T CELL MEMORY

Long-term Persistence of Virus-specific Cytotoxic T Cells

By BETH D. JAMIESON and RAFI AHMED

From the Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024

Virus-specific CTL are an important factor in the control and elimination of viral infections (1–10). These cells recognize viral antigen in association with H-2 class I, or in some instances, class II molecules (1, 11, 12). In recent years, there has been considerable progress in documenting the biological significance of CTL and in defining and mapping the antigenic epitopes recognized by such cells (1–16). However, relatively little is known about the life span of virus-specific CTL.

We and others (10, 17, 18) have shown that a chronic lymphocytic choriomeningitis virus (LCMV)[1] infection can be eliminated by adoptive transfer of $CD8^+$ T cells from LCMV-immune mice. However, it is not known for how long the transferred donor CTL retain biological activity and persist in the cured carrier host. To address this question, we have used congenic mice (Thy-1.1 and Thy-1.2) in the adoptive transfer experiments, making it possible to distinguish between activity due to immune donor T cells (Thy-1.1) and carrier host T cells (Thy-1.2). In this study we show that the transferred CTL persist indefinitely in the cured carrier mice. These donor $CD8^+$ T cells can be recovered as late as 9 mo post-transfer and still exhibit biological activity against LCMV both in vitro and in vivo. They kill LCMV infected cells in vitro, are capable of massive proliferation in vivo, and can control an LCMV infection upon transfer into a second infected recipient. These results suggest that virus-specific CTL have the potential to persist for the life span of the host as memory T cells.

Materials and Methods

Mice. C57BL/6 (H-2^b; Thy-1.2) and B6.PL-Thy-1^a/cy (H-2^b; Thy-1.1) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. These two strains of mice will be referred to as B6.Thy-1.2 and B6.Thy-1.1, respectively. Both neonatally and congenitally infected B6.Thy-1.2 mice were used. Neonatally infected carriers were made by injecting 10^4 plaque-forming units (PFU) of LCMV intracerebrally into mice within 24 h of birth. The congenital carrier colony was derived from these neonatally infected mice and bred at UCLA, Los Angeles, CA. LCMV-immune mice were made by injecting 6–12-wk-old B6.Thy-1.1 mice

This work was supported by Public Health Service grant NS-21496 from the National Institutes of Health and by an award (R86LA018) from the University of California Systemwide Task Force on AIDS to R. Ahmed. R. Ahmed is a Harry Weaver Neuroscience Scholar of the National Multiple Sclerosis Society. Address correspondence to R. Ahmed, Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024.

[1] *Abbreviations used in this paper:* CTLp, CTL precursors; LCMV, lymphocytic choriomeningitis virus; LN, lymph nodes; PE, phycoerythrin; PFU, plaque-forming units.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/89/06/1993/13 $2.00 1993
Volume 169 June 1989 1993–2005

intraperitoneally with 10^5 PFU of LCMV. Spleen and lymph nodes (LN) were taken 30–90 d post-infection and used as LCMV-specific immune cells.

Virus. The Armstrong CA 1371 strain of LCMV (Arm-7) and a variant derived from this virus (Arm-13) were used in this study (6). Arm-7 was used for injecting the adult B6.Thy-1.1 mice to make immune mice, whereas Arm-13 was used to establish the B6.Thy-1.2 LCMV carrier colony. The An3739 strain of Pichinde virus was used in ^{51}Cr-release assays as a control for virus specificity of the effector cytotoxic T cells.

Antisera. The following mAbs were used in the study. The mAbs anti-Thy-1.2 (HO-13-4) and anti-Lyt-2.2 (Ad4[15]) were purchased in the form of ascites fluid from Cedarlane, Hornby, Ontario, Canada. Anti-Thy-1.1 (Tn-26a) was obtained from Miles Laboratories, Inc., Naperville, IL, as an ascites. All reagents were used at concentrations specified by suppliers. The rat anti-mouse hybridoma (GK1.5) that reacts with the CD4 antigen was obtained from the American Type Culture Collection, Rockville, MD, and grown in our laboratory (19). The GK1.5 antibody was partially purified from culture supernatants by precipitation with a 50% saturated solution of ammonium sulfate. The antibody concentration was determined by an immunodiffusion test with rat IgG standards. The rat anti-mouse mAb RL172.4 was kindly provided by Michael Bevan, Scripps Clinic and Research Foundation, La Jolla, CA. This antibody (IgM) also reacts with the CD4 surface molecule and is effective in in vitro complement-mediated depletion of $CD4^+$ cells (20). To deplete Thy-1.1^+ cells in vivo, the mAb 19E1.2 was used. Hybridomas producing this antibody were obtained from Michael Bevan, and grown in our laboratory at UCLA. To confirm the specificity of mAb 19E1.2, B6.Thy-1.1 and B6.Thy-1.2 mice were injected with 0.5 ml of supernatant from 19E1.2 hybridoma cells every other day, for a total of five injections. This treatment had no effect in B6.Thy-1.2 mice but depleted >90% of T cells from B6.Thy-1.1 mice. The specificities of mAbs AD4-15 (anti-Lyt-2), Tn-26a (anti-Thy-1.1), and HO-13-4 (anti-Thy-1.2) were confirmed by a functional assay. Spleen and LN cells of mice infected with LCMV 8 d previously were treated with either complement (C') alone or with mAb plus C'. The lymphocytes were then tested in a cytotoxicity assay. Treatment of cells with anti-Lyt-2 completely abrogated cytotoxic activity of lymphocytes from both B6.Thy-1.1 and B6.Thy-1.2 mice. Anti-Thy-1.1 treatment eliminated CTL activity of lymphocytes derived from Thy-1.1, but not Thy-1.2, mice. Anti-Thy-1.2 depleted specific activity only when effector cells were from B6.Thy-1.2 mice.

