Antigen Is Required for the Activation of Effector Activities, whereas Interleukin 2 Is Required for the Maintenance of Memory in Ovalbumin-specific, CD8$^+$ Cytotoxic T Lymphocytes

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Summary

The mechanisms that maintain memory in T cells are not completely understood. We have investigated the role of antigen and interleukin (IL)-2 in the growth and maintenance of CD8$^+$ T cells using a cytolytic T cell line specific for ovalbumin $(OVA)_{257\text{-}264}$ presented by H-2K^b. This line does not secrete IL-4 or IL-2; hence, stimulation with the OVA-transfected EL4 line (E.G7-OVA) does not induce proliferation without addition of exogenous growth factors. Furthermore, this line can be maintained continuously by weekly addition of irradiated, splenic filler cells and IL-2, with or without E.G7-OVA. Although IL-2 induced proliferation of these cytotoxic T lymphocytes (CTLs), production of interferon γ and tumor necrosis factor α required stimulation of the CTL with E.G7-OVA. The kinetics of lymphokine secretion after stimulation by E.G7-OVA were the same whether the CTL had been maintained with or without antigen (Ag). In addition, both CTL lines killed E.G7-OVA target cells within 4 h. Thus, the effector functions of these CTLs were rapidly induced by T cell receptor (TCR) occupancy. CTLs cultured with or without Ag also served as memory T cells when parked for 100 d in unirradiated, syngeneic recipients without OVA. In the absence of OVA, the precursor frequency was identical in spleens of normal and β_2-microglobulin knockout recipients, but significantly less in IL-2 knockout mice. The decline of memory in the absence of IL-2 supports data from other investigators, suggesting that cell cycling is important to the maintenance of CD8$^+$ T cell memory. These data also suggest that stimulation of OVA-specific CTLs by lymphokines seems to be more important to maintaining memory than stimulation of TCRs by cross-reactive peptides complexed to class I molecules.

Memory is a hallmark of the immune response to T cell–dependent antigens. Memory, in both B and T cells, is manifest by stronger and quicker responses upon secondary exposure to antigen. The mechanisms that maintain memory are not completely understood. In part, T cell memory reflects an increase in the frequency of precursor cells. Memory T cells are also qualitatively distinct from naive T cells in that they have less stringent requirements for activation and respond more rapidly than naive T cells (for review see reference 1). Naive and memory T cells are distinct from effector T cells in that the latter are actively engaged in lymphokine secretion and lytic function in the case of CD8$^+$ T cells. By contrast, both naive and memory T cells must be stimulated with antigen to express these effector functions. Naive T cells can be distinguished from effector T cells by differences in the expression of several cell-surface antigens. However, effector T cells cannot be easily distinguished from memory T cells because they both express activation antigens and increased density of adhesion molecules.

Several important questions remain concerning the maintenance of memory T cells. The question of whether antigen is required for memory T cell persistence has been the subject of considerable debate. The majority of studies on the requirement for antigen in maintaining CD8$^+$ memory T cells have been performed using polyclonal T cells activated by viruses such as lymphocytic choriomeningitis virus (LCMV), Sendai virus, or influenza. One of the major obstacles in studying memory in virus-specific, CD8$^+$ T cells is ruling out restimulation by persisting Ags. However, transfer of CD8$^+$ T cells from infected mice into irradiated, syngeneic recipients showed that memory persisted in the absence of virus, as measured by PCR (2–4). Another important issue is the relationship between effector and memory T cells. Whether memory T cells are derived from effector T cells in a linear fashion or whether

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they differentiate along separate pathways is not known. Signals that regulate development of memory T cells have not been identified.

We have recently demonstrated that soluble OVA emulsified in CFA (5) or nonionic block copolymer adjuvants primed $CD8^+$ CTL precursors in mice (6). These OVA-specific CTLs (OVA-CTLs) produced IFN-γ and TNF-α upon stimulation with E.G7-OVA or with $OVA_{257-264}$ in association with $H\text{-}2K^b$ (6). However, these OVA-CTL cells do not produce IL-2 or -4. We reasoned that such $CD8^+$ T cells would provide a good model for studying the memory function of CTLs because persistence of Ags would not be a problem. The results presented below compare the requirements for activation of CTLs versus maintenance of memory CTLs in vitro and in vivo. Our results show that $CD8^+$ CTLs specific for the exogenous Ag, OVA, have the same potential to persist as memory T cells specific for viruses and tumors. Moreover, persistence of memory T cells was more dependent upon IL-2 than cross-reactive complexes of peptides and MHC class I antigens.

Materials and Methods

Animals. C57BL/6 ($H\text{-}2^b$), C57BL/10 ($H\text{-}2^b$), and BALB/c ($H\text{-}2^d$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Knockout mice bearing homozygous, targeted mutations for the β_2-microglobulin (β_2m)[1] gene (7), C57BL/6J-β_2m^{tm1Unc}, or the IL-2 gene (8), C57BL6J-$ll2^{tm1Hor}$, also were purchased from the Jackson Laboratory. All of the inbred, knockout mice express the $H\text{-}2^b$ haplotype and C57BL/6J mice were used as the controls for these experiments as recommended by the Jackson Laboratory. All mice were obtained from pathogen-free colonies and maintained in sterile microisolator cages. Furthermore, no pathogens were detected in sentinel mice housed in the same room with the experimental mice. Female mice, 8–12 wk old, were used in all these experiments. Mice were maintained on standard laboratory chow and water ad libitum in a temperature and light controlled environment. All procedures on animals were conducted according to the principles outlined in the guidelines of the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council.

