ± 0.2 |
| 8 | <2.0 | 2.9 ± 0.3 |
| 9 | <2.0 | <2.2‡ |

* n = 3/group.
‡ Only one of three animals had any detectable virus.

We next examined the magnitude of the in vivo antiviral CTL response mounted by the HY-transgenic mice. Using standard ex vivo CTL assays, it was observed that at 8 d after LCMV infection, when C57BL/6 animals displayed high levels of CTL activity, the HY animals displayed a more modest level of cytotoxicity (Fig. 1). Using lytic units (LU; calculated at 35% lysis) to evaluate the differences in the levels of CTL activity between these two populations, we found in four different experiments that wild-type animals had 5.5 ± 1.3 LU/10^6 spleen cells, whereas HY-transgenic mice had only 1.0 ± 0.4 LU/10^6 spleen cells. The LU calculated for the HY mice, however, also include the higher levels of lysis of uninfected syngeneic cells (see Fig. 1) and are probably an overestimation of the virus-specific CTL activity. These observations are consistent with the experiments performed by Doherty et al. that showed that transgenic CBA/Ca mice expressing only a single randomly selected TCR^b chain had lower CTL activity after LCMV infection than did wild-type mice (16) and those of Woodland et al., where it was observed that a large deletion in the TCR^b chain locus of BALB/c mice resulted in a reduction in the response to many common antigens (17).

Figure 1. Direct CTL activity mediated by spleen leukocytes 8 d after LCMV infection of C57BL/6 mice and HY-transgenic mice. Spleen cells were prepared as stated in Materials and Methods and tested against ^3HCr-labeled MC57G cells. Circles represent specific lysis by a single wild-type (open circle) and HY-transgenic mouse (closed circle) versus LCMV-infected targets. Triangles represent lysis by wild-type (open triangle) and HY-transgenic (closed triangle) mice against uninfected targets.
pCTL frequency per CD8 cell is within a factor of two of that observed at the height of the acute infection (18, 19). LDA's in one experiment showed that 240 d after LCMV infection, 1 in 158 CD8+ spleen leukocytes in a C57BL/6 mouse were LCMV-specific, whereas in a HY-transgenic mouse, only 1 in 1,598 CD8+ spleen leukocytes were LCMV-specific 58 d after LCMV infection. In a second experiment, the pCTL frequency in the C57BL/6 mouse was 1 in 85 at 411 d after infection and 1 in 580 in the HY-transgenic mouse at 118 d after LCMV. This 7–10-fold reduction in the number of pCTL is consistent with the less vigorous CTL response that takes place during acute infection.

Specificity of CTLs Generated during LCMV Infection of HY-transgenic Mice. To further characterize the CTL response in the HY-transgenic animals, we analyzed the specificity of the antiviral CTLs generated during acute infection. We first sought to determine which of the three well-defined LCMV immunodominant peptides, nucleoprotein (NP) 397 (20), glycoprotein (GP) 33 (21), or GP 278 (21), would sensitize target cells to lysis by CTLs generated in the HY-transgenic mouse during acute virus infection. As seen in Fig. 2, spleen cells and FACSFac-sorted CD8 cells from HY animals showed a tendency to lyse RMA-S cells coated with either of the immunodominant LCMV peptides GP 33 (KAVYNFATCGIFA) or NP 397 (QPQNGQFIHFY). Wild-type C57BL/6 mice also generated CTLs that lysed targets coated with these two peptides as well as cells coated with a third immunodominant peptide GP 278 (VENPGGYCFL), confirming studies by Whitton et al. (21). None of the HY-transgenic mice showed any lytic activity toward GP 278-coated targets, but it is possible that GP 278-restricted CTLs may have been generated at a frequency too low to detect in bulk assays. This pattern of lysis was consistent in four other experiments, but one animal out of six did not exhibit any NP 397-restricted CTL activity. These observations are in general agreement with Doherty et al. (16) who showed, upon LCMV infection of mice transgenic for a randomly selected TCR, that CTLs were generated with an epitope specificity that was similar to that of wild-type CBA/Ca mice.

