

T Cell Memory Is Short-lived in the Absence of Antigen

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Summary

Immunological memory has generally been ascribed to the development of long-lived memory cells that can persist for years in the absence of renewed antigenic encounter. In the experiments reported here, we have adoptively transferred memory T cells in the presence and absence of priming antigen and assessed their functional survival. The results indicate that, in contrast to the traditional view, the maintenance of T cell memory requires the presence of antigen, suggesting that memory, like tolerance, is an antigen-dependent process rather than an antigen-independent state.

Memory of a first encounter with antigen, one of the defining characteristics of the immune system, can persist for years. Three different mechanisms have been proposed to account for the persistence of memory. The first suggests that antigen induces the responding lymphocyte to differentiate into a "memory cell", which has a very long life span compared to its virgin precursor (1, 2). The second proposes that the difference between a memory cell and its precursor does not reside in life span but in thresholds for stimulation. A memory cell, having altered levels of various adhesion molecules (3, 4), can be more easily stimulated than the precursor and will be maintained by periodic activation by crossreacting environmental antigens (5, 6), or antiidiotypic interactions (7). The third attributes memory to the antigen rather than the responding cell, suggesting that the original antigen is periodically re-introduced by recurring infection (8), or persists in specialized reservoirs (9, 10), causing continued stimulation of memory clones. Although it is well accepted that, in the case of many common pathogens, antigen persistence and re-infection occur and may act to maintain memory (for example in many human childhood virus diseases [8, 11]), memory to nonreplicating, noninfectious agents is usually thought to reside with long-lived memory cells that persist for years in the apparent absence of antigen (1, 2). We recently showed, however, that B cell memory to the nonreplicating antigen, DNP, wanes rapidly if primed B cells are transferred without additional DNP into adoptive hosts (12), indicating that nonreplicating antigens must also persist if memory is to be maintained. We now present evidence that is also true for T cells. Both helper and cytotoxic T cells maintain their ability to generate secondary responses only in the presence of original priming antigen.

Materials and Methods

Animals

Congenic strains of PVG rats that differ only in their κ light chain allotype were used. PVG-K1^a (PVG-Ciu) and PVG-K1^b (PVG-RI-a²) (13) were bred in the Institute for Biomedical Research (Füllinsdorf, Switzerland). C57BL/6 were supplied by Iffa-Credo (Lyons, France) while B6-PL-Thy-1.1 (Thy-1 distinct congenics) were bred and maintained at the Basel Institute breeding facility (Kaiseraugst, Switzerland).

Immunizations, Cell Preparations, and Adoptive Transfer

Helper Cell Experiment. Donor PVG-K1^a rats were primed intraperitoneally with 50 μ g alum-precipitated keyhole limpet hemocyanin (KLH)¹ + 10⁹ *Bordetella pertussis*, and boosted 4 wk later with 50 μ g soluble KLH. At least 2 mo after boosting, the thoracic duct of these rats was cannulated; at this time there is no transfer of antigen with this source of cells (12). Cells from the first 24 h of drainage were collected, washed, and injected (10⁸ thoracic duct lymphocytes = 5 \times 10⁷ T cells) into syngeneic recipients that had been irradiated with 550 cGy 1 d previously. Half of the rats were given 10 μ g of soluble KLH upon cell transfer. After 1, 3, 6, or 12 wk, to assay for remaining KLH-specific T helper activity, the rats were injected with 2 \times 10⁷ B cells from PVG-K1^b rats that had been primed with DNP-BSA, and they were then immunized intravenously with 50 μ g soluble DNP-KLH. The B cells were thoracic duct lymphocytes (TDL) depleted of T cells using W3/13 and OX-19 mAbs coated onto Dynabeads (DynaL AS, Oslo, Norway) (12). These antibodies recognize leukosialin (14) and CD5 (15), respectively. The response of donor B cells to

¹ Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; TDL, thoracic duct lymphocytes.

DNP was measured by a solid phase RIA (12) using radiolabeled K1^b-specific mAb LOR-K1^b (Serotec Ltd., Oxford, UK). The kinetics of the response of the donor B cells is used as a measure of T cell memory, as nonprimed animals support only a slow primary response from these cells (Gray, D., unpublished data).

Cytotoxic T Cell Experiment. B6.PLThy-1.1 female mice were primed by an intraperitoneal injection of 10⁷ syngeneic Mitomycin C-treated male cells. 2 mo later, T cells were purified from spleen and lymph nodes by two consecutive rounds of panning with mAbs to MHC class II and mouse Ig and a final selection with anti-Ig and anti-class II-coated Dynabeads. Antibodies for preparing the panning plates were a mixture of M5/114 (rat γ 2b, anti-MHC class II [16]) and sheep anti-mouse Ig serum (Cappel Laboratories, Cochranville, PA), 10 μ g/ml each in PBS. The Dynabeads (anti-rat Ig) were coated with M5/114 overnight, washed, and used directly together with Dynabeads coupled with anti-mouse Ig at a ratio of 40 beads to 1 target cell. FACS[®] analysis revealed contamination of donor with <2% potential APC (assessed by staining for Ig and class II). 5 \times 10⁶ to 10⁷ purified T cells were injected intravenously into female B6 *nu/nu* recipients, half of which also received 10⁷ Mitomycin C-treated male stimulator spleen cells intraperitoneally. From 6 to 28 wk later, between three and five mice from each group were boosted with a second intraperitoneal injection of 10⁷ Mitomycin C-treated male cells, and 2 wk later their spleen cells were tested for the ability to generate CTL specific for H-Y, and as a measure of general responsiveness, to allogeneic stimulators. By FACS[®] analysis the T cells in the recovered spleen cells were >95% of Thy-1.1 donor origin. For FACS[®] staining we used FITC-labeled anti-Thy-1.1 and Thy-1.2 (Becton Dickinson & Co., Mountain View, CA). Mitomycin C (Sigma Chemical Co., St. Louis, MO) was freshly prepared (5 μ g/ml) and incubated with spleen cells (10⁷/ml) for 40 min at 37°C. After washing, these cells were used for immunization.