Flow Cytometry. Donor and host $CD4^+$ and $CD8^+$ T cells present in the spleens and LNs of cured carrier mice were quantitated by flow cytometry. Both the FITC-conjugated anti-mouse Thy-1.1 (Tn-26) and FITC-conjugated anti-mouse Thy-1.2 were obtained from ICN ImmunoBiologicals, Lisle, IL. Phycoerythrin (PE)-conjugated anti-mouse CD4 mAb (clone GK1.5), biotin-conjugated anti-mouse CD8 mAb (clone 53-6), and strepavidin-PE were purchased from Becton Dickinson & Co., Mountain View, CA.

Cells (10^6 per group) were costained simultaneously with one of the FITC-conjugated anti-Thy-1 mAbs and either PE-conjugated anti-CD4 or biotin-conjugated anti-mouse CD8. The cells were washed and strepavidin-PE was added to the groups stained for the CD8 antigen. After a final wash, all cells were fixed in 1% buffered formalin phosphate and 10^4 cells were analyzed on either an Epics V (Coulter Electronics Inc., Hialeah, FL) coupled to an Innova 90 argon laser (Coherent Inc., Palo Alto, CA), or in a FACS II (Becton Dickinson & Co.). The gates were set to include lymphocytes and blast cells.

Virus Titrations. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (6).

Immune Therapy of Persistently Infected Mice. Adult B6.Thy-1.1 immune mice (infected 30–90 d earlier with LCMV) were used as donors of spleen and LN cells. After death, their spleen and LNs were harvested and single cell suspensions were prepared in MEM supplemented with antibiotics and L-glutamine. Erythrocytes were removed by one cycle of treatment with 0.83% NH_4Cl solution. 2×10^7 spleen and LN cells were transferred intraperitoneally or intravenously into 6–12 wk-old B6.Thy-1.2 LCMV carrier mice. These treated carrier mice were then monitored for viral clearance. Various tissues were tested for the presence of infectious virus by plaque assay and for viral antigen by immunofluorescence (18).

Detection of LCMV-specific CTL in Carrier Mice (Thy-1.2) Receiving Adoptive Transfer of Immune Thy-1.1 Cells. At various times after immune therapy the treated carrier mice were killed

and their spleen and LN cells tested for the presence of LCMV-specific CTL after stimulation with virus for 8 d in vivo (in irradiated mice). About 2–5 × 10^7 pooled spleen and LN cells from treated carrier mice (Thy-1.1 → Thy-1.2) were injected intravenously into normal mice irradiated (650 rad) 1 or 2 d previously. Unless otherwise stated, these irradiated recipient mice were B6-Thy-1.2. At the time of cell transfer, irradiated recipients were challenged intravenously with 10^5 PFU of LCMV Arm 7. 8 d later, the recipient mice were killed and the spleen and axillary, inguinal, and cervical LNs were harvested and assayed for CTL activity. The phenotype (Thy-1.1 or Thy-1.2) of the CTL was determined by complement-mediated depletion using Thy-1.1- and Thy-1.2-specific mAbs as described previously (21).

T Cell Depletion. To deplete donor T cells, spleen and LN cells from cured carrier mice were treated in vitro with anti-Thy-1.1 (Tn-26) plus C′ and transferred intravenously into irradiated recipients for in vivo stimulation. The irradiated recipients were injected intraperitoneally with 0.5 ml of 19E1.2 at the time of cell transfer and again on day +2 and +3 to further deplete donor T cells in vivo. The depletion of host T cells was accomplished in vitro with two rounds of anti-Thy-1.2 (HO-13-4) plus C′. $CD4^+$ T cells were depleted in vitro with two rounds of RL172.4 plus C′ and the remaining spleen and LN cells were transferred intravenously into irradiated mice. $CD4^+$ cells were then further depleted by injecting the irradiated recipients with 0.5 ml of GK1.5 at the time of cell transfer and again on day 3.

LCMV-specific CTL Assay. Single-cell suspensions of spleen and lymph nodes, free of erythrocytes, were prepared in complete RPMI medium supplemented with 10% heat-inactivated FCS, antibiotics, and L-glutamine. The cells were then tested for cytotoxicity on uninfected, LCMV-infected, or Pichinde virus–infected MC57 (H-2^b) or BALB CL-7 (H-2^d) fibroblasts. MC57 cells were infected 48 h before use with the indicated virus at a multiplicity of infection of 0.5 PFU/cell. BALB CL-7 cells were infected at a multiplicity of infection of 0.5 PFU with the indicated virus 24 h before use. The target cells were labeled with ^{51}Cr and the cytotoxicity assay was performed as previously described (6). Test duration was 6 h and samples were run in triplicate. The percent specific ^{51}Cr release was calculated as follows: 100× [(Sample release − spontaneous release [cpm])/(maximum − spontaneous release [cpm])].