The generation of CTLs capable of killing not only virus-infected targets, but also uninfected allogeneic targets, is a facet of acute LCMV infection (2). We thus performed CTL assays to determine if the restricted TCR repertoire in HY-transgenic mice precluded the generation of allospecific CTLs during acute infection. Fig. 3 shows the pattern of allogeneic target cell lysis exhibited by unsorted cells and by FACSFac-sorted CD8+ cells from HY animals and unsorted effector cells from a C57BL/6 mouse. While the C57BL/6 animal displayed lysis against both P815 (H-2d) and L929 (H-2d) targets, the HY-transgenic animal exhibited preferential lysis of targets expressing the H-2k allele. This pattern of CTL activity was consistent in six different experiments. In three of the six experiments, this H-2k killing in the HY mice was equal to or greater than the observed lytic activity seen against LCMV-infected syngeneic targets. The inability to generate an anti-H-2d response was not due to a specific lesion in the T cell repertoire because HY-transgenic mice generated comparable levels of both an anti-H-2d (58% specific lysis of L929 cells in a C57BL/6-H4 anti-C3H culture at 10^6 responders/well) as well as an anti-H-2k (54% specific lysis of P815 cells in a C57BL/6-H4 anti-BALB/c culture at 10^6 responders/well) CTL response in allogeneic mixed lymphocyte cultures in vitro. This selective stimulation of H-2d, but not H-2k-specific CTLs in the HY-transgenic mice during acute LCMV infection, is consistent with the concept that the generation
of allospecific CTL is antigen-driven and is not the consequence of bystander activation.

Thus, it appears that the HY-transgenic mouse clears LCMV more slowly than wild-type C57BL/6 mice (Table 1) and with diminished CTL diversity (Fig. 3) and less stimulation of HY-specific T cells does not occur.

Figure 4. HY-specific T cells fail to enter into blastogenesis during acute LCMV infection. A FACS profile of spleen cells from an HY-transgenic mouse stained with anti-CD8-PE, T3.70-biotin and streptavidin-FITC 8 d after LCMV infection is shown on the right. Relative cell size as measured by FSC is shown on the left. The R1 gate shows the HY-specific (T3.70+/CD8+) T cells and the R2 gate (T3.70+/CD8+) represents the remaining CD8 cells.

The absolute numbers of the HY-specific T cells present in the spleen were reduced in number by day 8. 18 uninfected HY mice had an average of 4.2 ± 1.1 × 10^6 HY-specific cells per spleen; this number dropped in three experiments representing six mice to 2.9 ± 0.3 × 10^6 HY-specific T cells by day 8 after infection. Concomitantly with this reduction in HY-specific cells was an increase in the remaining CD8+ cells from 6.1 ± 2.1 × 10^6 to 10.3 ± 2.4 × 10^6 at day 8 after LCMV. Thus, there appeared to be a selection for the responding HY-nonspecific CD8+ cells entering into blastogenesis, while those HY-specific cells remained small in size and appeared to be diluted out as the immune response progressed. These data argue in favor of the concept that the profound stimulation seen during LCMV infection is not a consequence of bystander activation of non-virus-specific naive T cells present at the site of a vigorous immune response.

Stimulation of HY-specific T cells in male mice. This lack of stimulation of HY-specific T cells seen during viral infection was not due to the fact that these HY-specific T cells were incapable of responding to antigen because 4 d after adoptive transfer of spleen cells from a female HY-transgenic mouse into a male C57BL/6 mouse, the CD8+/T3.70- cells entered into blastogenesis as indicated by an increase in mean FSC from 488 to 624. This is in agreement with Rocha and von Boehmer, who showed proliferation of HY-specific T cells after transfer into male mice (22).