Tumor Cells and Cell Cultures. The MHC class II negative, $H\text{-}2^b$ thymomas, EL4 and E.G7-OVA, were used as target and stimulator cells. E.G7-OVA was produced transfection of EL4 cells with the cDNA gene encoding chicken egg OVA (9; provided by M.J. Bevan, University of Washington, Seattle, WA). All cell cultures were maintained in RPMI 1640 medium supplemented with 10% bovine calf serum, 1 mM l-glutamine, 1 mM Na pyruvate, 50 μM 2-mercaptoethanol (2-ME), and antibiotics, at 37°C in 6% CO_2 in air.

Generation of Ag-specific CTLs. Generation of CTL lines from C57BL/10 (B10) were described previously (5, 6). In brief, spleen cells were obtained from mice primed 10 d earlier with 200 μg OVA in CFA. Spleen cells were incubated with irradiated (20,000 rad) E.G7-OVA for 1 wk as described by Moore et al. (9). Effector T cells resulting from these cultures recognized $OVA_{257-264}$ (SIINFKEL) peptide in the context of $H\text{-}2K^b$ (5, 6). Allogeneic B10 α BALB/c CTLs were initiated by incubating spleen cells of B10 ($H\text{-}2^b$) with irradiated (2,000 rad) spleen cells from BALB/c ($H\text{-}2^d$) mice. The resulting CTLs recognize allogeneic P815 ($H\text{-}2^d$), but not syngeneic EL4 ($H\text{-}2^b$) targets (5, 10). To establish long-term CTL lines, OVA-CTLs were stimulated weekly with irradiated E.G7-OVA, irradiated, syngeneic splenic filler cells and 5% supernatant from Con A–stimulated rat spleen cells as a source of IL-2 (9, 11). Similarly, B10 α BALB/c CTLs were maintained by weekly stimulation with irradiated spleen cells from BALB/c mice and IL-2 (10).

Cytofluorometric Analysis. OVA-CTLs were incubated with filler cells and IL-2 in the presence or absence of E.G7-OVA stimulators for 1 wk. Viable T cells were isolated by Ficoll-Hypaque gradient centrifugation (12) and then stained with the following mAbs: FITC-conjugated M1/9.3.4 (anti-CD45; reference 13), 145-2C11 (anti-CD3; reference 14), or PE-conjugated 3C7 (anti–IL-2R; reference 15). FITC- or PE-conjugated rat IgG or hamster IgG were used as isotype-matched control Abs, respectively. After incubation at 4°C for 30 min, cells were washed three times and then fixed with 0.5% paraformaldehyde. Cell staining was analyzed on a FACScan® cytofluorimeter using LYSIS-II software (Becton Dickinson, San Jose, CA). Forward angle, light scatter was used to exclude dead and aggregated cells. The results are presented as fluorescence histograms with the relative number of cells on a linear scale plotted versus the relative fluorescence intensity on a log scale.

Cytotoxicity Assay. The ^{51}Cr–release assay used in these experiments has been described in detail elsewhere (10). In brief, target cells were labeled with Na_2CrO_4 (DuPont, Boston, MA) at 37°C for 60 min in Tris-phosphate buffer, pH 7.4, containing 10% FBS. After washing, ^{51}Cr-labeled target cells were added to effector cells in 96-well round-bottomed plates. After a 4-h incubation at 37°C, supernatants were collected and radioactivity was detected in a gamma counter (Wallac, Turku, Finland). Percent specific lysis was calculated as: $100 \times$ ([release by CTL − spontaneous release] / [maximal release − spontaneous release]). Maximal release was determined by addition of 1% Triton X-100 (EM Science, Gibbstown, NJ). Spontaneous release in the absence of CTLs was generally <15% of maximal release.

Measurement of Cytokine Production. Cytokine production was tested by two-site sandwich ELISA using a mouse IFN-γ kit (Genzyme, Cambridge, MA) or TNF-α kit (Endogen, Inc., Boston, MA) (16, 17). In brief, supernatants from OVA-CTL cultures were added to microtiter plates precoated with anti–IFN-γ or anti–TNF-α capture Ab and incubated overnight at 4°C. After addition of biotinylated detecting Ab and incubation at room temperature for 45 min, avidin-peroxidase was added and incubated at room temperature for 30 min. Plates were washed extensively with 1% Tween in PBS between each step. Finally, ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]) substrate containing H_2O_2 was added and the colorimetric reaction was read at an absorbance of 450 nm using an automatic microplate reader (Molecular Devices Corp., Menlo Park, CA). The concentrations of IFN-γ (U/ml) or TNF-α (pg/ml) were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

Adoptive Cell Transfer. OVA-CTL or allogeneic B10 α BALB/c CTLs were harvested 7 d after stimulation as described above. Viable CTLs recovered from Ficoll were washed and resuspended in serum-free HBSS medium. Donor T cells, at 5×10^6 to 1×10^7 per mouse, were injected into the tail veins of naive, syngeneic recipient mice. Recipient mice were killed after various time periods as indicated. Spleen cells were serially titrated in 96-well

[1] *Abbreviation used in this paper:* β_2m, β_2-microglobulin.

microtiter plates and stimulated in vitro with 2×10^4 irradiated E.G7-OVA, 2.5×10^5 syngeneic filler cells, and 5% Con A supernatant. For frequency analysis of donor CTLs within recipient spleen cells, each serial dilution of spleen cells from recipient mice were plated in 24 wells of 96-well microtiter plates. Cytolytic activity was tested using 10^4 of ^{51}Cr–labeled E.G7-OVA targets. The data are displayed as the log of the percentage of negative wells on the ordinate versus number of spleen cells per well on the abscissa. Wells were judged positive if lysis was equal to or greater than the average of the background lysis of ^{51}Cr-labeled E.G7-OVA targets plus 3 SD of the background in the absence of target cells.