Results

Long-term Persistence of Adoptively Transferred LCMV-specific CTL. The lifelong chronic LCMV infection of congenitally infected carrier mice can be eliminated by adoptive transfer of $CD8^+$, MHC class I–restricted, virus-specific T cells derived from adult immune mice (10, 17, 18, 22). To determine the life span of the donor T cell population in the cured carrier host, we examined the time course during which donor-derived T cells and donor cytotoxic T cell activity could be recovered. To allow us to identify the donor T cells and to distinguish between their activity and the activity of the host T cells, we used mice congenic at the Thy-1 locus in adoptive transfer experiments. Immune cells from B6.Thy-1.1 (donor) mice were adoptively transferred into B6.Thy-1.2 carrier (host) mice. Viral clearance from treated carrier mice was then monitored by determining the level of infectious virus in blood and several other tissues. At various times post-transfer, mice with no detectable viremia were killed and the presence of LCMV-specific CTL activity was checked in their spleen and LN cells. Freshly isolated lymphocytes from treated carrier mice did not exhibit CTL activity and showed minimal or no killing of virally infected cells (data not shown). However, an LCMV-specific CTL response was readily detectable when these lymphocytes were stimulated with virus for 8 d in irradiated recipient mice. This protocol of in vivo stimulation with virus was used in all experiments to monitor the presence of virus-specific CTL in treated carrier mice. The data from several such experiments are summarized in Fig. 1 and details of one particular experiment are shown in Table I. As demonstrated in Fig. 1, an LCMV-specific CTL response was

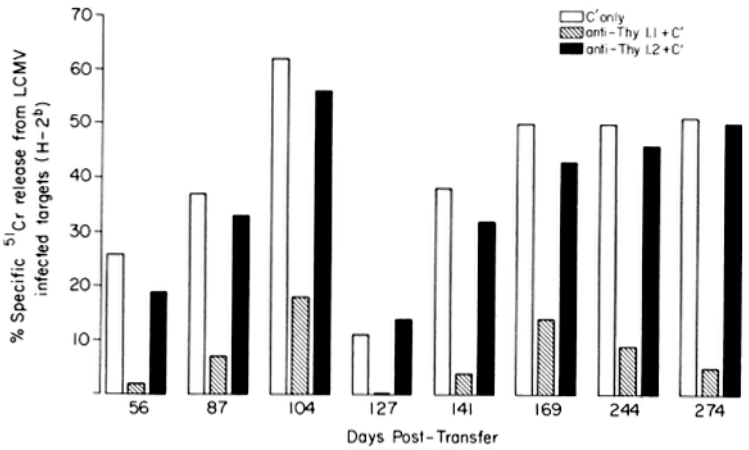

FIGURE 1. Persistence of donor-derived LCMV-specific CTL (Thy-1.1) in carrier mice (Thy-1.2). Spleen and LN cells were harvested from cured carrier mice (Thy-1.2) at the indicated times after transfer of immune T cells (Thy-1.1) and tested for CTL activity after in vivo stimulation with virus as described in Materials and Methods. The phenotype (Thy-1.1 or Thy-1.2) of the CTL was determined by complement-mediated depletion using Thy1.1- and Thy1.2-specific mAbs. The data shown is percent specific killing of infected targets at an E/T ratio of 50:1.

easily detectable and most of the killing was mediated by the donor Thy-1.1 T cells present in cured carrier mice. Donor-derived CTL activity was present at all time points checked and could be recovered as late as 274 d post-transfer. Although there is some variation in the magnitude of the response, no decline in CTL activity was observed at the later time points. Table I shows in detail recovery of donor Thy-1.1 CTL activity from cured carrier mice 274 d post-transfer. Depletion of host Thy-1.2$^+$ T cells from the effector population had no effect on the magnitude of the CTL response. In contrast, depletion of either donor Thy-1.1$^+$ or CD8$^+$ T cells completely abrogated LCMV-specific cytotoxicity, demonstrating that the effectors were CD8$^+$ T cells of donor Thy-1.1 origin. These results show that as late as 274 d post-transfer, donor Thy-1.1 T cells are still present in the cured carrier host and retain CTL activity against LCMV. The CD8$^+$ Thy-1.1$^+$ T cells were MHC class I restricted (no killing of LCMV-infected H-2^d cells) and virus specific as they did not lyse uninfected or Pichinde virus-infected H-2^b targets (data not shown).

Long-lived Donor T Cells Retain In Vivo Antiviral Activity against LCMV. In addition to mediating in vitro CTL activity, these long-lived donor T cells are biologically active in vivo and can eliminate virus from a second infected recipient. When Thy-1.1$^+$ donor T cells isolated from cured carrier mice are transferred into irradiated mice infected with LCMV, they can control the viral infection. The results of one such experiment are shown in Table II. No detectable virus was found in mice that received the Thy-1.1 T cells, in contrast to the high levels of infectious LCMV in organs of irradiated mice receiving virus but no cells.

In Vivo Proliferation of Long-lived Donor CD8$^+$ T Cells. The cured carrier spleens and LNs were examined by flow cytometry for the relative percentages of donor and host CD4$^+$ and CD8$^+$ T cells both before and after in vivo stimulation with LCMV. Single cell suspensions of pre- and post-stimulation lymphocytes were co-stained for the Thy-1.1 or Thy-1.2 antigen and either the CD4 or CD8 surface marker

