Long-lasting CD8 T Cell Memory in the Absence of CD4 T Cells or B Cells

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Summary

The cellular basis of immunological memory has been a debated issue. It is not clear whether CD8 T cell memory is maintained by long-lived cells or by specific or nonspecific restimulation. Here, we have approached the question from a different angle, asking whether the cellular interactions that are required to maintain memory are the same as those necessary to activate cytotoxic T lymphocytes. We studied the CD8 memory response to the male antigen H-Y in mice deficient in CD4 cells or B cells and found that memory in these mice was virtually unimpaired. These results suggest that CD8 memory is CD4 independent and that there is no requirement for long term retention of immune complexes on follicular dendritic cells, nor for B cells as antigen-presenting cells.

Despite 30 years of study and recent renewed efforts, the mechanisms responsible for the induction and maintenance of antigen-specific immunological memory are not yet understood. Two models have been proposed to account for long-term maintenance of memory. One postulates that cells involved in a primary response switch to a long-lasting memory state whose maintenance requires no additional input. The other envisions that memory cells need to be restimulated periodically. According to the first model, virgin and memory cells have different life spans. The newly developed virgin T or B cell is short lived. It circulates through the body for a few weeks and then dies to make room for new cells. However, upon encounter with antigen, it changes to a long-lived experienced cell that can circulate for years in the absence of any further stimulation. The second model sees the maintenance of memory as a dynamic process. The idea is that virgin and experienced cells have similar life spans, and immunological memory is the result of periodic restimulation by antigen, either because of reinfection by the original pathogen or other, cross reactive environmental antigens (1, 2), or because of persisting antigen–antibody complexes on follicular dendritic cells (FDCs)[1] (3, 4).

To distinguish between these two alternatives, several groups used cell transfer experiments to ask whether memory persists in the absence of the original priming antigen. For CD4 cells the results were clear, but no such picture has yet emerged for CD8 cells. For example, when primed experienced lymphocytes were removed from the original animal and transferred to new, antigen-free hosts, CD4 help and B cell memory waned within a few weeks (5–9), and in some cases CTL memory was also lost (9, 10). However, in several other cases, CTL memory persisted (11–14) in the absence of any residual trace of the original priming antigen (11).

One possible explanation for the different results is that memory to some antigens can be maintained by reactions to cross reactive environmental antigens (1, 2). If this is the case (and it is hard to see how such cross reactions would not occur), it would be very difficult to predict the cross-reactivity of any particular antigen in any particular environment. Thus, when memory persists in the absence of the original antigen, it is virtually impossible to know whether environmental antigens are involved or whether antigen is entirely unnecessary.

We therefore tried another approach.

If memory is due to long-lived cells, without any requirement for restimulation, then the persistence of CTL memory should be independent of any other cell type. However, if experienced CD8 CTLs need to be periodically restimulated, then one might expect to find a need for other cells, in particular, Th cells (for those antigens to which CTLs are helper dependent), or B cells (to produce the antibodies necessary for the formation of long-term reservoirs of antigen attached to FDCs) (3, 4).

We therefore investigated whether CD8 CTLs to the male antigen H-Y could persist in the absence of CD4 help or B cells. We used H-Y for two reasons. First, memory to H-Y is lost in adoptive transfer experiments, suggesting that there are few if any cross-reactive environmental antigens able to maintain CTL memory to this antigen (9). Second, CD8 CTLs to H-Y are completely dependent on

[1] *Abbreviations used in this paper:* FDC, follicular dendritic cell.

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help from CD4 cells (15, 16) and in fact become tolerant if they encounter H-Y in the absence of help (17–19).

We report here that CD8 memory to H-Y is maintained for 9 mo in mice depleted of CD4 cells or deficient in B cells, suggesting that CD8 memory persistence depends neither on help by CD4 cells nor on antigen presentation or antibody secretion by B cells.

Materials and Methods

Mice. C57Bl/6 mice (B6) (6–8 wk old) were purchased from Taconic Laboratories (Germantown, NY). μMT mice (20) were backcrossed for seven generations to the B6 background and were bred at the National Institute of Allergy and Infectious Diseases (Bethesda, MD), which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Antibodies. The anti-CD4 rat IgG2b mAbs GK1.5 (21) and YTA3.1.2 (22) were purified from ascites. For FACS analysis, the following mAbs were used: anti-TCR–FITC (H57, PharMingen, San Diego, CA), anti-CD4–PE (RM4-4, PharMingen), the binding of which is blocked by neither GK1.5 nor YTA3.1.2 at the concentrations used (data not shown), anti-CD8α–Red 613 (53-6.7; GIBCO BRL, Gaithersburg, MD), anti-rat IgG2b FITC (G15-337, PharMingen), anti-CD45R/B220–PE (RA3-6B2; PharMingen), and anti-FcγR (2.4G2; American Type Culture Collection, Rockville, MD) (23).

Cytofluorimetric Analysis. Determination of B220-positive and TCR-, CD4-, and CD8-positive cells in the spleen was done by staining with anti-TCR–FITC, anti-CD4–PE, and anti-CD8α–Red 613 mAbs followed by analysis with a FACScan® (Becton Dickinson, San Jose, CA). The anti-FcγR mAb 2.4G2 was used to block nonspecific staining. Determination of rat IgG2b mAb in the serum was done by FACS® analysis of normal spleen cells incubated with titrated dilution of serum from each animal, followed by anti–rat IgG2b FITC.

In Vivo Immunization. Female mice were immunized by an intraperitoneal injection of 2×10^6 male splenocytes, prepared in PBS. A dose of 5×10^7 male splenocytes intraperitoneally was used for secondary challenge.

Anti-CD4 Depletion In Vivo. Mice were injected intraperitoneally with 0.5 mg of GK1.5 and 0.1 mg of YTA3.1.2, two synergistic anti-CD4 antibodies, in 0.2 ml of PBS. The depletion treatment started 1 mo after priming with five daily consecutive injections. To maintain the depleted state, the mice were injected again 2 wk later and then every 10 d until the end of the experiment. Controls were injected with PBS on a similar schedule.

Cytotoxicity Tests. B6, CD4-depleted B6, and μMT female spleen cells from each mouse were stimulated in vitro with B6 male spleen cells and tested 5 or 6 d later for killing activity by the JAM Test (24). In brief, $4–6 \times 10^6$ responder spleen cells were cultured for 5–6 d against 2×10^6 irradiated (1,500 rads) stimulator spleen cells in 2 ml of IMDM supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, 50 μg/ml gentamycin sulfate, 4 mM glutamine, and 50 μM 2-mercaptoethanol.