Results

Requirements for Growth and Activation of CTLs In Vitro. OVA-CTLs were primed in vivo with OVA in CFA and stimulated in vitro with E.G7-OVA stimulators, syngeneic filler cells, and IL-2 (5). Some studies have shown that allogeneic CTLs could be maintained by recombinant lymphokines in filler cell–free culture systems (18–20). However, the requirement for antigen in the maintenance of OVA-specific CTLs has not been reported. Thus, we tested culture conditions under which the CTL growth would be optimal. OVA-CTLs were cocultured with various combinations of stimulators, filler cells, or IL-2, and their growth was determined 1 wk later. As shown in Fig. 1 *A*, OVA-CTLs grew well in IL-2 alone. The growth rate of OVA-CTLs in cultures containing IL-2 was somewhat greater if E.G7-OVA cells were present, presumably because antigen increases the expression of IL-2R (21, 22). OVA-CTLs not only grew well in the presence of IL-2, exogenous IL-2 was absolutely for the growth of OVA-specific CTL lines and B10 α BALB/c lines (not shown).

The requirements for activation of the effector functions of CTLs were measured using IFN-γ production because this OVA-CTL line does not secrete detectable IL-2. Although exogenous IL-2 was necessary for growth of the CTL in vitro, neither IL-2 nor filler cells were required for IFN-γ production by CTLs activated with E.G7-OVA (Fig. 1 *B*). By contrast, E.G7-OVA were absolutely essential for the activation of OVA-CTLs, indicating that stimulation of TCRs by complexes of peptides and MHC class I molecules was required to induce lymphokine secretion. In spite of some reports that exogenous IL-2 may enhance spontaneous production of IFN-γ by $CD8^+$ T cells (23, 24), we did not find any significant production of IFN-γ by OVA-CTLs cultured with IL-2 or filler cells in the absence of E.G7-OVA stimulators. These results indicate that growth of OVA-CTLs can be maintained by IL-2 without receptor-mediated stimulation. Activation of the effector function of CTLs required antigen and this response to antigen could be enhanced by IL-2.

Responses to IL-2 can be enhanced by antigen because antigen induces an increase in the density of IL-2R on $CD8^+$ CTLs (21, 22). To verify that the IL-2–induced enhancement of lymphokine secretion that was observed in

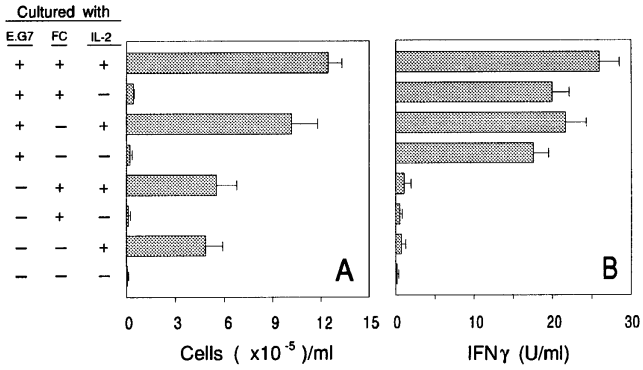

Figure 1. Requirements for growth and activation of CTLs in vitro. OVA-CTLs (2×10^5/ml) were cocultured in 24-well plates with various combinations of irradiated E.G7-OVA (10^5), syngeneic filler cells (2.5×10^6) or 5% Con A supernatant as a source of IL-2. (*A*) After 1 wk, viable CTLs were counted by the trypan blue exclusion method. (*B*) After 24 h, supernatants were tested for IFN-γ production by ELISA. The results are the mean of triplicates ± SD from a representative experiment repeated twice with similar results.

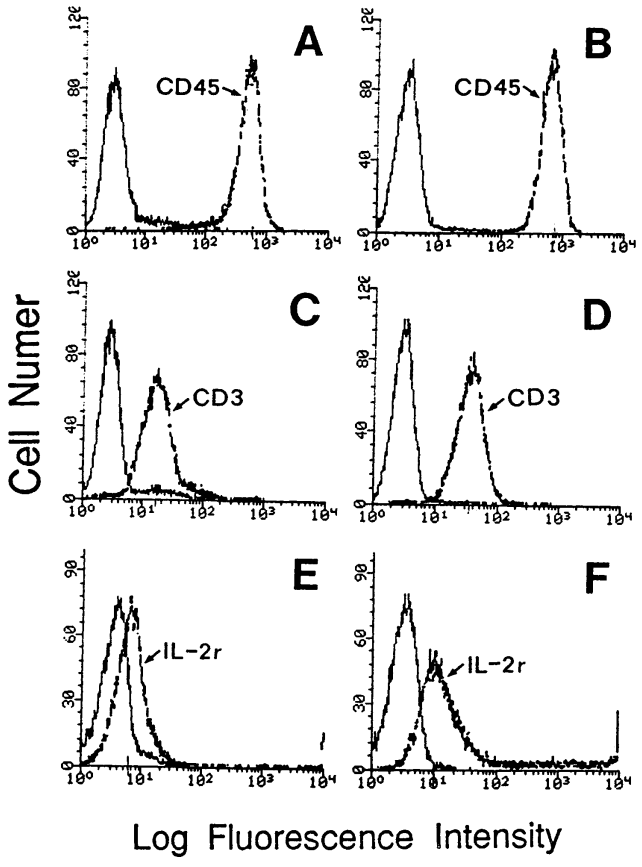

Figure 2. Fluorescence flow cytometric analysis of OVA-CTLs. OVA-CTLs were cocultured with irradiated, syngeneic filler cells and Con A supernatant in the presence (*A–C*) or absence (*D–F*) of irradiated E.G7-OVA stimulators, as described in Fig. 1. After incubation at 37°C for 1 wk, viable T cells were stained with (*A* and *D*) FITC-conjugated M1/9.3.4 (anti-CD45) or normal rat IgG, (*B* and *E*) FITC-conjugated 145-2C11(anti-CD3) or normal hamster IgG, or (*C* and *F*) PE-conjugated 3C7 (anti–IL-2R) or normal rat IgG. The results are presented as fluorescence histograms with the relative number of cells on the ordinate and the relative fluorescence intensity on the abscissa.