TABLE I

Recovery of Donor LCMV-specific Cytotoxic T Cells 274 d after Cell Transfer

Group	Treatment	CTL response (percent specific ^{51}Cr release from MC57 [H-2^b] targets)			
		LCMV infected E/T ratio			Uninfected E/T ratio
		5.5:1	16.6:1	50:1	50:1
Cured carrier* (Thy-1.1→Thy-1.2)	C′	15	31	52	1
	Anti-Thy-1.1 + C′	1	2	5	0
	Anti-Thy-1.2 + C′	12	29	51	1
	Anti-Thy-1.1 + C′ + anti-Thy-1.2 + C′	0	1	2	0
	Anti-CD8 + C′	2	1	2	0
B6.Thy-1.1‡	C′	12	32	47	2
	Anti-Thy-1.1 + C′	1	4	12	1
	Anti-Thy-1.2 + C′	14	34	50	3
	Anti-Thy-1.1 + C′ + anti-Thy-1.2 + C′	0	1	6	2
	Anti-CD8 + C′	0	1	3	0
B6.Thy-1.2‡	C′	11	32	52	4
	Anti-Thy-1.1 + C′	11	29	47	2
	Anti-Thy-1.2 + C′	0	0	1	0
	Anti-Thy-1.1 + C′ + anti-Thy-1.2 + C′	0	0	0	0
	Anti-CD8 + C′	0	5	10	0

* Spleen and LN cells from cured carrier mice (274 d post-transfer) were stimulated with LCMV in vivo in irradiated mice and CTL activity determined 8 d post-stimulation.

‡ 6-8-wk-old normal B6.Thy-1.2 and B6.Thy-1.1 mice were injected intravenously with 10^5 PFU of LCMV 8 d before testing for CTL activity as controls for specificity of the anti-Thy-1.2 and anti-Thy-1.1 mAbs.

(see Materials and Methods). Although the donor T cells represented only a minor population of the freshly isolated resting lymphocytes from cured carrier mice, this population underwent tremendous expansion when stimulated with LCMV. The results presented in Fig. 2 and Table III show that this expansion of donor T cells occurred almost exclusively within the donor $CD8^+$ subset. In experiment 1 (see Fig. 2 and Table III), carrier mice that had received Thy-1.1^+ T cells 274 d earlier were used. When their pooled spleen and LN cells were analyzed by flow cytometry,

TABLE II

Donor (Thy-1.1) T Cells Recovered from Cured Carriers Can Eliminate Virus from a Second Infected Recipient

Types of cells transferred	Infectious virus 8 d post-transfer			
	Serum	Liver	Spleen	Lung
	log_{10} PFU/ml			
None	3.6 ± 0.34	4.7 ± 0.34	4.6 ± 0.6	3.8 ± 0.19
Thy-1.1^+ T cells from cured carriers	<1.6	<1.6	<1.6	<1.6

Donor Thy-1.1 T cells were obtained from cured carrier mice (61 d after cell transfer) after complement-mediated depletion of host Thy-1.2 T cells. This enriched population of donor (Thy-1.1) T cells was transferred into irradiated mice infected with LCMV. The amount of infectious virus in various tissues was determined 8 d later. LCMV-infected mice that received no cells served as controls. Virus titers are the averages of two to six mice.

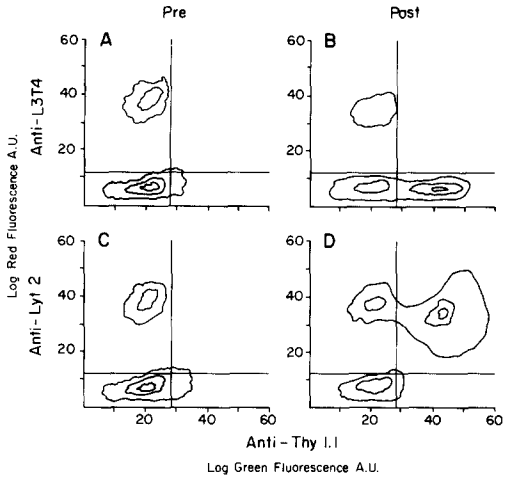

FIGURE 2. Proliferation of donor Thy-1.1$^+$ CD8$^+$ T cells after stimulation with LCMV. Spleen and LN cells were obtained from cured carrier (Thy-1.2) mice 274 d after transfer of LCMV-immune Thy-1.1 T cells. Cells were costained for the Thy-1.1 antigen (green) and either the CD4 or CD8 (red) surface marker and analyzed by flow cytometry. The "pre" sample is the freshly isolated resting lymphocytes from cured carrier mice (274 d post-transfer). The "post" sample is the same cell population after 8 d of in vivo stimulation with LCMV in irradiated normal B6 Thy-1.2 mice. Note the expansion of the Thy-1.1 CD8$^+$ T cells. The expansion is not seen if the cells are transferred into uninfected normal B6 Thy-1.2 mice (data not shown).

the donor Thy-1.1$^+$ CD8$^+$ T cells comprised only 3% of the total population, but after stimulation with LCMV, this minor population of T cells expanded within 8 d to comprise the majority (52%) of the pooled spleen and LN cells. In this particular experiment, there was a sevenfold increase in the total number of Thy-1.1$^+$ CD8$^+$ T cells based on the total number of cells initially transferred into irradiated mice and the number recovered 8 d later. This sevenfold increase is an underestimate of the actual expansion because not all post-stimulation cells were recovered. Only the spleen and the axillary, cervical, and inguinal LNs were recovered from each irradiated recipient, and some donor T cells would have been left in the remaining LNs and in the blood. The data from experiment 2 (Table III) show basically the same pattern; the donor CD8$^+$ T cells initially constitute a minor population but proliferate in vivo upon exposure to virus and become the major cell type present.