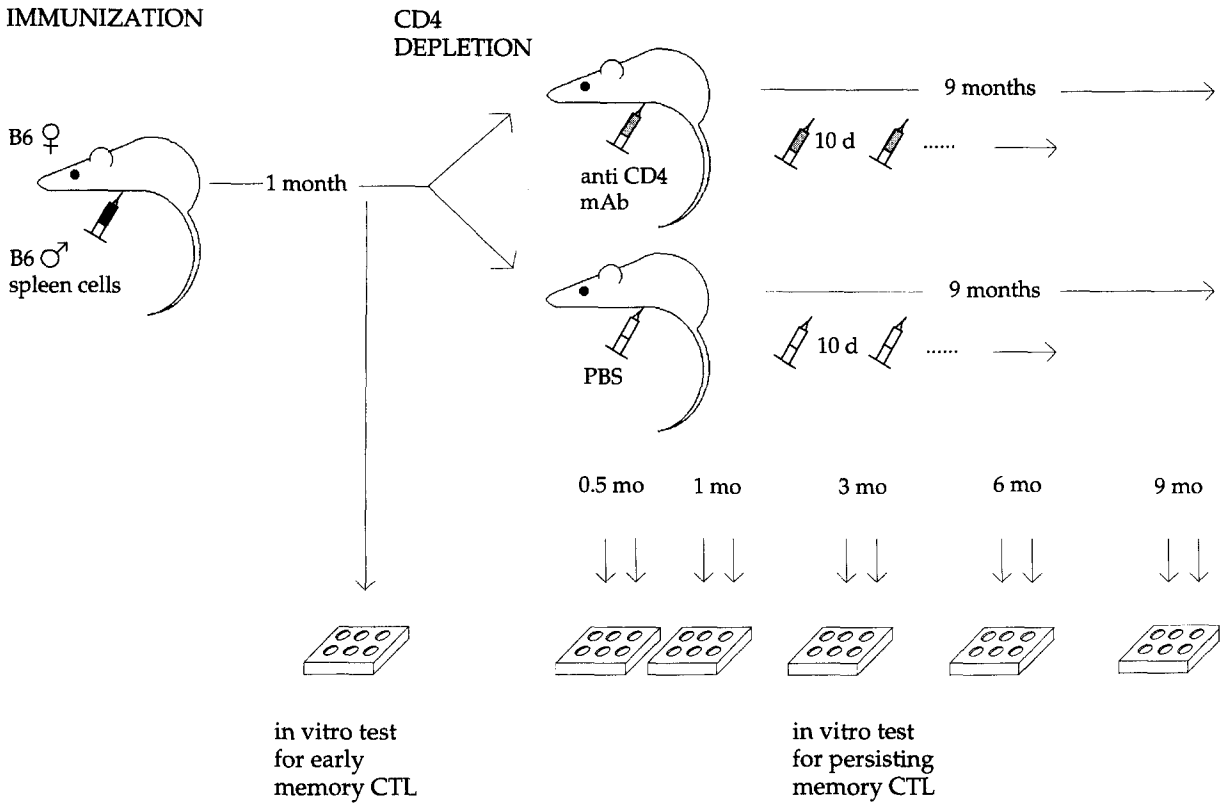

Figure 1. Scheme of the experimental protocol to investigate CD4 dependence of CD8 memory to H-Y. We immunized B6 female mice with male spleen cells, tested some of them 1 mo later for anti–H-Y killing, and divided the remaining mice into two groups. We depleted one group of their CD4 cells by multiple anti-CD4 mAb injections and maintained the depletion by further injections every 10 d. At five different time points later, we tested batches of 5–10 mice for anti–H-Y killing, in two separate experiments for each single time point.

At the end of the 5-d culture, the responders were harvested and tested for cytotoxic activity against [^{3}H]thymidine-labeled B6 male and B6 female Con A–activated blasts. In the experiments comparing normal versus CD4-depleted B6 mice, each anti-H-Y culture was performed either with or without optimal concentrations of conditioned supernatant (rat T-Stim™ without Con A; Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA) as a substitute source of T cell help. In the experiments comparing B6 and μMT mice, supernatant was added to each anti–H-Y culture.

Results

Testing CD4 Dependence: Overall Scheme

To test the CD4 dependence of CTL memory, we depleted CD4 cells from mice primed to H-Y and tested the mice over the next 9 mo for their ability to generate CTLs in vitro. Since unprimed female mice do not make in vitro responses to H-Y, this assay assesses the persistence of experienced CTL.

We followed the protocol outlined in Fig. 1. We immunized 150 B6 female mice to the H-Y antigen by i.p. injection of 2×10^6 male spleen cells and checked for priming 3 wk later by testing some of the mice for their ability to generate CTLs in vitro. 4 wk after priming, we divided the immunized mice into two groups and depleted one group of their CD4 cells by five daily injections of the two synergistic anti-CD4 mAbs, GK1.5 and YTA3.1.2, leaving the other group as undepleted controls. We then tested the mice for anti–H-Y CTL activity at five different time points after the start of CD4 depletion: 2 wk, then 1, 3, 6, and 9 mo.

To ensure that the CD4 cells did not return, we reinjected the depleted mice with the anti-CD4 mAbs every 10 d for the length of the experiment, and, to assess the efficacy of depletion, we examined each mouse for the presence of CD4 cells, both phenotypically by FACS® analysis and functionally by an in vitro test for help. In addition, we tested two lots of mice for CD4 helper function in vivo at different times after depletion.

Long-lasting CD8 Memory Does Not Require CD4 Cells

Fig. 2 shows the CTL responses, over a period of 9 mo, of primed normal mice, and primed and CD4-depleted mice. Because the lack of CD4 help would prevent the activation of anti-H–Y CTLs in vitro (9, 25), we added helper factors to the cultures in the form of conditioned supernatant from Con A–stimulated rat spleen cells. We found that the absence of CD4 cells did not have an effect on CTL memory. Both the normal and CD4-depleted groups were able to generate CTLs to H-Y in the presence of conditioned supernatant for the duration of the experiment, and, though some mice responded better than others, the variability in response was the same in the two groups. These data suggest that CD8 memory to H-Y can last for quite long periods in the CD4-depleted mice.

This result left us with two possibilities. Either CD8 memory is independent of CD4 help, or the in vivo depletion of CD4 cells might have been insufficient, leaving a residue of effective T helpers. To test the second possibility, we measured the presence of residual CD4 cells functionally and phenotypically.