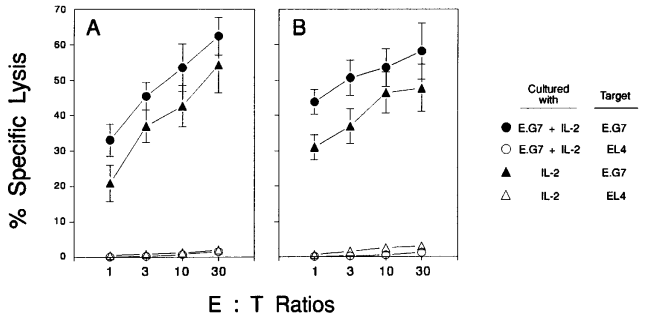

Figure 3. Comparison of cytolytic activity between stimulated and unstimulated CTLs. Stimulated OVA-CTLs were stimulated weekly with irradiated E.G7-OVA, syngeneic filler cells, and Con A supernatant. Unstimulated OVA-CTLs were maintained by weekly replenishment with irradiated filler cells and Con A supernatant, but without E.G7-OVA. After (*A*) 50 or (*B*) 100 d, stimulated and unstimulated OVA-CTLs cultured with IL-2 (*triangles*) or E.G7-OVA plus IL-2 (*circles*) were tested for cytolytic activity using ^{51}Cr-labeled EL4 and E.G7-OVA targets at various E/T ratios. After incubation at 37°C for 4 h, supernatants were harvested and radioactivity was counted. The results are the mean of triplicates ± SD from a representative experiment repeated several times with similar results.

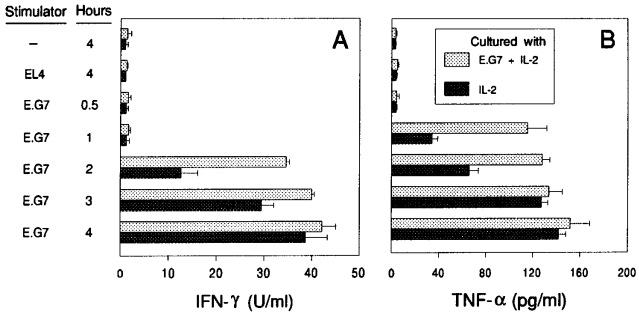

Figure 4. Comparison of cytokine production between stimulated and unstimulated CTLs. Stimulated OVA-CTLs were stimulated weekly with irradiated E.G7-OVA, syngeneic filler cells, and Con A supernatant. Unstimulated OVA-CTLs were maintained by weekly replenishment with irradiated filler cells and Con A supernatant, but without E.G7-OVA. After 100 d, 10^6 of stimulated or unstimulated OVA-CTLs were incubated with 10^5 of EL4 or E.G7-OVA, or without targets. After incubation at 37°C, supernatants were tested for production of (*A*) IFN-γ and (*B*) TNF-α by ELISA. The results shown are the mean of triplicates ± SD of a representative experiment that has been repeated twice.

the previous experiment resulted from the upregulation of IL-2R by antigen, we cocultured OVA-CTLs with filler cells and IL-2 in the presence or absence of E.G7-OVA stimulators for 1 wk and then stained them with mAbs specific for CD45, CD3, or the α chain of IL-2R. There were no significant changes in the expression of CD45 between OVA-CTLs incubated with or without E.G7-OVA (Fig. 2, *A* and *B*). Expression of CD3 was slightly increased by stimulation with E.G7-OVA (Fig. 2, *C* and *D*). However, the IL-2R level expressed by stimulated OVA-CTLs (Fig. 2 *F*) was two to threefold higher than unstimulated CTLs (Fig. 2 *E*). Thus, triggering TCR by antigen upregulated IL-2R expression permitting IL-2 to enhance growth and lymphokine production.

CTLs Cultured with IL-2, but No Antigen, Retained Antigen-specific Reactivity. Based on the observation that IL-2 was sufficient for maintaining CTL growth in short-term cultures, we grew the OVA-CTLs by repeated stimulation with filler cells and IL-2, but without E.G7-OVA. After culturing for 50 and 100 d, the cytolytic activity of these unstimulated OVA-CTLs was tested. No significant difference in cytolytic activity of OVA-CTLs maintained without antigen was observed by comparison to OVA-CTLs that were restimulated weekly with E.G7-OVA (Fig. 3). The specificity of the CTL was maintained as shown by killing of E.G7-OVA but not EL4 targets. These data suggest that Ag is not required to maintain T cell memory in vitro.

We also investigated whether there were any differences in cytokine production between these OVA-CTL lines maintained with or without Ags. OVA-CTLs were incubated with or without stimulator cells. Supernatants were collected at various times and tested for IFN-γ or TNF-α production by ELISA. A latent phase of 1–2 h was observed before IFN-γ (Fig. 4 *A*) or TNF-α (Fig. 4 *B*) were detected in the supernates of the OVA-CTLs that had been stimulated weekly with E.G7-OVA. Activation of the OVA-CTLs that had been maintained with filler cells and IL-2 occurred a bit later and with slightly slower kinetics than seen with OVA-CTLs maintained with Ag. However, comparable levels of IFN-γ or TNF-α were produced by both cultures of OVA-CTLs after 4 h of incubation with E.G7-OVA. Neither of the cultures produced lymphokines after stimulation by EL4. These results confirm that OVA-CTLs, cultured with filler cells and IL-2 for long periods of time, retained their biological functions as well as their antigenic specificity.