HY-chimeric mice. The lack of bystander activation of HY-specific cells seen during LCMV infection could have been due to the requirement for TCR engagement needed for T cells to proliferate, or it could have been due to the fact that the antiviral immune response in the HY animals was not as vigorous as that seen in wild-type mice, and therefore was below the threshold required for inducing bystander stimulation. To compensate for this, we created an environment in which the HY-specific T cells would be present in a milieu where a robust immune response mimicking that seen in the wild-type animal would take place. This was done by creation of a HY normal mouse chimera. HY-chimeric mice were HY-transgenic mice adoptively reconstituted with 5 × 10^7 spleen cells from normal C57BL/6 female mice. This led to a T cell population that contained the HY-specific T cells and the adoptively transferred T cells that expressed a wide variety of TCRs. These adoptively transferred cells could respond to viral infection with the intensity nearly equal to that of wild-type mice, thus mimicking a normal host response to viral infection.
As can be seen in Fig. 5, in the environment of the HY-chimeric mice, the HY-specific T cells (R1 gate) failed to enter into blastogenesis 7 d after LCMV infection, even though many of the remaining CD8+ T cells increased in both size and number. The HY-specific cells also remained small in size at day 3, 5, 9, and 11 after infection (data not shown). Here again, the HY-specific T cells expressed higher levels of CD8 than did the other CD8+ cells, providing further evidence for their lack of participation in this antiviral response. We next examined the spleens of HY-chimeric mice at day 3, 5, 7, 9, 11, and 31 and determined the absolute number of HY-specific transgenic CD8 T cells (CD8+/T3.70+) and the remaining (CD8+/T3.70-) CD8 T cells (Fig. 6). As expected, little expansion in the total CD8 T cell number was seen until between day 7 and 9, at which time there was nearly a threefold expansion in the number of non-HY-specific CD8+ T cells (closed bars). Concomitant with this increase in CD8+ T cells was a reduction in the number of HY-specific CD8 T cells (open bars). This suggests that a nonresponding cell population not entering into blastogenesis is diluted in the spleen by those cells responding to viral antigen. This dilution, however, is not permanent. By day 31 after LCMV infection and beyond, well after infectious virus was cleared, the HY-specific cells once again were detected in the spleen of HY-chimeras. It is not clear whether these HY-specific T cells found after virus infection represent those cells that were forced out of the spleen during acute LCMV-infection or whether they were recent thymic emigrants.

We next attempted to look for bystander activation of memory cells by exposing spleen cells from HY-transgenic mice to male antigen in vivo and determining their fate during acute LCMV infection. This was accomplished using a method similar to Bruno et al. (23) by adoptive transfer of 4 × 10^7 spleen cells from female HY-transgenic mice into irradiated (C57BL/6 × bm12)F1 male mice. The (C57BL/6 × bm12)F1 mice provide both the HY antigen as well as allogeneic MHC class II molecules that can be recognized by CD4+ T cells that, in turn, provide T cell help that promotes the survival of HY-specific memory cells. After 10 d 3 × 10^7 (experiment 1) or 4 × 10^7 (experiment 2) spleen cells from the reconstituted (C57BL/6 × bm12)F1 male mice were then adoptively transferred into irradiated (600 Rad) female α/β-TCR knockout C57BL/6 mice. At the time of transfer into the female mice, these antigen-experienced, HY-specific T cells were larger than naive cells, with FSC values of 520 to 551 (versus 417–425 for naïve HY-specific CD8+ cells) and comprised 90% (experiment 1) to 82% (experiment 2) of all the CD8+ T cells in the spleen. 3–4 wk later, some of these recipient mice were challenged with LCMV, and some recipients were rendered chimeric with normal C57BL/6 splenocytes as in Figs. 5 and 6 and then challenged with LCMV. The absolute number of antigen-experienced HY-specific cells and the remaining CD8+ T cells per spleen were monitored, and the CTL activity was examined 8 d after infection. Very low levels of CTL activity were detected in nonchimeric animals (<1% specific lysis, n = 4), whereas at an E/T ratio of 100:1, specific lysis ranged from 9 to 41% in chimeric recipient mice (n = 6).