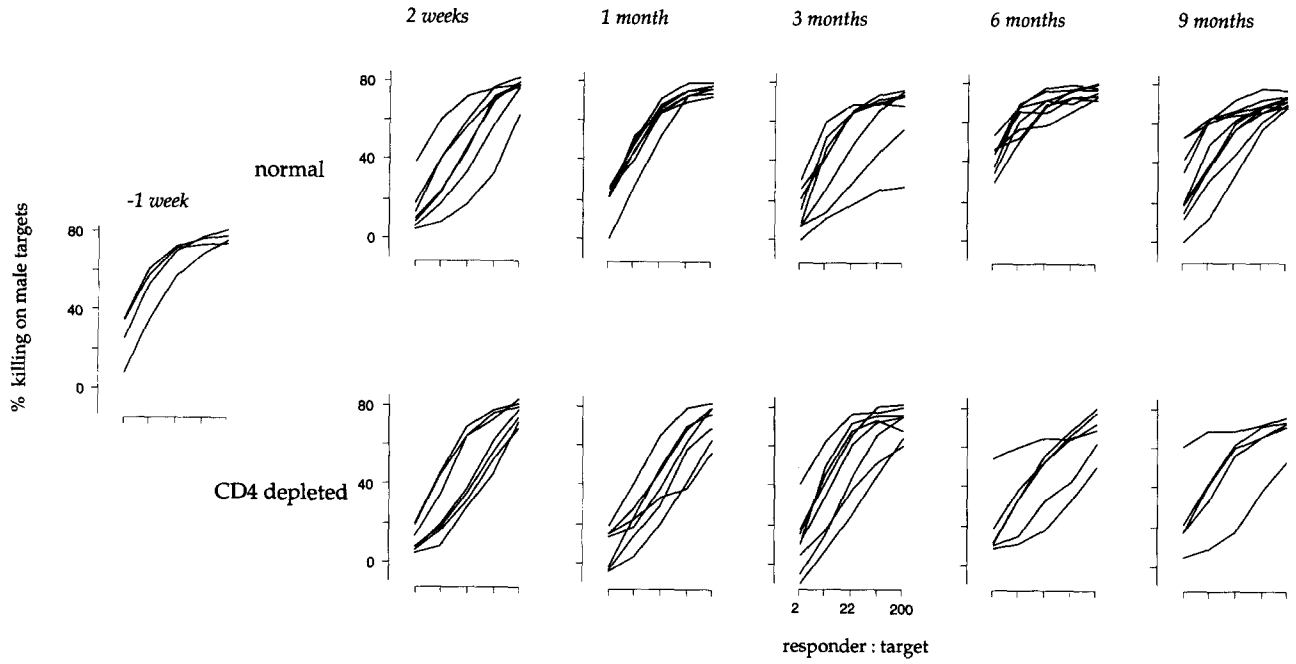

Figure 2. CD8 memory to H-Y persists in the absence of CD4 cells. Primed B6 female mice were tested for anti–H-Y killing activity 1 wk before the start of the CD4 depletion and at five different time points afterward. The panels represent the titrated killing responses of spleen cells from individual mice cultured against B6 male targets in the presence of helper supernatants.

Tests for CD4 Depletion

Phenotype. To determine whether the 10-d interval between antibody injections was adequately spaced to maintain high levels of the depleting GK1.5 and YTA3.1.2 anti-CD4 mAbs in the CD4-depleted mice, we checked for the levels of the depleting antibodies remaining in the serum by indirect immunofluorescence with anti–rat IgG2b and found them to be consistently high, still detectable at a dilution of 1:100 (data not shown). Thus, our treated mice had consistently high levels of anti-CD4 mAb in their serum.

Each unprimed, primed normal, and primed CD4-depleted mouse that we tested for anti–H-Y responsiveness was also tested for CD4 depletion. We stained spleen samples from each mouse with anti-CD4, anti-CD8, and anti-TCR antibodies. To ensure that we would not miss any CD4 cells because of blocking by the GK1.5 and YTA3.1.2 anti–CD4-depleting mAbs, we used the anti-CD4 mAb RM4-4, which is not blocked by either of the depleting antibodies at the concentrations used (data not shown). Representative profiles at the different time points, depicted in Fig. 3, show that the initial depleting regimen brought the number of labeled splenic CD4 T cells down from about 15% to less than 1%, and the continued injections kept the numbers down for months. Moreover, the residual CD4 cells may not have been T cells, since they had very low levels of TCR on their surface (Fig. 3 and data not shown). In Table 1, we report the mean percentages and SD of TCR^+ CD4,TCR^+ CD8, and TCR^+ double-negative cells in all of the mice. The CD4-depleted mice are almost completely lacking in TCR^+ CD4 cells, and we observed no concomitant increase in the number of TCR^+ double-negative cells, ruling out the possibility that the CD4 cells were present but had simply downregulated their CD4 molecules. On the whole, the CD4-depleted mice did not show a consistent increase in the proportion of TCR^+ CD8 cells. This is due to the abundance of other cell types and to the variability in the percentages of both CD4 and CD8 from mouse to mouse.

Function. The initiation of primary (15–18) and secondary CD8 CTL responses to H-Y are strictly helper dependent in vivo. To test the helper dependence of the secondary in vitro response, we cultured responder cells from H-Y-primed CD4-depleted mice against irradiated male stimulators in the presence or absence of conditioned supernatant. Fig. 4 *A* shows that mice depleted of CD4 cells by repeated injections for 6 mo were unable to generate H-Y–specific CTLs unless conditioned supernatant was added to the culture, whereas the supplement was not required by normal, undepleted, primed responders. Although the addition of supernatant to the culture also increased the killing on female targets, there remained an easily detectable level of male-specific killing. Fig. 5 summarizes the data for each mouse used over the period of 9 mo, showing the killing activity at a single responder-to-target ratio for each mouse. Though the CD4-depleted animals begin to show some weak CTL activity at 9 mo, we found that they were dependent on the addition of conditioned supernatant to fully develop their cytotoxic activity against male targets, with the exception of only one mouse, out of 34 tested, which was not considered for further analysis. These results show that our CD4-depleted mice were not only lacking in detect-