The rapidity with which TNF-α and IFN-γ were secreted into the supernates of the CTLs after stimulation with Ags (Fig. 4) raised a question about whether the secreted lymphokines were newly synthesized or released from preexisting stores. However, cycloheximide inhibited the secretion of IFN-γ (Fig. 5 *A*) and TNF-α (Fig. 5 *B*) stimulated by E.G7-OVA, suggesting that these proteins are synthesized in response to antigenic stimulation. The finding that actinomycin D also inhibited secretion of the lymphokines (Fig. 5) suggests that new messenger RNA synthesis was also required for lymphokine secretion. Thus, cultured OVA-specific CTLs upregulate lymphokine gene expression within minutes after stimulation by antigen. By contrast, lysis of targets by OVA-specific CTLs was relatively insensitive to inhibitors of protein and RNA synthesis (Fig. 5 *C*) as would be expected because the lytic proteins, perforin and granzymes, are stored in granules for release upon target cell recognition.

Persistence of Donor CTLs in Recipients. CTL lines that are stimulated weekly with antigen and/or IL-2 do not revert to a resting state. Such cell lines should be considered to be effector cells rather than memory T cells. To test whether such CTL lines could persist in vivo in the absence of an antigenic stimulus, OVA-CTLs were harvested

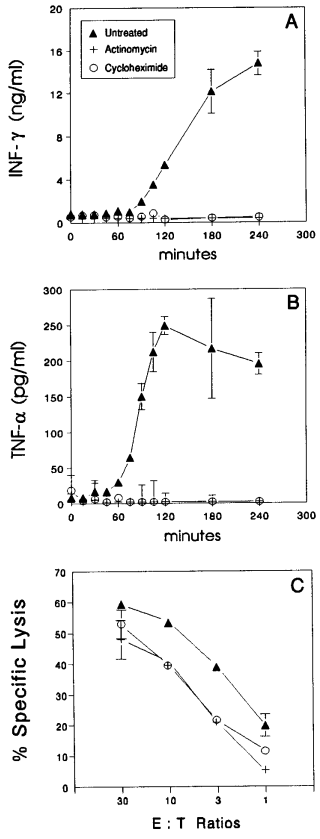

Figure 5. Metabolic inhibitors prevent cytokine production, but not cytolytic activity of CTLs. OVA-CTLs (10^6/ml) were pretreated with 5 μg/ml actinomycin D, 15 μg/ml cycloheximide, or medium at 37°C for 1 h. The cells were then washed and incubated with 10^5 of E.G7-OVA. After incubation at 37°C for various periods of time, supernatants were tested for production of (*A*) IFN-γ and (*B*) TNF-α by ELISA. In *C*, pretreated OVA-CTLs were incubated with ^{51}Cr-labeled targets. After 4 h, supernatant fluids were collected and counted. The results of one of two similar experiments are shown as the mean of triplicates ± SD.

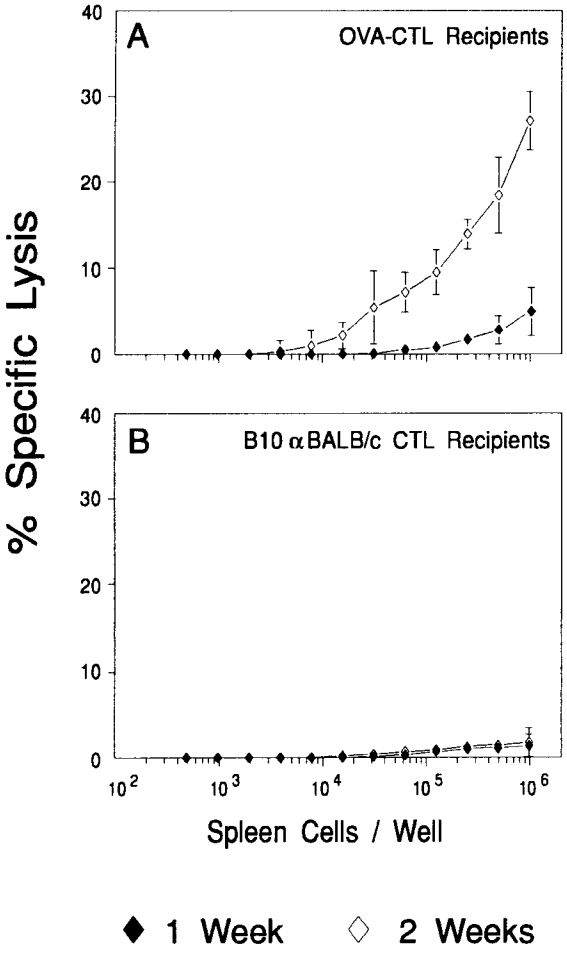

Figure 6. Persistence of donor CTLs in recipients. Unirradiated, syngeneic mice were injected intravenously with 10^7 (*A*) OVA-CTLs or (*B*) allogeneic B10 anti-BALB/c CTLs. After 100 d, recipients were killed and spleen cells were titrated in 96-well plates. Irradiated E.G7-OVA, syngeneic filler cells, and Con A supernatant were added to the cultures. After incubation at 37°C for 1 wk, the cytolytic activity of half of the wells was tested using ^{51}Cr-labeled E.G7-OVA targets. The other half of the cultures were restimulated as described above and tested for cytolytic activity after an additional week in culture. Results shown are the averages of 12 wells ± SD.