In experiment 1, the number of antigen-experienced HY-transgenic splenocytes in uninfected recipient (n = 1), LCMV-infected recipient (n = 2), and LCMV-infected chimeric recipient (n = 4) mice averaged 6, 7.2, and <2.5 × 10^5, respectively, with two of four chimeric recipient mice having no detectable HY-transgenic cells. The remaining
CD8⁺ cell number averaged 8, 11, and 34 × 10⁵. In experiment 2, the antigen-experienced HY splenocyte numbers averaged 24 (n = 1), 14 (n = 2), and 8 × 10⁵ (n = 2) cells, respectively, whereas the remaining CD8⁺ splenocytes numbers were 36, 25, and 140 × 10⁵. This indicates that as the nontransgenic CD8⁺ T cells responded to the LCMV infection and increased in number, the antigen-experienced HY cells decreased in number much like they did in the HY-chimeric mice in Fig. 6. These results indicate that there was no significant bystander activation of these antigen-experienced cells.

Lack of Bystander Activation in LCMV-carrier Mice. Another system was used to look for bystander activation of T cells during an LCMV-specific immune response. This system involved the adoptive transfer of spleen cells from an LCMV-immune mouse into LCMV-carrier mice. LCMV-carrier mice are persistently infected with LCMV due to the fact that they have been infected in utero and have clonally deleted LCMV-reactive T cells (24). King et al. demonstrated that transfer of T cells from LCMV-immune mice into persistently infected mice resulted in clearance of infectious virus from the thymus by day 8 after transfer (24). Using Thy1 congenic mice, it was possible to distinguish the immune donor T cells (CD8⁺/Thy1.2⁺) from the host T cells (CD8⁺/Thy1.2⁻), and to observe if the activation and proliferation of the immune donor cells resulted in bystander activation of host T cells. This differs from the HY-transgenic experiments in that the LCMV carriers are housed conventionally and have both naive as well as memory T cells as candidates for bystander activation.

6 d after adoptive transfer of Thy1.2⁺ spleen cells from a control mouse into Thy1.2⁺ LCMV-carrier mice, few, if any, donor CD8⁺/Thy1.2⁺ cells were found in the spleen (Fig. 7). However, 6 d after adoptive transfer of spleen cells from a Thy1.1⁺ LCMV-immune mouse into a Thy1.2⁺ LCMV-carrier mouse (Fig. 8), both donor Thy1.2⁺ and host Thy1.2⁺ cells were observed in the spleen. Comparing the relative cell size of these two populations, the host Thy1.2⁺ (R1 gate) cells appeared equal in size relative to cells in the mouse that received naive Thy1.1⁺ cells, whereas the Thy1.2⁺ donor cells derived from LCMV-immune mice (R2 gate) were of large size and probably proliferating.

Table 2 provides a summary of three adoptive transfer experiments. After transfer of LCMV-immune spleen cells into the carrier mice, the donor CD8⁺ cells had greater forward scatter than did the host cells (P < 0.005). The number of host CD8⁺ cells present in the spleen dropped concomitantly with an increase in the numbers of CD8⁺ donor cells (Table 2). Thus, there was no demonstrable bystander proliferation of host cells in this context of vigorous immune response.

The cell surface phenotype of the donor and host cells was examined to look for any changes in activation molecules that may have occurred in either the donor or host cell populations. Fig. 9 shows the expression of MEL-14 (L-selectin) and CD44 on the donor and on the host CD8⁺ cells 6 d after adoptive transfer of either naive Thy1.2⁺ (naïve transfer) or LCMV-immune Thy1.2⁺ spleen cells (immune transfer). (Fig. 9, left) MEL-14 expression on host cells remained at the same high levels regardless of whether naive or immune spleen cells were transferred, whereas the donor cell population consisted of mostly MEL-14low cells. This demonstrates that the donor CD8⁺ cells are activated since MEL-14 expression is decreased on LCMV-induced activated CD8⁺ cells (5). (Fig. 9, right) The expression of CD44 on the donor and host cells is shown. Donor cells were predominantly CD44high, whereas host cells showed little change in their moderate cell surface expression of CD44. Table 2 summarizes the expression of MEL-14 and CD44 in three adoptive transfer experiments. It can be seen that the mean fluorescent intensity for MEL-14 and CD44 on host CD8⁺ cells changed little after adoptive transfer of LCMV naive or immune spleen cells. However, the donor CD8⁺ cells expressed less MEL-14 (P < 0.009) and higher levels of CD44 (P < 0.13), consistent with them being activated cells. All data shown are from day 6 after transfer.
Table 2. Effects of Immune Cell Transfer in LCMV-carrier Mice