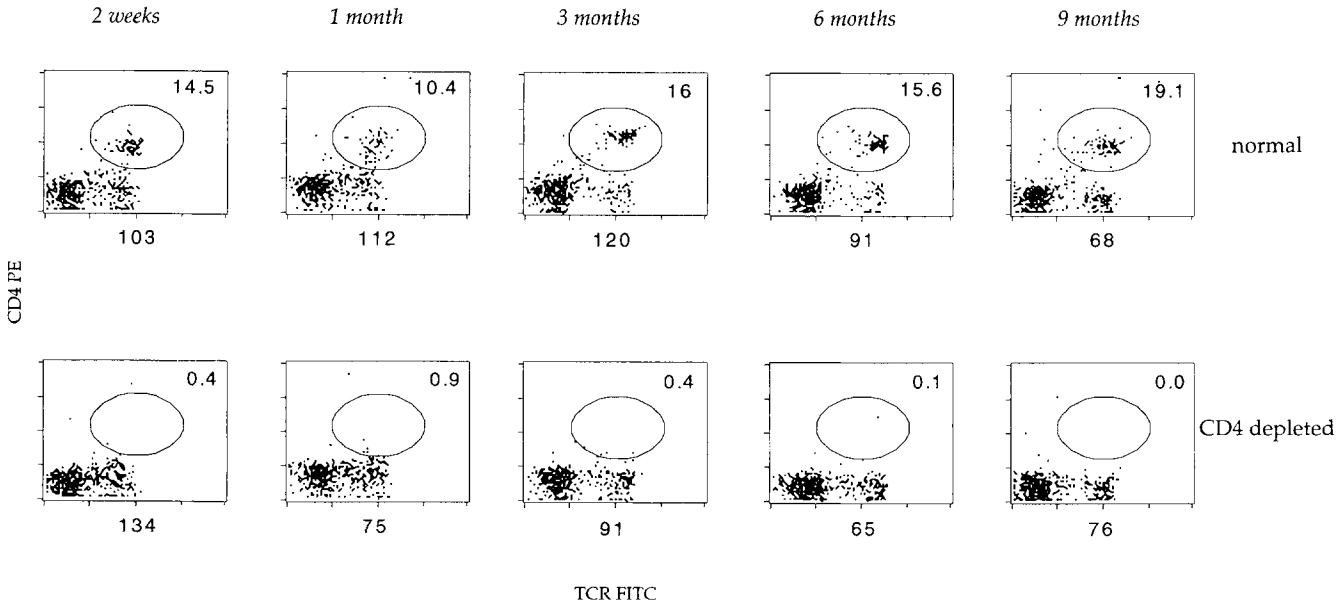

Figure 3. Spleen cell cytograms of primed normal and primed CD4-depleted B6 female mice. TCR and CD4 staining profiles are shown for one representative mouse per group at each time point. The scales on the x and y axes are logarithmic, in arbitrary units. The numbers at the top right corner of each panel are the percentages of cells in the delineated region of $CD4^+$ TCR^+ cells. Below each panel are the total numbers of spleen cells of each mouse, in millions.

Table 1. *Percentages of TCR$^+$ CD4, CD8, and Double-negative Splenocytes in Unprimed Control and in Primed Normal and CD4-depleted B6 Female Mice*

Time Point	Percent TCR$^+$ CD4$^+$			Percent TCR$^+$ CD8$^+$			Percent TCR$^+$ CD4$^-$8$^-$		
		Primed			Primed			Primed	
	Unprimed	Normal	CD4-depleted	Unprimed	Normal	CD4-depleted	Unprimed	Normal	CD4-depleted
2 wk	14	14.8 (2.4)	0.8 (0.4)	10.1	11.2 (0.9)	11.7 (2.4)	3.8	2.8 (1.3)	3.9 (1.8)
1 mo	10.8	10.9 (3.4)	1.3 (0.9)	8.8	9.1 (1.5)	11.8 (2)	5.5	6.1 (1.6)	5.9 (0.8)
3 mo	13.3	14.8 (1.5)	0.1 (0.2)	9.5	11.3 (1.1)	16.1 (1.6)	2.6	2.1 (0.8)	3.6 (0.8)
6 mo	16.1	16.6 (3.1)	0.3 (0.4)	11.1	12 (3.2)	19.6 (1.8)	2.6	2.6 (0.5)	3.9 (0.4)
9 mo	16	14.6 (3.3)	0.5 (0.5)	12	9.2 (3.3)	15.1 (3.5)	2.6	2.0 (0.5)	1.3 (0.8)

Spleen cells were stained with mAbs to TCR, CD4, and CD8 and analyzed by FACS® at five different time points after the start of the CD4-depleting treatment. The numbers are the mean percentages and SD of CD4, CD8, and double-negative splenocytes that are TCR$^+$. SD is not given for the unprimed group, which only comprises two mice per time point. The primed normal and primed CD4-depleted groups comprise 5–10 mice per time point. The cytofluorimetric analysis was performed on each mouse tested for anti-H-Y CTL activity in the 9-mo experiment.

able CD4 cells; they also lacked in vitro detectable helper activity. Since the number of CD4 cells does not increase with time in the depleted mice (Fig. 3 and Table 1), the slight increase in helper activity found at 9 mo is most likely due to double-negative cells, as found in the CD4 knockout mice (26).

To test for helper activity in vivo, we analyzed two sets of our primed and primed CD4-depleted mice for in vivo anti-H-Y secondary responses, one at the start of CD4-depleting treatment (Fig. 4 *B*) and the other a month later (data not shown). The primed mice were injected with a second dose of male spleen cells and then were analyzed in vitro for their anti-H-Y CTL activity. As expected, the primed normal mice showed 3–30-fold increased anti-H-Y responses after challenge, compared with mice that received the antigen only once. We did not observe this en-