7 d after stimulation with irradiated E.G7-OVA. No viable E.G7-OVA remain in these cultures, as shown by the failure of these cultures to stimulate fresh CTLs (not shown). Dead cells and debris were removed from the CTLs by Ficoll-Hypaque centrifugation. Viable OVA-CTLs and B10 α BALB/c CTLs were adoptively transferred to normal, syngeneic mice without secondary challenge. To provide a physiological environment, the recipient animals were not irradiated. Spleen cells from these recipients were harvested 100 d after transfer and stimulated in vitro with E.G7-OVA. After stimulation for 1 and 2 wk, OVA-specific cytolytic activities were detected in spleen cells from mice receiving OVA-specific CTLs (Fig. 6 *A*). Spleen cells from mice receiving control B10 α BALB/c CTLs did not develop OVA-specific responses (Fig. 6 *B*), which is consistent with the previous reports that OVA-specific CTLs cannot be induced by stimulation with E.G7-OVA in vitro without previous priming (9). These results suggest that CTLs persisted in vivo in the absence of viable antigen-bearing tumor cells.

One of the main problems with previous studies on the persistence of memory in $CD8^+$ T cells is that the antigens used frequently were viruses that potentially could be carried over into the recipient animals. Contaminating viruses could activate host CTLs that could be confused with the transferred memory T cells. The OVA-CTLs provide an advantage over virus-specific CTLs in that the antigen used to stimulate the CTLs in vitro is E.G7-OVA that had been irradiated with 20,000 Rad. Furthermore, E.G7-OVA do not survive being cultured with OVA-CTLs for 7 d, as illustrated by the fact that no tumor cells grow from these cultures when unirradiated E.G7-OVA were used as stimulators (not shown). Although no intact antigen-bearing tumor cells were transferred with the CTLs, it is conceivable that some persisting antigenic fragments might have been transferred. Therefore, we sought a method to test the OVA-CTLs for the presence of antigen. One approach would be to irradiate the OVA-CTLs before transfer to prevent their growth in the recipient mice. Development of CTLs by cultures of the recipient spleen cells would then reflect stimulation of host T cells by antigenic carry over. In fact, no CTL activity was primed in such mice (data not shown). However, the interpretation of this experiment is compromised by the fact that irradiated E.G7-

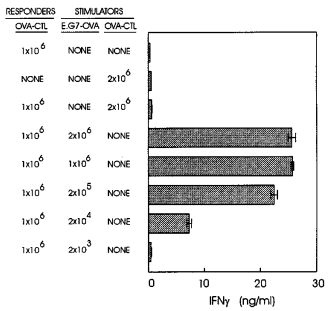

Figure 7. Antigenic carryover in OVA-CTLs. OVA-CTLs were cultured in 24-well plates with media, the indicated concentrations of irradiated E.G7-OVA, or irradiated OVA-CTLs harvested 7 d after stimulation with irradiated E.G7-OVA. After overnight incubation, the supernatants were collected and analyzed for IFN-γ production using an ELISA with a recombinant IFN-γ standard. The results are the mean of triplicates ± SD from a representative experiment repeated twice with similar results.

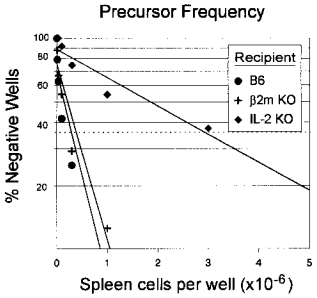

Figure 8. Precursor frequency of donor CTLs in recipient spleen cells. OVA-CTLs (5×10^6) were adoptively transferred into unirradiated, syngeneic normal, $\beta_2m^{-/-}$ or IL-$2^{-/-}$ mice. After 5 wk, recipients were killed and spleen cells from several mice were pooled and titrated in 24 wells of 96-well plates. Irradiated E.G7-OVA, syngeneic filler cells, and Con A supernatant were added to the cultures. After incubation at 37°C for 1 wk, the wells were restimulated as described above. After an additional week in culture, the cytolytic activity of the recovered cells was tested using ^{51}Cr-labeled E.G7-OVA targets. The log of the percentage of negative wells was plotted on the ordinate and the number of spleen cells on the abscissa.

OVA also failed to prime CTLs (data not shown and reference 9). Thus, we cannot judge whether there was carry over of antigen by testing the priming of CTL in vivo using irradiated cells.

In contrast to naive animals, OVA-CTL lines can be stimulated to produce IFN-γ by irradiated E.G7-OVA, as illustrated in Fig. 1 *B*. This observation provides a sensitive method to gauge the amount of antigen remaining in the OVA-CTLs. 7 d after stimulation with E.G7-OVA, the OVA-CTLs were irradiated and added to fresh unirradiated OVA-CTLs. Activation was detected by secretion of IFN-γ. The results verify that OVA-CTLs do not produce IFN-γ without addition of exogenous antigen (Fig. 7), suggesting that little contaminating Ag remains in these cultures. No IFN-γ was produced by irradiated OVA-CTLs, nor did the irradiated OVA-CTLs stimulate IFN-γ production by unirradiated OVA-CTLs. Titration of irradiated E.G7-OVA established a standard curve for stimulation. From this experiment, we estimate that adoptive transfer of 5×10^6 OVA-CTLs would contain $<5 \times 10^3$ cell equivalents of E.G7-OVA. In other words, <0.1% of the normal immunizing dose of E.G7-OVA might have been transferred as antigenic fragments to adoptive recipients. Hence, we conclude that the CTL activity recovered from B6 recipients of the OVA-CTLs is due to persistence of the CTLs as memory T cells rather than host CTLs primed by contaminating antigen.