The expansion of CD8$^+$ cells was not matched by the Thy-1.1$^+$ CD4$^+$ T cell subset. Fig. 2 and Table III (experiments 1 and 2) show that the Thy-1.1$^+$ CD4$^+$

TABLE III

In Vivo Proliferation of Long-lived Donor CD8$^+$ T Cells

Exp.	Stimulation with LCMV	Percent of total spleen and LN cells from cured carrier mice (Thy-1.1→Thy-1.2)*			
		Donor		Host	
		Thy-1.1$^+$CD4$^+$	Thy-1.1$^+$CD8$^+$	Thy-1.2$^+$CD4$^+$	Thy-1.2$^+$CD8$^+$
1‡	− (Pre)	4.0	3.0	19.0	16.0
	+ (Post)	3.0	**52.0**	11.0	16.0
2§	− (Pre)	2.1	2.0	18.5	13.9
	+ (Post)	5.5	**28.2**	14.6	25.8

* Aliquots of pre- and post-stimulation (8 d after infection) spleen and LN cells were double stained with labeled mAbs for the indicated surface markers and analysed by flow cytometry as described in Materials and Methods.

‡ Cured carrier mice 274 d after transfer of donor T cells were used.

§ These mice were from two groups of cured carriers 82 and 110 d post-cell transfer.

T cells do not exhibit any major expansion after stimulation with LCMV. This subset represented 4% (experiment 1) and 2.1% (experiment 2) of the cured carrier lymphocytes before stimulation and 3% (experiment 1) and 5.5% (experiment 2) 8 d after stimulation with LCMV.

Effect of CD4$^+$ T Cell Depletion on Donor CD8$^+$ Proliferation, Generation of a CTL Response, and Viral Clearance. To investigate the role of CD4$^+$ T cells in the induction of anti-LCMV responses mediated by these long-lived donor CD8$^+$ T cells, both host and donor CD4$^+$ cells were depleted before, and during, in vivo stimulation of the cured carrier lymphocytes. As shown in Table IV, depletion of CD4$^+$ T cells had no detectable effect on the proliferation of donor CD8$^+$ T cells and minimal to no effect on the generation of LCMV-specific CTL responses and viral clearance. Depletion of CD4$^+$ T cells was quite effective and there were <1% CD4$^+$ T cells in the CD4-depleted mice as determined by flow cytometry. Despite this reduction in number of CD4$^+$ T cells, the average numbers of lymphocytes recovered per irradiated recipient were equal for both the CD4-depleted and mock-treated groups (data not shown). The equal proliferation, the potent CTL response, and effective viral clearance by the CD8$^+$ cells in the CD4-depleted group demonstrates a minimal requirement for CD4$^+$ T cells in the induction of the anti-LCMV activities of these long-lived donor CD8$^+$ T cells.

Immunodominance of Donor T Cells over Host-derived LCMV-specific CTL Response. We have previously reported that the cured carrier mice can make a host-derived (Thy-1.2) LCMV-specific CTL response (21). In these earlier studies, donor (Thy-1.1) T cells

TABLE IV

Donor CD8$^+$ T Cells Proliferate, Mediate an LCMV-specific CTL Response, and Control Virus Infection Even After Depletion of CD4$^+$ T Cells

	Proliferation of donor cells	Donor-derived LCMV-specific CTL activity (percent specific ^{51}Cr release at indicated E/T ratio)				Control of LCMV infection (LCMV titer)		
	Percent	LCMV infected			Uninfected			
Group (percent CD4$^+$ T cells)	Thy-1.1$^+$CD8$^+$ cells	5.5:1	16.6:1	50:1	50:1	Serum	Lung	Liver
						log$_{10}$ PFU/ml		
Mock (16.4)	27.4	10.4	33.8	54.9	0	<1.6	<1.6	<1.6
						<1.6	<1.6	<1.6
						<1.6	<1.6	<1.6
						<1.6	<1.6	<1.6
CD4 depleted (<1.0)	29.1	7.8	23.9	50.5	0	<1.6	<1.6	<1.6
						<1.6	<1.6	<1.6
						<1.6	<1.6	<1.6
						<1.6	<1.6	<1.6

Spleen and LN cells from nine cured carrier mice (95 d post-transfer of Thy-1.1$^+$ T cells) were pooled and then divided into two groups of equal numbers of cells. To deplete CD4$^+$ T cells (CD4-depleted group), cells were treated in vitro with two rounds of RL172.4 plus C′ and transferred intravenously into infected irradiated mice for in vivo stimulation with LCMV. Each irradiated recipient was injected intraperitoneally with 0.5 mg of GK1.5 at the time of cell transfer and again on day 3 to further deplete any remaining CD4$^+$ T cells. In the mock group cells were treated twice in vitro with C′ only before transfer into irradiated recipients. Proliferation of CD8$^+$ T cells, LCMV-specific CTL response, and virus titers were determined 8 d post-infection as described in Materials and Methods.