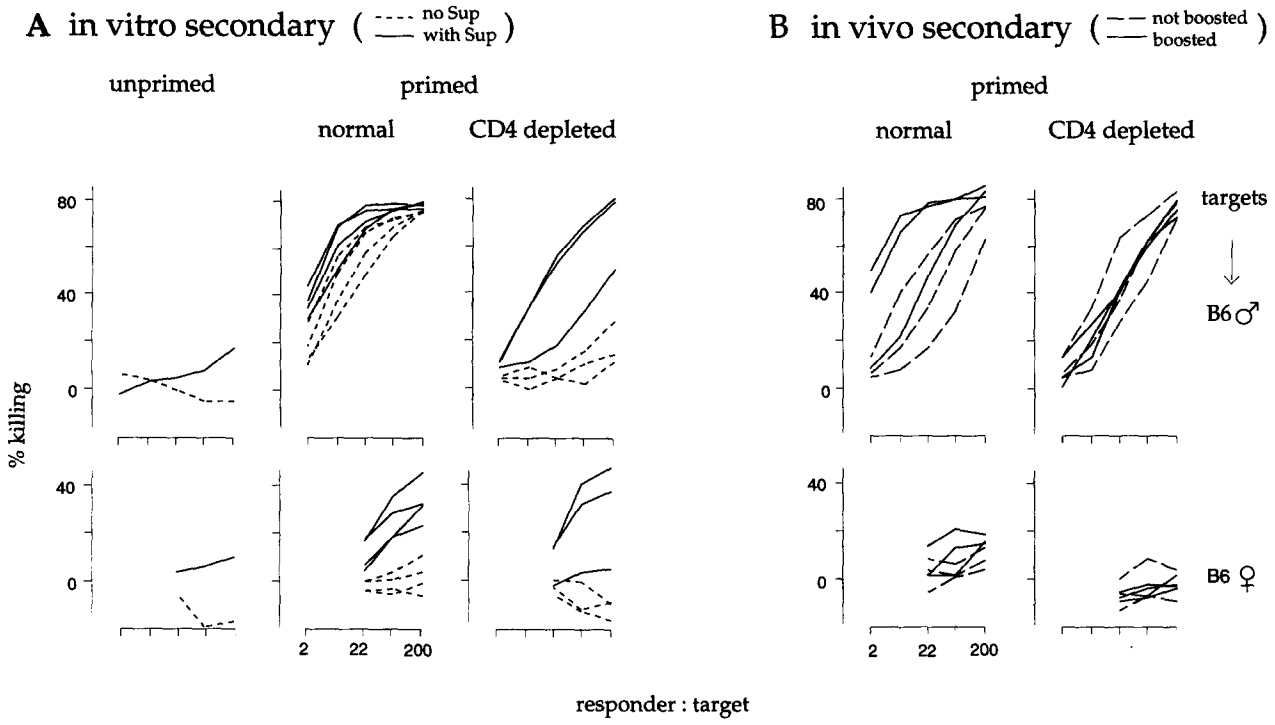

Figure 4. The CD8 secondary response to H-Y is dependent on help from CD4 cells. The panels represent the titrated responses, against B6 male and female targets, of spleen cells from individual mice cultured for 5 d in vitro against male stimulators, with or without conditioned supernatant. (*A*) Killing from an unprimed mouse and of mice primed once in vivo and then depleted or not of CD4 cells. (*B*) Killing from primed mice that were then depleted or not of CD4 cells and subsequently challenged i.p. with 5×10^7 B6 male spleen cells. Their in vitro responses were analyzed 2 wk later and compared with those of primed but not boosted mice. The responses obtained by stimulation with male spleen cells in the presence of supernatant are shown.

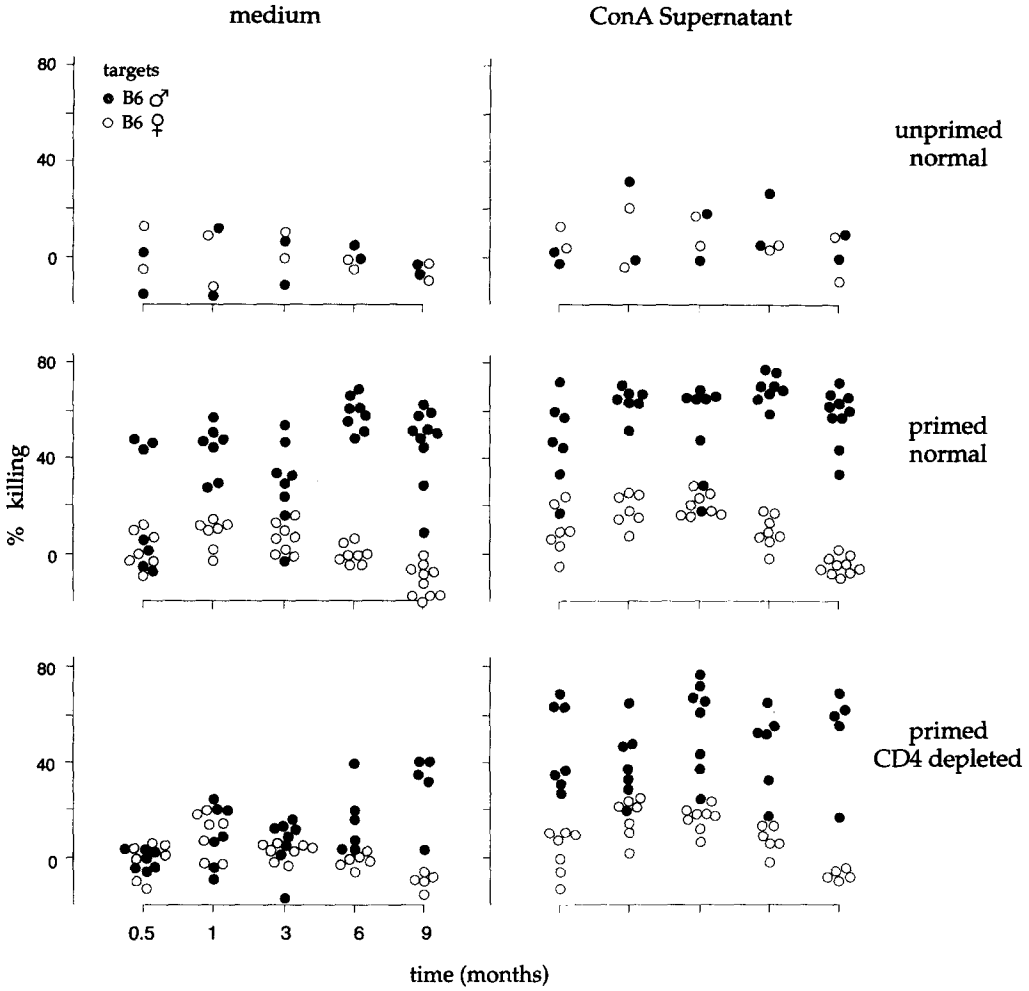

Figure 5. The in vitro secondary response of CD4-depleted mice to H-Y is dependent on supernatant as a source of help. Spleen cells of unprimed and H-Y–primed B6 female responders (same as Fig. 2) were tested for their specific killing either on male or female targets, after a 5-d stimulation culture with male spleen cells, in the presence or absence of supernatant. The panels represent the percentage killing from individual mice at a 22:1 responder-to-target ratio.

hancement of anti–H-Y CTL activity in the primed CD4-depleted mice that were challenged with male spleen cells a second time.