<table>
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<th>Treatment*</th>
<th>n</th>
<th>CD8 Cell No. $\times 10^6$</th>
<th>Mean FSC</th>
<th>CD44 MFI$^a$</th>
<th>MEL-14 MFI$^a$</th>
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<tr>
<td>Host (naive transfer)</td>
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<td>15</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Host (immune transfer)</td>
<td>3</td>
<td>12 $\pm$ 3</td>
<td>642</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Donor (immune transfer)</td>
<td>3</td>
<td>5.3 $\pm$ 2</td>
<td>717</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Host (naive transfer)</td>
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<td>10 $\pm$ 1</td>
<td>578</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Host (immune transfer)</td>
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<td>4.5 $\pm$ 1</td>
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<tr>
<td>Host + poly I:C (naive transfer)</td>
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<td>10 $\pm$ 1</td>
<td>578</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>4.0 $\pm$ 0.6</td>
<td>565</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Donor + poly I:C (immune transfer)</td>
<td>3</td>
<td>4.0 $\pm$ 0.6</td>
<td>720</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Experiment No. 1 (2 $\times 10^7$ cells transferred)

| Host (naive transfer) | 2  | 7.8 $\pm$ 1                 | 498      | 533 $\pm$ 18 | 479 $\pm$ 7     |
| Host (immune transfer) | 4  | 4.9 $\pm$ 0.7               | 494      | 52 $\pm$ 11   | 505 $\pm$ 30    |
| Donor (immune transfer) | 4  | 3.7 $\pm$ 0.8               | 630      | 96 $\pm$ 15   | 385 $\pm$ 21    |
| Host + poly I:C (naive transfer) | 2  | 7.0 $\pm$ 2                | 496      | 505 $\pm$ 16  | 485 $\pm$ 6     |
| Host + poly I:C (immune transfer) | 3  | 3.8 $\pm$ 0.6              | 471      | 447 $\pm$ 22  | 457 $\pm$ 19    |
| Donor + poly I:C (immune transfer) | 3  | 3.8 $\pm$ 0.7              | 627      | 666 $\pm$ 22  | 350 $\pm$ 27    |

Experiment No. 2 (4 $\times 10^7$ cells transferred)$^b$

Experiment No. 3 (3 $\times 10^7$ cells transferred)$^c$

| Host (naive transfer) | 2  | 12 $\pm$ 2                 | 563      | 760 $\pm$ 24  | 449 $\pm$ 19    |
| Host (immune transfer) | 3  | 6.1 $\pm$ 1                | 578      | 723 $\pm$ 85  | 433 $\pm$ 12    |
| Donor (immune transfer) | 3  | 4.5 $\pm$ 1                | 726      | 846 $\pm$ 54$^d$ | 318 $\pm$ 5     |
| Host + poly I:C (naive transfer) | 2  | 10 $\pm$ 1                | 578      | 749 $\pm$ 2   | 435 $\pm$ 7     |
| Host + poly I:C (immune transfer) | 3  | 4.0 $\pm$ 0.6              | 565      | 783 $\pm$ 74  | 423 $\pm$ 12    |
| Donor + poly I:C (immune transfer) | 3  | 4.0 $\pm$ 0.6              | 720      | 885 $\pm$ 6   | 316 $\pm$ 7     |

| Host (naive transfer) | 2  | 7.8 $\pm$ 1                 | 498      | 533 $\pm$ 18 | 479 $\pm$ 7     |
| Host (immune transfer) | 4  | 4.9 $\pm$ 0.7               | 494      | 512 $\pm$ 11  | 505 $\pm$ 30    |
| Donor (immune transfer) | 4  | 3.7 $\pm$ 0.8               | 630      | 696 $\pm$ 15  | 385 $\pm$ 21    |
| Host + poly I:C (naive transfer) | 2  | 7.0 $\pm$ 2                | 496      | 505 $\pm$ 16  | 485 $\pm$ 6     |
| Host + poly I:C (immune transfer) | 3  | 3.8 $\pm$ 0.6              | 471      | 447 $\pm$ 22  | 457 $\pm$ 19    |
| Donor + poly I:C (immune transfer) | 3  | 3.8 $\pm$ 0.7              | 627      | 666 $\pm$ 22  | 350 $\pm$ 27    |

*LCMV-carrier mice received the indicated number of either LCMV-immune (immune transfer) or naive spleen cells (naive transfer) as described in Materials and Methods. They were then either left untreated or treated with 100 $\mu$g poly I:C intraperitoneally after transfer. 6 d after adoptive transfer, the spleens were harvested and stained as stated in the text.