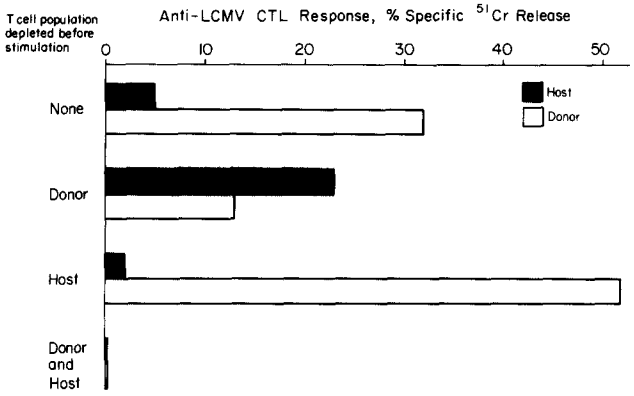

FIGURE 3. Depletion of immune donor T cells is required for detection of a host-derived LCMV-specific CTL response. Spleen and LN cells harvested from cured carrier mice 61 d after transfer of immune Thy-1.1 cells were tested for their ability to make an LCMV-specific CTL response. Before in vivo stimulation with virus, the indicated T cell population(s) were depleted as described in Materials and Methods. Percent specific killing at an E/T ratio of 50:1 is shown.

had been depleted by in vivo treatment of cured carrier mice with an mAb against Thy-1.1 (19E1.2) before testing the host CTL response (21). In the results presented in this paper, as shown in Fig. 1 and Table I, stimulation of cured carrier lymphocytes containing both donor and host T cells resulted in the generation of CTL responses mediated predominantly by donor T cells. These findings suggested the dominance of the donor CTL response over the generation of host CTL activity. This hypothesis was tested by the following experiment. Spleen and LN cells from cured carrier mice were pooled and then divided into four groups containing equal numbers of cells. From one group, donor T cells were depleted, from one, host T cells, from one, both host and donor T cells, and one group was mock treated (i.e., no cells depleted). These four cell populations were then tested for their ability to generate an LCMV-specific CTL response and to determine if it is mediated by host or donor T cells. As shown in Fig. 3, a host (Thy-1.2)-derived LCMV-specific CTL response was observed only in the donor T cell-depleted group. When both host and donor T cells were present ("no depletion" group) the donor (Thy-1.1) CTL response was dominant over the potential CTL response of the cured carrier host. This experiment has been repeated two additional times with similar results (data not shown).

Discussion

This study provides direct evidence that virus-specific CTL can persist indefinitely in vivo. This was accomplished by transferring Thy-1.1 T cells into Thy-1.2 recipient mice to specifically identify the donor T cell population and to characterize its antigenic specificity and function by using a virus-specific CTL assay. The transferred LCMV-specific CTL persist in the recipient mice in small numbers and comprise only a minor fraction of the total T cells. But on exposure to virus these cells proliferate in vivo to become the predominant cell population. These $CD8^+$ T cells can be recovered up to a year post-transfer and still retain antigenic specificity and biological function. They kill LCMV-infected cells in vitro and can eliminate virus upon transfer into a second infected host. These results strongly suggest that virus-specific CTL can persist for the life span of the host as memory cells.

In several instances natural infections, such as with measles virus, varicella, etc., and also vaccinations with certain viruses, result in long-lived T cell immunity (23–25). However, it is not clear if this is due to persistence of the initially primed T cells

and their progeny, or due to subsequent re-exposure to virus and the priming of new (virgin) T cells. By adoptively transferring marked (Thy-1.1) mature T cells from LCMV-immune mice into Thy-1.2 recipients, we have unequivocally shown that primed virus-specific CTL and/or their progeny can indeed persist for a long and indefinite period of time. Thus, our results show that priming of new T cells is not necessary for maintaining long-term T cell immunity.

In our experiments we have examined the longevity of virus-specific CTL in cured carrier mice, which at the time of adoptive transfer contained vast amounts of virus. With the exception of the kidney, no viral antigen was detectable in the tissues of the cured carrier mice at the time donor T cells were recovered. However, we cannot rule out the possibility that the presence of minute amounts of viral antigen not detectable by conventional assays, or virus within the kidney, provides continuous antigenic stimulation to the donor T cells causing proliferation and maintenance of this T cell population. It will be of interest to determine if these long-lived LCMV-specific CTL require antigen for their persistence. A recent study examining hapten-specific antibody responses has shown that B cell memory is short lived in the absence of antigen (26). Experiments are currently in progress to investigate the life span of virus-specific CTL in an antigen "free" environment.

Our study also provides direct evidence for the in vivo proliferation of $CD8^+$ T cells during the induction of a CTL response. This is in contrast to the accepted view of cytotoxic T cell development, in which proliferation is thought to occur primarily within the $CD4^+$ T cell population with $CD8^+$ T cells undergoing only limited expansion (27). Several months after adoptive transfer, the donor $CD8^+$ T cells represented only a minor population (2.0–3.0% of the total spleen and LN cells) of the freshly isolated resting lymphocytes from cured carrier mice. However, after in vivo stimulation with virus, these cells expanded at least 5–10-fold in number to become the predominant T cell population (27.4–52.0% of the total cells). In addition, the virus-specific CTL activity resided almost exclusively within this subset of cells (i.e., Thy-1.1^+ $CD8^+$ cells). Taken together, these results suggest that memory CTL are present in small numbers in a "resting" stage and are capable of expansion after exposure to viral antigen.

Our data also suggest that these long-lived $CD8^+$ CTL are not dependent on help from $CD4^+$ T cells, since depletion of $CD4^+$ T cells had minimal or no effect on the biological properties (proliferation, CTL response, viral clearance) of the donor $CD8^+$ T cells. These results extend our previous finding that the primary CTL response against LCMV does not require $CD4^+$ T cells (28). Thus, it appears that both the primary and memory LCMV-specific CTL responses can function in the absence of $CD4^+$ T cells. It is possible that these long-lived $CD8^+$ CTL are autocrine in nature and capable of producing the lymphokines necessary for their own growth and activation. Alternatively, in the absence of $CD4^+$ T cells, help may be provided by $CD8^+$ helper cells within the donor and/or host T cell population. The existence of $CD8^+$ Th cells and the ability of $CD8^+$ T cells to produce IL-2 has been shown in several systems (29–31).