These results indicate that memory CD8 cells, restimulated with the H-Y antigen either in vivo or in vitro, depend on help to develop a secondary response and that the CD4-depleted mice appeared to be deficient in functional helper activity both in vivo and in vitro. Thus, the persistence of CTL memory does not seem to result from the persistence of functional CD4 help in the face of CD4 depletion, indicating that CD8 CTL memory can persist for quite long periods in the absence of CD4 help.

Long-lasting CD8 Memory Does Not Require B Cells and Antibodies

We have previously shown that B cells are not required for the primary response of virgin T cells to H-Y (27). There are, however, three possible ways in which B cells might contribute to T cell memory. First, they produce antibody and therefore can favor antigen retention as immune complexes by FDCs. Though no antibody has yet been found to the H-Y antigen, the complement-dependent and fluorescence labeling tests for its presence have all relied on surface expression. Since H-Y is an intracellular protein (28, and Ways, J. and P. Matzinger, personal communication), the presence of anti–H-Y antibody could easily have been missed. Second, though B cells cannot present antigen to turn on virgin T cells (29–31), they are able to stimulate memory T cells (32–36), and antigen-specific B cells can present high concentrations of the original antigen as well as any potentially cross-reactive antigens that may exist in the environment. Last, they might contribute to memory maintenance by presenting their own idiotypic peptides and thus stimulating cross-reactive memory T cells.

To investigate the role of B cells and antibody in the maintenance of CD8 memory, we compared the memory anti–H-Y responses of normal B6 females with those of μMT mice, which lack peripheral B cells (20). We immu-

nized B6 and μMT females with either B6 or μMT male spleen cells to check the role of both host B cells and the antigen-presenting B cells in the immunizing inoculum. We then analyzed their anti–H-Y CTL activity at 2 wk, and at 3, 6, and 9 mo after priming. Figs. 6 and 7 show that the CTL responses of the B6 and μMT females were indistinguishable and that both B cell–deficient and normal male spleen cell inocula were able to elicit long-lasting CTL memory. We also did FACS analysis of spleen cells from each μMT mouse, looking for B220 expression, and confirmed that all of them lacked mature $B220^+$ cells (27, and data not shown). These results suggest that neither antigen-presenting B cells nor antibody are required for the maintenance of CD8 memory to H-Y.

Discussion

In this study, we asked whether the cellular interactions necessary for activation of CD8 T cells are also necessary for their persistence as memory cells. Using the CD8 response to H-Y, in which activation is critically dependent on help from CD4 cells, we found that the persistence of memory to H-Y was CD4 independent. In addition, using B cell–deficient mice, we found that CD8 memory persisted in the absence of B cells and antibody, suggesting that there is no requirement for long-term retention of immune complexes on FDCs, nor for B cells as APCs. These results suggest that the stimuli involved in CD8 T cell activation may be different from those that maintain memory.

The most straightforward interpretation of these results is that experienced, H-Y–specific, CD8 CTLs are long lived and do not need antigenic stimulation or interactions with other lymphocytes. The problem with this interpretation, however, is that, though it can explain the retention of long-term memory found to several viruses in the absence of antigen, it does not account for previous data from cell transfer experiments on CTL memory to H-Y, nor to some of the data gathered with responses to other viruses, where memory disappeared in the absence of antigen.

Is it possible to reconcile all of the data? Let us begin with the data on the response to H-Y, before we broaden the scope. In an earlier study (9), we found that memory to H-Y waned over a space of about 3 mo when a purified population of 10–20 million mixed CD4 and CD8 T cells was transferred from primed animals into naive nude recipients. We concluded that the memory CTLs needed to be periodically restimulated by antigen. Since the activation of killers to H-Y has been shown to need CD4 help, we would have predicted that memory would wane in the ab-

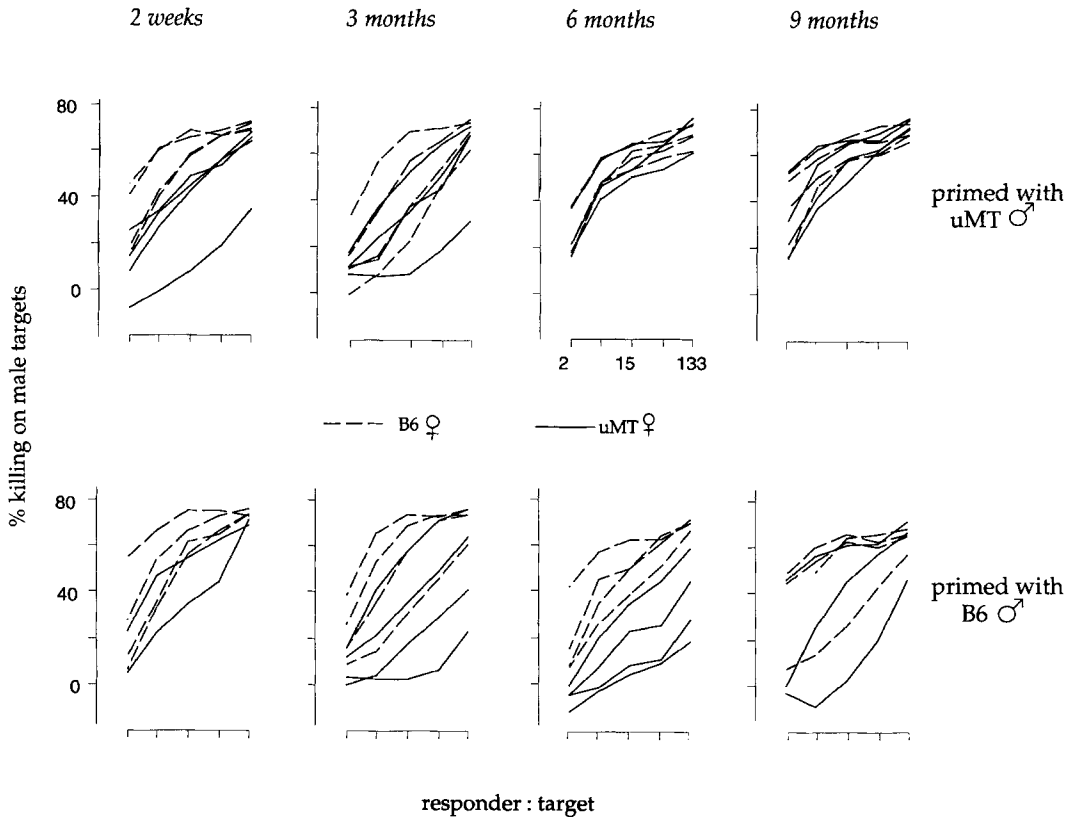

Figure 6. CD8 memory to H-Y persists in the absence of B cells. B6 and μMT females were immunized with 2×10^6 B6 male or 2×10^6 μMT male spleen cells i.p. and tested for anti-male responses at four different time points after the immunization. Conditioned supernatant was added to all cultures, so that they were equally supportive of CTL development, irrespective of CD4 content.