$^a$ MFI, mean fluorescent intensity.

$^b$ The differences between the mean FCS and MEL-14 MFI of host (naive transfer) and donor cells are all significant at $P < 0.005$ for FSC and $P < 0.009$ for MEL-14, as determined by Student’s t test.

$^c$ Different ($P < 0.013$) from host (naive transfer).

$^d$ Different from both host (naive transfer) ($P < 0.02$) and host + poly I:C (naive transfer) ($P < 0.05$).

Adoptive transfer, which was the peak of the donor cell response. LCMV-carrier mice were also examined on days 5, 7, and 8 after adoptive transfer. At none of these days was there any indication of an increase in number, size, or change in cell surface markers (MEL-14 and CD44) in the host CD8 cells. It should be pointed out that there was no increase in the frequency of the population of host cells that express the memory antigenic phenotype (CD44$^+$), nor was there any increase in their cell size as measured by FSC.

It has recently been suggested that IFN may be a nonspecific polyclonal stimulator of memory CD8 T cells (25). One distinction between the immune response engendered by adoptive transfer of T cells into the LCMV-carrier mice and that occurring during an acute LCMV infection is that high levels of IFN-α/β are generated during the acute infection. To mimic this induction, the carriers were given the IFN-α/β inducer polyinosinic:cytidylic acid (poly I:C), which we have previously shown induces IFN and NK cell proliferation in normal and LCMV-carrier mice (26). The poly I:C treatment with or without immune cell transfer did not increase the number or size of host cells, nor did it reduce host MEL-14 expression or raise host CD44 expression, which are associated with CD8 cell activation. The only noticeable effect of poly I:C treatment was that it slightly reduced the total number of spleen cells from 1.0 $\times 10^8$ in untreated mice to 0.7 $\times 10^7$ in animals that received poly I:C and LCMV-immune spleen cells. Poly I:C treatment reduced the absolute number of host CD8$^+$ cells, while not dramatically affecting the number of donor CD8$^+$ cells, and in one experiment, slightly decreased, rather than increased, CD44 expression on host CD8 cells. The reduction in the number of host CD8 cells indicated that this regimen of poly I:C was functioning in vivo, and we confirmed in parallel experiments that the carrier mice synthesized IFN in response to the poly I:C treatment.

To show that LCMV-carrier host spleen cells could indeed undergo blastogenesis, these mice were infected with $5 \times 10^8$ PFU of PV and examined for T cell activation 8 d later. In two separate experiments involving five mice, the
mean FSC of the CD8 cells increased an average of 58 ± 5, whereas the mean fluorescent intensity of MEL-14 dropped by 45 ± 12 and the mean fluorescent intensity of CD44 increased by 22 ± 13. This indicates that the CD8 cells in the LCMV-carrier mice become activated in response to infectious agents to which they are not tolerant.

Discussion

In this study we have provided evidence that the massive expansion in CD8 T cells during an acute LCMV infection can not be accounted for simply by bystander activation and therefore must involve TCR-specific recognition. Many viruses, such as VV, MCMV, PV (3), and EBV (27) can generate CTLs capable of lysing MHC-mismatched cells upon infection, arguing in favor of a general, nonspecific induction of allospecific T cells during viral infection (3). Yet, CTL clones cross-reactive between uninfected allogeneic targets and virus-infected syngeneic targets have been observed in several systems (3) and the allospecific induction of allospecific T cells during viral infection (3). Yet, CTL clones cross-reactive between uninfected allogeneic targets and virus-infected syngeneic targets have been observed in several systems (3) and the allospecific induction of allospecific T cells during viral infection (3).