The requirements for persistence of OVA-CTLs were examined next. To test whether memory was maintained by stimulation with cross-reactive peptides complexed to MHC class I, β_2m knockout recipients were used. Because OVA-CTLs do not produce IL-2, the potential role of host IL-2 in long-term persistence memory could be addressed using IL-2 knockout mice. 5 wk after transfer, spleen cells from recipient mice were restimulated in vitro with E.G7-OVA, syngeneic filler cells, and IL-2. The frequencies of donor OVA-CTLs in the host spleen cells were then analyzed. As shown in Fig. 8, the frequency of donor OVA-CTLs in spleen cells from $\beta_2m^{-/-}$ mice was in the same order of magnitude as in normal recipients ($\sim 1/10^5$ spleen cells), suggesting that stimulation by MHC class I–bearing cells was not required for the persistence of donor CTLs in vivo. In contrast, the frequency of donor OVA-CTLs in spleen cells of IL-$2^{-/-}$ mice ($\sim 1/3 \times 10^6$ spleen cells) was much lower than in normal recipients, indicating that bystander IL-2 provided essential signals for the long-term maintenance of donor CTL in vivo.

Discussion

This study provides evidence that CTLs specific for exogenous protein Ags can persist for long periods both in vitro and in vivo without antigenic stimulation. Unlike some alloreactive, virus- or tumor-specific $CD8^+$ CTLs that produce IL-2 (25–28), the OVA-CTLs cultured from mice primed with OVA in CFA are dependent on exogenous IL-2 for their growth. The finding that T cells that have been grown in the presence of exogenous IL-2 develop into IL-2–dependent lines is not surprising. Similar observations been reported by many investigators beginning with the seminal studies of the CTLL line reported by Gillis et al. (9). Moreover, the inability of these CTLs to produce endogenous IL-2 upon activation provided us the opportunity to analyze the requirements for growth and activation separately.

Our in vitro experiments demonstrate that IL-2 was required for CTL growth, but not for the activation of effector functions. This is consistent with the observations from Baker et al. (29) and Cheever et al. (28) that CTLs can be maintained in vitro in an IL-2–dependent proliferative state virtually indefinitely. Proliferation that was induced by IL-2 could be enhanced by the addition of Ag (E.G7-OVA) because Ag upregulated the expression of IL-2Rs. $CD8^+$ CTLs that were maintained for prolonged periods with IL-2 and filler cells but no Ag, retained the ability to be activated by specific antigen to produce lymphokines and to kill targets. After activation by Ag, lymphokines could be detected in the supernatant fluid within 1–2 h and reached a plateau within 4 h. Lymphokine production by the CTLs that had been maintained with IL-2 lagged only slightly behind the cultures that had been maintained with Ags. Lymphokine secretion by the activated CTLs was abrogated by inhibitors of both protein and RNA synthesis. By contrast

cytolytic activity, which is largely dependent upon release of lytic factors stored in granules (30), was largely resistant to these inhibitors. These results show that Ag-specific, $CD8^+$ T cells maintained for prolonged periods with IL-2 but not Ag, respond to Ag with rapid kinetics similar to that of CTLs that had been recently stimulated with Ag.

These studies also show that activation of effector functions by the OVA-specific CTLs required exposure to antigen (E.G7-OVA). Antigen was not only necessary, but sufficient to stimulate lymphokine secretion. Neither filler cells, IL-2, or filler cells plus IL-2 induced lymphokine secretion in the absence of Ag, nor did they significantly enhance lymphokine secretion that was induced by Ag. Ag induced messenger RNA synthesis and secretion of lymphokines within 1–2 h of incubation with CTLs. This rapid response is compatible with previous observations that primed T cells are able to respond to antigen much more quickly than naive T cells (for review see reference 1).

Maintenance of memory T cells in vivo has often been examined by adoptive transfer of T cells into irradiated syngeneic recipients. Irradiation of recipients is thought to provide room for transferred lymphocytes to take up residence in the appropriate niches. Indeed, mature peripheral T cells have the capacity to expand to a constant steady state level in irradiated adoptive recipients in the absence of antigen (31). Under physiological conditions, however, memory T cells are maintained in the presence of a vast excess of irrelevant T cells. Therefore, we transferred cultured CTLs into unirradiated, syngeneic recipients to study their capacity to serve as memory T cells. OVA-specific or B10 anti-BALB/c CTLs were injected intravenously into B6 mice where they were parked without stimulation by exogenous antigen. After 100 d, the spleen cells from the recipient mice were cultured for 7 d with E.G7-OVA and assayed for specific lytic activity. It is important to note that OVA-specific CTL activity cannot be stimulated in spleen cells from normal, unprimed mice even after several cycles of stimulation with E.G7-OVA plus IL-2 in vitro (5, 9). Likewise, no OVA-specific CTL activity was detected in cultures of spleen cells from mice that received B10 anti-BALB/c CTLs after stimulation with E.G7-OVA. Low, but significant, levels of cytolytic activity were detected in cultures of spleen cells from mice that received the OVA-specific CTLs. The low level of CTLs detected 1 wk after culture initiation was greatly expanded upon restimulation in vitro. Thus, the donor OVA-CTLs persisted, as memory T cells, in the adoptive recipients for several months in the absence of exogenous antigen.

That OVA-specific, memory CTLs persisted in vivo supports the results of Cheever et al. who demonstrated that long-term cultured T cells survived in adoptive recipients where they were able to kill adoptively transferred tumor cells (28). Our data also confirm the studies of Lau et al. who showed that CTLs specific for lymphocytic choriomeningitis virus persisted for indefinite periods in a secondary, uninfected host (4), and those of Bruno et al. who showed that H-Y–specific memory cells persist in the absence of male cells (32). Thus, $CD8^+$ CTLs specific for a variety of antigens including viruses, tumors, and exogenous proteins persist in the absence of the cognate Ag in vivo and in vitro.