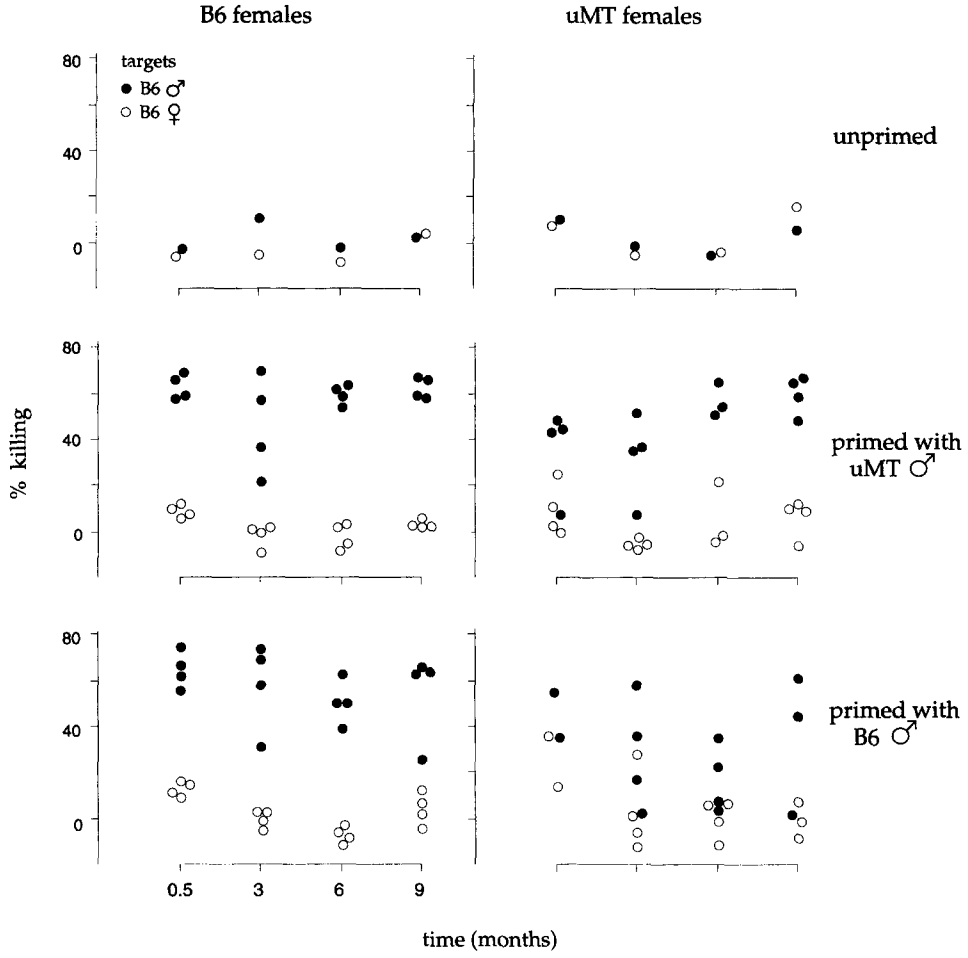

Figure 7. The specific CD8 response to H-Y persists in the absence of B cells. Spleen cells of unprimed B6 and μMT females and H-Y–primed responders (same as Fig. 6) were tested for their specific killing either on male or female targets, after a 5-d stimulation culture with male spleen cells and supernatant. The panels represent the percentage of specific killing of individual mice at the responder-to-target ratio of 15:1.

sence of help, yet in the present study, we found that the memory seems to be helper independent. There are four possible ways to reconcile these two sets of data.

H-Y–specific CD8 Killers Are Long Lived in the Absence of Antigen but Require CD4 Cells to Be Stimulated; CD4 Cells Are Not Long Lived and, in the Absence of CD4 Cells, Stimulation Leads to Tolerance

In the adoptive transfer experiments, we waited for various lengths of time after the transfer and then boosted the mice in vivo with an injection of male spleen cells 2 wk before testing for their ability to generate killers in vitro. If there were any residual memory CTLs, we reasoned that this boost should induce their expansion such that they would be easily seen in the in vitro assay. This detail of the experimental protocol opens the possibility that the loss of memory was due to loss of CD4 help rather than CD8 killers. The killers in mice that had lost CD4 memory would have lasted until they were boosted, at which time they would have become tolerant by the recognition of antigen in the absence of help (18). Since we did not boost the mice in the present study, the loss of help would not, by itself, result in the loss of killer precursors able to be restimulated in culture.

H-Y–Specific Killers Are Not Particularly Long Lived; They Are Maintained by Cross-Reactive Antigens That Do Not Require CD4 Help

It has been proposed that cross-reactive antigens, either as self peptides (1) or common environmental antigens (1, 2), can restimulate memory CD8 cells. According to this view, the maintenance of CD8 memory in adoptive transfer experiments is due to restimulation of CD8 memory cells by cross reaction. We suggest that the nature of the cross-reactive antigen may determine whether its effect is helper dependent or not.

The literature on help for killers shows that killers to normal cell antigens, such as H-Y (15–18), Qa (17, 18, 37, 38), or minor H antigens (39), are absolutely dependent on help from CD4 cells, whereas killers to many viruses are partially or completely independent (40, 41). Yet the killer precursor cannot know that its antigen is a viral or normal cellular peptide. Therefore the difference must reside elsewhere. We (17, 18, 42) and others (43) have suggested that the difference lies in the APCs. On the basis of the evidence that T helper cells can activate APCs (44, 45), we proposed that help for killers is normally routed through the APCs, rather than given directly. We started with the idea that APCs do not normally express costimulatory sig-

nals for naive CTLs but can be induced to do so by Th cells. In effect, the Th cell signals the APC, which then becomes activated and expresses distinct, CTL-specific, costimulatory signals. The activated APC can now turn on CTL precursors in the absence of the original helper. In this way, the Bretscher–Cohn type of antigen-specific helper signal (46) is translated into a Lafferty–Cunningham type of costimulation (47). In the case of virally infected APCs, we would suggest that the infection itself might upregulate the relevant costimulatory molecules, and thus the activation of killers would be independent of CD4 help. Thus, killers that are CD4 dependent when responding to normal APCs presenting H-Y may become independent when responding to APCs infected with a cross-reactive virus. This scenario leads to two possible ways in which H-Y memory could have been lost in the transfer experiments yet maintained in the original host depleted of CD4 cells.