Virus-specific CTL unresponsiveness is a hallmark of the LCMV carrier state (6, 21, 22, 32, 33). In this report, we have again demonstrated that mice cured of congenitally acquired chronic LCMV infection can generate a host-derived virus-specific CTL response. This confirms our recent finding that mice previously in-

fected by and unresponsive to the virus can become immunocompetent and are then protected against reinfection (21). The present study also extends the earlier finding by showing that despite the ability of the cured carrier host to generate a competent LCMV-specific CTL response, donor-derived T cells must be removed before stimulation of cured carrier lymphocytes before a significant host CTL response can be detected. This clearly demonstrates the dominance of the long-lived donor T cells over the host response. This finding could be explained as the dominance of a secondary (donor) vs. a primary (host) response. A differential requirement by primary and secondary populations for lymphokines and antigens has been described for MLCs (34). Primary host T cells may have more stringent requirements for lymphokines or antigenic stimulation than the secondary donor T cell population. In addition, donor CTL precursors (CTLp) may exist in higher numbers than host CTLp. Thus, the higher precursor frequencies and/or differential lymphokine requirements would allow the donor cells to proliferate and respond more rapidly than the host, therefore, competing more successfully for antigen and growth factors required to activate the host CTLp. This would allow the donor population to effectively deal with the virus before the host cells were extensively exposed and stimulated.

T cell immunity is crucial in defense against viral, parasitic, and intracellular bacterial infections, and also in surveillance of tumors (35–37). Failure of T cell immunity often leads to chronic infections. Our results showing that antigen-specific CTL can persist indefinitely and retain the ability to mediate viral clearance has implications towards understanding the immunological mechanisms effective in controlling chronic infections and tumors. It is conceivable that one of the limiting factors in the successful resolution of certain chronic infections are these long-lived T cells capable of persisting and mediating clearance for months or even years. Strategies to restore and/or increase the function and numbers of such T cells may be effective in the treatment of chronic infections.

Immunological memory is one of the most fascinating but least understood aspects of the immune system. A major obstacle in studying T cell memory has been the difficulty in obtaining "pure" populations of in vivo generated and selected memory cells. The system we have developed may allow us to overcome this problem. Based on our results, it is highly likely that the majority of Thy-1.1$^+$ CD8$^+$ T cells that persist in cured carrier mice (Thy-1.2) are LCMV specific. Thus, it is possible to obtain a highly enriched population of in vivo selected memory CTL and to examine their biological and physical characteristics. This should allow us to address some fundamental questions about memory T cells.

Summary

This study documents that virus-specific CTL can persist indefinitely in vivo. This was accomplished by transferring Thy-1.1 T cells into Thy-1.2 recipient mice to specifically identify the donor T cell population and to characterize its antigenic specificity and function by using a virus-specific CTL assay. Thy-1.1$^+$ T cells from mice previously immunized with lymphocytic choriomeningitis virus (LCMV) were transferred into Thy-1.2 mice persistently infected with LCMV. The transferred LCMV-specific CTL (Thy-1.1$^+$ CD8$^+$) eliminate virus from the chronically infected carriers and persist in the recipient mice in small numbers, comprising only a minor fraction of the total T cells. Upon re-exposure to virus, these long-lived "resting"

$CD8^+$ T cells proliferate in vivo to become the predominant cell population. These donor $CD8^+$ T cells can be recovered up to a year post-transfer and still retain antigenic specificity and biological function. They kill LCMV infected H-2-matched cells in vitro and can eliminate virus upon transfer into a second infected host. In addition, these long-lived $CD8^+$ T cells appear not to be dependent on help from $CD4^+$ T cells, since depletion of $CD4^+$ T cells has minimal or no effect on their biological properties (proliferation, CTL response, viral clearance). These donor CTL also exhibit an immunodominance over the host-derived LCMV-specific CTL response. When both host and donor T cells are present, the donor CTL response is dominant over the potential CTL response of the cured carrier host. Taken together, these results suggest that virus-specific CTL can persist for the life span of the host as memory cells.

We thank Rita J. Concepcion for excellent technical assistance, Roger Bohman for the flow cytometry, and Keisuke F. Iwamoto for the irradiation procedures. We would also like to thank Lori Kenton and Jeannie Martinez for help in preparing the manuscript.

Received for publication 6 January 1989 and in revised form 15 March 1989.

References

1. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* 27:51.
2. Finberg, R., and B. Benacerraf. 1981. Induction, control and consequences of virus specific cytotoxic T cells. *Immunol. Rev.* 58:157.
3. Sethi, K. K., K. Omata, and K. E. Schneweis. 1983. Protection of mice from fatal herpes simplex virus type I infection by adoptive transfer of cloned virus-specific and H-2 restricted cytotoxic T lymphocytes. *J. Gen. Virol.* 64:443.
4. Reddehase, M. J., W. Mutter, K. Munch, H.-J. Buhring, and U. H. Koszinowski. 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J. Virol.* 61:3102.
5. Cannon, J., E. J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. *Virology.* 62:133.
6. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med.* 160:521.
7. Lukacher, A. E., V. L. Braciale, and T. J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160:814.
8. Byrne, J. A., and M. B. A. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus *in vivo*. *J. Virol.* 51:682.
9. Zinkernagel, R. M., and R. M. Welsh. 1976. H-2 compatibility requirement for virus-specific T cell mediated effector functions *in vivo*. I. Specificity of T cells conferring antiviral protection against lymphocytic choriomeningitis virus is associated with H-2K and H-2D. *J. Immunol.* 117:1495.
10. Jamieson, B. D., L. D. Butler, and R. Ahmed. 1987. Effective clearance of a persistent viral infection requires cooperation between virus-specific $Lyt2^+$ T cells and nonspecific bone marrow-derived cells. *J. Virol.* 61:3930.