Despite the data showing that memory T cells persist in the absence of cognate antigen, the precise requirements for the maintenance of these T cells are still not clear. A number of possibilities could account for the long-term survival of memory T cells, the most straight forward being that they are long-lived cells. Indeed, studies by Tough and Sprent demonstrated that a sizable proportion of T cells with a memory phenotype remained in interphase for prolonged periods (33). However, these authors also showed that a large portion of the T cells with a memory phenotype were rapidly dividing. The nature of the stimulus that drives proliferation of the memory T cells, in the absence of Ag, has not been defined. It is possible that they are stimulated via their TCR by cross-reactive peptides associated with MHC class I, as discussed by Beverley (34). Alternatively, they could be stimulated by ligands, such as costimulatory molecules (for review see reference 1) or cytokines (35, 36), whose receptors are upregulated on memory T cells.

The mice used in our adoptive transfer experiments were not exposed to OVA, experimentally or environmentally. To address the possibility that memory was maintained by cross-reacting peptides presented by K^b, we compared the recovery of OVA-specific memory T cells from normal or $\beta_2m^{-/-}$ adoptive recipients. The precursor frequencies of CTLs from these mice were identical. Similar results were reported by Hou et al. (3) for persistence of virus-specific $CD8^+$ T cells in $\beta_2m^{-/-}$ mice. However, in the latter experiments, memory T cells were transferred into irradiated recipients where the lack of homeostatic mechanisms (31) could have contributed to expansion and hence the persistence of the memory pool. We minimized this problem by adoptively transferring the OVA-specific CTLs into unirradiated hosts.

Studies of T cell memory in $\beta_2m^{-/-}$ mice by Hou et al. (3) were previously criticized by Matzinger (37) on the grounds that $\beta_2m^{-/-}$ mice express low levels of free MHC class I antigens on cell surfaces that are sufficient to prime MHC class I–specific, $CD8^+$ CTLs (38). Theoretically, such peptide-loaded, MHC class I molecules could stimulate cross-reactive memory T cells. However, in the absence of β_2m, free H-$2D^b$ molecules are transported to the cell surface but H-$2K^b$ molecules are not (39). Moreover, Zugel et al. have shown that H-$2D^b$–restricted peptides, but not H-$2K^b$–restricted peptides, stabilize MHC class I expression on the surface of $\beta_2m^{-/-}$ macrophages and that the H-$2D^b$ peptide complexes were recognized by $CD8^+$ CTLs (40). Since OVA-specific, $CD8^+$ CTLs used in our experiments recognize $OVA_{257\text{-}264}$ presented by H-$2K^b$ (5), the expression of H-$2D^b$ molecules in these mice is irrelevant.

Another potential complication in the adoptive transfer of Sendai virus–specific CTLs into $\beta_2m^{-/-}$ mice (3) is that unfractionated spleen cells were transferred. Since the spleen cells contain stem cells, it was reasoned that they

could have repopulated the recipient $\beta_2m^{-/-}$ mice with sufficient MHC class I–positive cells to stimulate cross-reactive memory (37). In our experiments, OVA-specific CTL lines that had been maintained for >2 yr in vitro by culturing with irradiated splenic filler cells were transferred into the $\beta_2m^{-/-}$ mice. Thus, no contaminating stem cells were transferred in our experiments. In addition, the OVA-specific CTLs were transferred intravenously into unirradiated, $\beta_2m^{-/-}$ recipient mice where the transferred cells would be diluted by a vast excess of viable host cells. Thus, we consider the possibility that the OVA-specific CTLs bearing MHC class I could have presented cross-reacting peptides to themselves to be highly unlikely. We conclude that maintenance of $CD8^+$ memory T cells does not require stimulation of TCRs by cross-reactive, peptides complexed to MHC class I.

The possibility that some ligands interacting with other receptors that are upregulated on memory T cells may play a role in the maintenance of memory has also been addressed by our studies. The frequency of OVA-specific CTLs recovered from adoptively transferred IL-$2^{-/-}$ mice was significantly lower than that obtained from normal mice. We conclude that bystander IL-2 provides one mechanism of support for memory T cells.

The observation that IL-2 helps to sustain memory in OVA-CTLs suggests that the OVA-CTL memory cells express IL-2R. Expression of IL-2R generally reflects activation of T cells via TCR–CD3, which raises the question of how IL-2R were stimulated in the absence of complexes of peptide and MHC class I in the $\beta_2m^{-/-}$ mice. Low levels of IL-2Rs have been reported to be expressed by some $CD8^+$ memory T cells (for review see reference 34). IL-2R are expressed by OVA-CTLs maintained by weekly stimulation in vitro (Fig. 2). Thus, expression of IL-2R may have been maintained in the adoptive recipients. Unutmaz et al. have recently reported that resting T cells could be stimulated to proliferate by IL-2 in the absence of TCR occupancy if they were stimulated with a combination of TNF-α and IL-6, which upregulated the IL-2R (36). Hence, cytokines produced during bystander T cell activation could activate IL-2R expression in the OVA-CTL in the absence of β_2m and TCR stimulation. In addition, Siefken et al. recently reported that certain antibodies to CD28 can induce expression of IL-2 and IL-2R by resting T cells in the absence of ligation of the TCR complex (41). Thus, stimulation through other cell surface receptors might have activated IL-2R gene expression in the $\beta_2m^{-/-}$ adoptive recipients.

Although the frequency of memory cells recovered from IL-$2^{-/-}$ mice was lower than from normal mice, significant numbers were obtained. This raises the possibility that other cytokines that activate memory T cells, such as IL-15 (42), may also provide signals that support memory T cells. Alternatively, the memory CTLs recovered from IL-$2^{-/-}$ mice could represent the long-lived, nonproliferating population that remains in interphase for prolonged periods (33). Experiments to examine these possibilities and to determine whether IL-2 is required for maintenance of memory T cells specific for other antigens are in progress.

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