The first possibility is that environmental antigens may differ from place to place. In this case, one might expect to find that memory to certain antigens will persist in one mouse colony and be lost in another, an explanation that has also been invoked to deal with the differences in the life span of cells with a virgin phenotype in different colonies (Sprent, J., personal communication). Thus, we might speculate that in our colony in Bethesda, there is a helper-independent antigen, able to restimulate anti–H-Y CD8 memory cells by cross-reaction, and that H-Y cross-reactive antigens were missing in the colony in Basel.

Second, since CD4 and CD8 cells recognize different determinants, presented by different classes of MHC molecules, any particular cross-reactive environmental antigen may maintain one population but not the other. Thus, in the transfer experiments CD4 helpers may have slowly died away in the absence of H-Y, leaving a set of persisting killers maintained by an environmental virus or other antigen that initiates CD4-independent responses. However, these persisting killers would have been tolerized by the in vivo boost with male spleen cells, since this is a reaction that requires CD4 cells.

The Signals That Maintain Memory Are Helper Independent and Are Different from Those That Result in Activation

There are two possibilities here. The first is antigen dependent but helper independent. The second is independent of both.

Antigen dependent. If the persistence of memory T cells requires only that they be rescued from death, but not that they be activated, one could postulate the involvement of different APCs or perhaps different signals, which were missing in the transfer to nude recipients. According to this view, an APC that is specialized for memory maintenance may be able to present the H-Y antigen and provide the complete array of stimuli required for renewal of memory CD8 cells in the absence of signals from CD4 cells, either because the CD8 T cells are able to make enough of their own cytokines or because the signals needed for persistence are different from those needed for response. This has previously been suggested for B cell memory, from studies in which it was found that overexpression of the *bcl-2* gene prolongs memory B cell lifespan and overrides the need for persisting antigen in the maintenance of B cell memory (48, 49). Moreover, though it is not known whether some APCs are better than others at presenting antigen for the maintenance of memory, it has been suggested that FDCs may be involved (3, 4). In our experiments, however, antigen retained by FDCs is unlikely to account for memory to H-Y, since memory was not impaired in mice lacking B cells (and therefore lacking antibody and the ability to generate antigen–antibody complexes attached to FDCs).

Antigen independent. CD8 memory cells may be kept alive and possibly cycling by nonspecific stimuli present in the lymphoid environment, such as cytokines or membrane interactions with cells other than CD4 and B cells. This antigen-independent effect of cytokines on the peripheral T cell pool maintenance has been proposed on the basis of in vitro results (50), but has not yet been shown in vivo. To have a specific effect on memory vs. virgin cells, one need only postulate that memory, but not virgin, cells carry receptors for these cytokines or that memory cells can traffic to specialized areas of lymphoid tissue, where virgin cells have no access (51, 52), and that there they receive signals for renewal. If this were the case, the loss of memory in the transfer to nude recipients could be due to the dearth of such memory environments in nudes repopulated with small numbers of memory cells.

The Intrinsic Lifespans of Virgin and Memory Cells Are the Same but the Population Sizes Are Different, So That Memory Lasts Longer

Many studies have attempted to define life span and division rate of virgin vs. memory cells by taking advantage of chromosome aberrations induced by radiation therapy (53, 54). However, these studies rely on the assumption that abnormal di- and acentric chromosomes can be produced only at the time of the initial event of radiation exposure, and this is not the case (discussed in 2). Therefore, we don't yet know the life span of a lymphocyte, and we can think of at least two ways in which it could be limited. It could have an intrinsic limit, like a red cell or a radioisotope, or it could be capable of living for a long time, given the right environment. Since populations of lymphocytes are hugely expandable, but the number remains relatively constant throughout life, the environment must be limited compared to the number of potential lymphocytes, a situation that could result in a form of musical chairs (55). Imagine, for example, that lymphocytes must traffic through certain areas or interact with certain stromal cells, that these resources are limited, and that the traffic is random. Cells expanding under the influence of environmental antigens would have an advantage over resting memory (and virgin) cells, which would slowly lose in the competition for the resource. Eventually, even long-lived resting memory cells would disappear. In this scenario, the persistence of memory is a result of the clonal expansion induced by the original immunization, not of any change in intrinsic lifespan.

In the adoptive transfer experiments, where we transferred 10–20 million T cells, the memory would disappear faster than in the original host, which contains 200 million T cells, because clones specific for other antigens expand and dilute the few transferred memory cells of interest (56). Conversely, memory would last forever in those cases where an environmental antigen cross-reacts.

These four possibilities to explain anti–H-Y memory maintenance can also be applied to the experimental results obtained by others (11–13). There are two cases that deserve comments. The first is a set of data in which it was found that the cells from an anti–H-Y transgenic mouse were able to persist in the absence of male antigen (14). This study suffers from the fact that cells are able to express two TCR-α chains. The most likely possibility is that the cells persist because the second set of α chains confer specificity to a wide range of environmental antigens. These types of studies should be repeated after obtaining the expression of the TCR transgene in a RAG1 or RAG2 Knockout background.

The second is a transfer study in which it was found that protective CTL memory cells disappeared in the absence of antigen (10). However, the authors show that a low level of memory cells can be seen in limiting dilution assays long after priming, though the ability to confer in vivo protection to a massive dose of virus is lost. We suggest that there are two functionally different categories of memory. The first is the ability to make a faster and more appropriate secondary response. The second is the ability to maintain a set of activated cells or a high level of antibodies. Protection from some pathogens requires persistently elevated levels of antibody. For example, protection from rabies virus (and tetanus toxin) disappears as the serum levels of specific antibodies drop. This is the reason that we revaccinate our dogs against rabies every two years and ourselves against tetanus every ten. In addition, as pointed out by Oehen et al. (10), protection from massive doses of some viruses, such as lymphocytic choriomeningitis virus, requires the presence of recently activated effector CTLs. Thus memory as assessed by protection will give different results depending on whether the challenge is one that allows the immune system time to gear up or whether it can only be met by an ongoing response.

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