Those results did not, however, rule out the possibility that a component of the allospecific CTL stimulation, as well as the stimulation of the majority of the T cells whose specificity was unknown, could be due to a nonspecific bystander activation. The results provided here indicate that bystander stimulation does not account for this expansion of T cell number. This conclusion is based on the observations that only one of two potential allospecific CTL specificities are induced by LCMV infection in a milieu with a restricted CTL repertoire (Fig. 3), that the activation and proliferation of transgenic T cells not specific for LCMV are not induced in the wake of a vigorous T cell response (Fig. 6), and that the induction of an active T cell response in an immunologically tolerant host fails to activate and expand the host T cells (Figs. 8 and 9).

We chose to examine the HY-specific T cell as one particular T cell with a clonotypic TCR not cross-reactive with LCMV to observe its fate during the course of viral infection. Our results show that such HY-specific T cells failed to enter into blastogenesis during acute infection of HY-transgenic mice or even in the HY-chimeric animals, which mounted more vigorous responses to LCMV than did the HY-transgenic animals. In fact, the nonresponding HY-transgenic cells were diluted in number during the course of an LCMV infection, even though much proliferation was found in the nontransgenic T cells. The fact that the HY-specific T cells do return to normal numbers after viral clearance may indicate that as T cells are removed from the spleen by apoptosis during the terminal phase of the acute response (28), naive cells repopulate the spleen so that they can respond to future infectious agents. Using the LCMV-carrier mice we showed that, while the adoptively transferred immune cells expressed an activation and blastogenesis phenotype, the host cells, much like the HY-transgenic cells, remained small in size and nonactivated (Figs. 8 and 9). In the LCMV-carrier system, the nonresponding host cells represent a diverse T cell population containing memory cells and T cells with a wider diversity of TCR specificities than do the HY-transgenic T cells.
An explanation for this lack of proliferation among the HY-specific cells and the LCMV-carrier T cells may be that these cells are not stimulated via the TCR and therefore do not receive the proper intracellular signals that are required for a productive T cell response, regardless of the diversity and concentration of cytokines present. If this assumption is correct, then it is likely that those T cells that become activated during acute LCMV infection are indeed LCMV-specific at some level and have TCRs that have some affinity for LCMV-peptide–MHC complexes. The observation that upon LCMV infection of HY-transgenic mice, CTL lysed H-2k but not H-2d allogeneic targets even though these mice have pCTL to H-2b, indicates that this is not an indiscriminate bystander activation response. Here the restrictions of the limited TCR repertoire apparently precluded the generation of CTLs cross-reactive between H-2d and virus-modified H-2b.

It has recently been suggested that IFN induced during virus infections may contribute to a bystander stimulation of memory CD8 cells (25). Memory cells have a wider diversity of cytokine receptors and adhesion molecules on their surfaces than do naïve cells, and their activation is much less dependent upon costimulatory molecules (29). Experiments have, in fact, demonstrated that infections of LCMV-immune mice with serologically unrelated viruses, such as VV, PV, or MCMV can indeed reactivate LCMV-specific memory cells easily detectable in bulk cultures (3, 10). However, analyses of CTL clones isolated from LCMV-immune mice infected with PV or VV have revealed clones with dual specificity, cross-reactive between the two viruses. This argues that even the activation and expansion of putatively unrelated memory CTLs may involve some level of cross-reactivity and may not simply be by bystander activation. Our studies with the LCMV-carrier mice, which were kept in a conventional environment and should have memory cells specific for environmental antigens, support the concept that any bystander activation of memory cells contributes little to the T cell expansion seen during acute LCMV infection. The injection of poly I:C to induce higher levels of IFN in the recipient carrier mice also did not increase the size or number of the host cells. It should be noted that during the acute infection, the peak in T cell proliferation parallels the levels of IL-2 and comes several days after the peak in type I IFN (30). Taken together, the data presented here support the notion that bystander activation of T cells not specific for the virus contributes little to the polyclonal T cell response to LCMV infection, indicating that antigen recognition at the level of the TCR must be the decisive event.

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