11. Nabholz, M., and H. R. MacDonald. 1983. Cytolytic T lymphocytes. *Annu. Rev. Immunol.* 1:273.
12. Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. Fan, and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163:903.
13. Townsend, A. R. M., J. Rothbard, F. Gotch, G. Bahadur, D. C. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell.* 44:959.
14. Bastin, J., J. Rothbard, J. Davey, I. Jones, and A. Townsend. 1987. Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 165:1508.
15. Gotch, F., J. Rothbard, K. Howland, A. Townsend, and A. McMichael. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (Lond.).* 326:881.
16. Oldstone, M. B. A., J. L. Whitton, H. Lewicki, and A. Tishon. 1988. Fine dissection of a nine amino acid glycoprotein epitope, a major determinant recognized by lymphocytic choriomeningitis virus-specific class I-restricted H-2D^b cytotoxic T lymphocytes. *J. Exp. Med.* 168:559.
17. Oldstone, M. B. A., P. Blount, P. J. Southern, and P. W. Lampert. 1986. Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature (Lond.).* 321:239.
18. Ahmed, R., B. D. Jamieson, and D. D. Porter. 1987. Immune therapy of a persistent and disseminated viral infection. *J. Virol.* 61:3920.
19. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch. 1983. Characterization of the murine antigenic determinant designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74:29.
20. Ceredig, R., J. W. Lowenthal, M. Nabbolz, and H. R. MacDonald. 1985. Expression of IL-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (Lond.).* 314:98.
21. Jamieson, B. D., and R. Ahmed. 1988. T-cell tolerance: exposure to virus in utero does not cause a permanent deletion of specific T cells. *Proc. Natl. Acad. Sci. USA.* 85:2265.
22. Volkert, M. 1963. Studies on immunological tolerance to LCM virus. 2. Treatment of virus carrier mice by adoptive immunization. *Acta. Pathol. Microbiol. Scand.* 57:465.
23. Mims, C. A., and D. O. White. 1984. Viral Pathogenesis and Immunology. Blackwell Scientific Publications, Inc., Cambridge, MA. 398 pp.
24. Fields, B. N. 1985. Virology. Raven Press, New York. 1614 pp.
25. Notkins, A. B., and M. B. A. Oldstone. 1987. Concepts in Viral Pathogenesis II. Springer-Verlag New York Inc., New York. 409 pp.
26. Gray, D., and H. Skravall. 1988. B-cell memory is short-lived in the absence of antigen. *Nature (Lond.).* 336:70.
27. Klein, J. 1986. Natural History of the Major Histocompatibility Complex. Wiley-Interscience. New York. 338 pp.
28. Ahmed, R., L. D. Butler, and L. Bhatti. 1988. T4$^+$ T helper cell function *in vivo*: differential requirement for induction of antiviral cytotoxic T-cell and antibody responses. *J. Virol.* 62:2102.
29. Mizouchi, T., H. Golding, A. S. Rosenberg, L. H. Glimcher, T. R. Malek, and A. Singer. 1985. Both L3T4$^+$ and Lyt2$^+$ helper T cells initiate cytotoxic T lymphocyte responses against allogeneic major histocompatibility antigens but not against trinitro phenyl-modified self. *J. Exp. Med.* 162:427.

30. Mizouchi, T., A. W. Hugin, H. C. Morse III, A. Singer, and R. M. L. Buller. 1989. Role of lymphokine-secreting $CD8^+$ T cells in cytotoxic T lymphocyte responses against vaccinia virus. *J. Immunol.* 142:270.
31. Kasaian, M. T., and C. A. Biron. 1989. The activation of IL-2 transcription in $L3T4^+$ and $Lyt\text{-}2^+$ lymphocytes during virus infection *in vivo*. *J. Immunol.* 142:1287.
32. Lehmann-Grube, F., L. M. Peralta, M. Bruns, and J. Lohler. 1983. Persistent infection of mice with the lymphocytic choriomeningitis virus. *In* Comprehensive Virology, Vol. 18. Virus-Host Interactions: Receptors, Persistence and Neurological Diseases. H. Fraenkel-Conrat and R. R. Wagner, editors. Plenum Publishing Corp., New York. 43–103.
33. Ahmed, R., C.-C. King, and M. B. A. Oldstone. 1987. Virus-lymphocyte interaction: T cells of the helper subset are infected with lymphocytic choriomeningitis virus during persistent infection *in vivo*. *J. Virol.* 61:1571.
34. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717.
35. Mims, C. A. 1987. The Pathogenesis of Infectious Disease. Academic Press Limited, London. 342 pp.
36. Cheever, M. A., D. B. Thompson, J. P. Klarnet, and P. D. Greenberg. 1986. Antigen-driven long term-cultured T cells proliferate in vivo, distribute widely, mediate specific tumor therapy, and persist long-term as functional memory T cells. *J. Exp. Med.* 163:1100.
37. Bloom, B. R., and V. Mehra. 1984. Immunological unresponsiveness in leprosy. *Immunol. Rev.* 80:6.