CTL Assay

2 wk after in vivo boosting, spleen and lymph cells were stimulated in standard in vitro cultures at 4 \times 10⁶ cells per well against 2 \times 10⁶ irradiated (3,000 cGy) stimulator spleen cells. Stimulators were B6 male (for H-Y responses) and DBA/2 (H-2^d, Mls^s, and minor antigen different for allogeneic responses) spleen cells. After 5 d, the cultures were harvested and serial threefold dilutions of the responder cells were tested for lysis of 10⁴ ⁵¹Cr-labeled targets (spleen cells cultured for 2 d with 2 μ g/ml Con A). Targets were B6 male, B6.PL Thy-1.1 female (not shown; used as a syngeneic specificity control, lysis was never >10%), and DBA/2 female. The titration of effectors, shown as responder dilution, begins with a maximum E/T ratio of 66–135:1 calculated from the original number of responder cells initially cultured. The percent specific lysis was calculated as: 100 \times (experimental release – spontaneous release)/(maximal release – spontaneous release).

Replacement of T Cell Help during In Vitro MLR

B6 female spleen cells primed 2 mo earlier against H-Y were depleted of CD4 helper cells by a one-step treatment with rabbit complement at 1:25 and 1 μ g/ml each of two noncompetitive mAbs specific for CD4: GK1.5 and YTA 3.1.2 (a generous gift from Herman Waldmann, Cambridge, UK) used as a 50% ammonium sulfate cut of ascites fluid. After washing, the recovered cells were plated at 2 \times 10⁶ (or 4 \times 10⁶ for untreated cells) per well in 2-ml cultures along with varying numbers of irradiated (1,000 rad) Marilyn cells and 2 \times 10⁶ irradiated B6 male stimulators. Marilyn is a 3-yr-old old CD4⁺ T cell clone isolated from a female

(CBA/N \times B6)F₁ mouse that had been primed to F₁ male cells. It recognizes processed H-Y in the context of A^b and crossreacts on Mls^s. After this titration, we used Marilyn to replace any potential lack of T cell help in the chimeric mice. Thus, 4 \times 10⁶ spleen cells from recipients that had received H-Y-primed T cells without antigen 5 mo earlier were boosted and tested 2 wk later in culture for the generation of H-Y-specific killer cells in the presence or absence of 2,000 irradiated Marilyn cells.

Results and Discussion

Experimental System. To ascertain the importance of persisting antigen in the maintenance of T cell memory, we evaluated the decay of memory responses from populations of murine T cells that were removed from their original environment, purified of any residual antigen or antigen-carrying presenting cells and “parked” in adoptive hosts for various lengths of time. For both CTL and helper cells, we chose secondary responses that measured functional characteristics that are classically used to distinguish them from primaries. For T helper responses, we measured the rate and level of antibody produced in vivo by indicator B cells to the hapten-carrier complex, DNP-KLH, and for CTL, we tested the generation of in vitro cytotoxic responses to the minor histocompatibility antigen, H-Y.

Maintenance of Helper T Cell Memory. Fig. 1 A shows the protocol to test T helper activity. Taking advantage of two rat strains, PVG.K1^a and PVG.K1^b, which differ only by their antibody light chains, we were able to use a standard hapten-carrier system to selectively test T helper activity in whole lymphocyte populations transferred into adoptive hosts. TDL from KLH-primed PVG.K1^a rats were collected 2 mo after immunization, at which time they consist of B and T cells but few, if any, antigen-carrying nonlymphoid cells. TDL were transferred into irradiated syngeneic hosts with or without a simultaneous injection of KLH. The sublethal dose of irradiation used (550 cGy) is sufficient to create space in lymphoid tissues for transferred cells but allows regeneration of the host peripheral lymphocytes from the bone marrow or surviving peripheral cells within 7 d (12). 1–12 wk after the transfer, we supplied the recipients with DNP-primed indicator B cells from PVG.K1^b rats, challenged them immediately with DNP-KLH, and followed the production of DNP-specific antibody of the K1^b allotype. If T cell memory requires the presence of antigen, secondary responses to KLH should decay rather rapidly in the antigen-free hosts but persist in hosts supplied with KLH at the time of initial transfer. However, if memory is an antigen-independent, long-lived differentiation state, a secondary response should persist in both groups.

Fig. 1 B shows the rate and levels of K1^b anti-DNP antibody produced by primed indicator B cells injected after 1, 3, 6, and 12 wk. At 1 wk, there was little difference between rats that were or were not given KLH at the time of transfer: both groups responded with classical, rapid, secondary kinetics. By 3 wk, responses from the antigen-free group were clearly weaker, and by week 6, their response reverted to the slow production expected of a primary response. This pri-

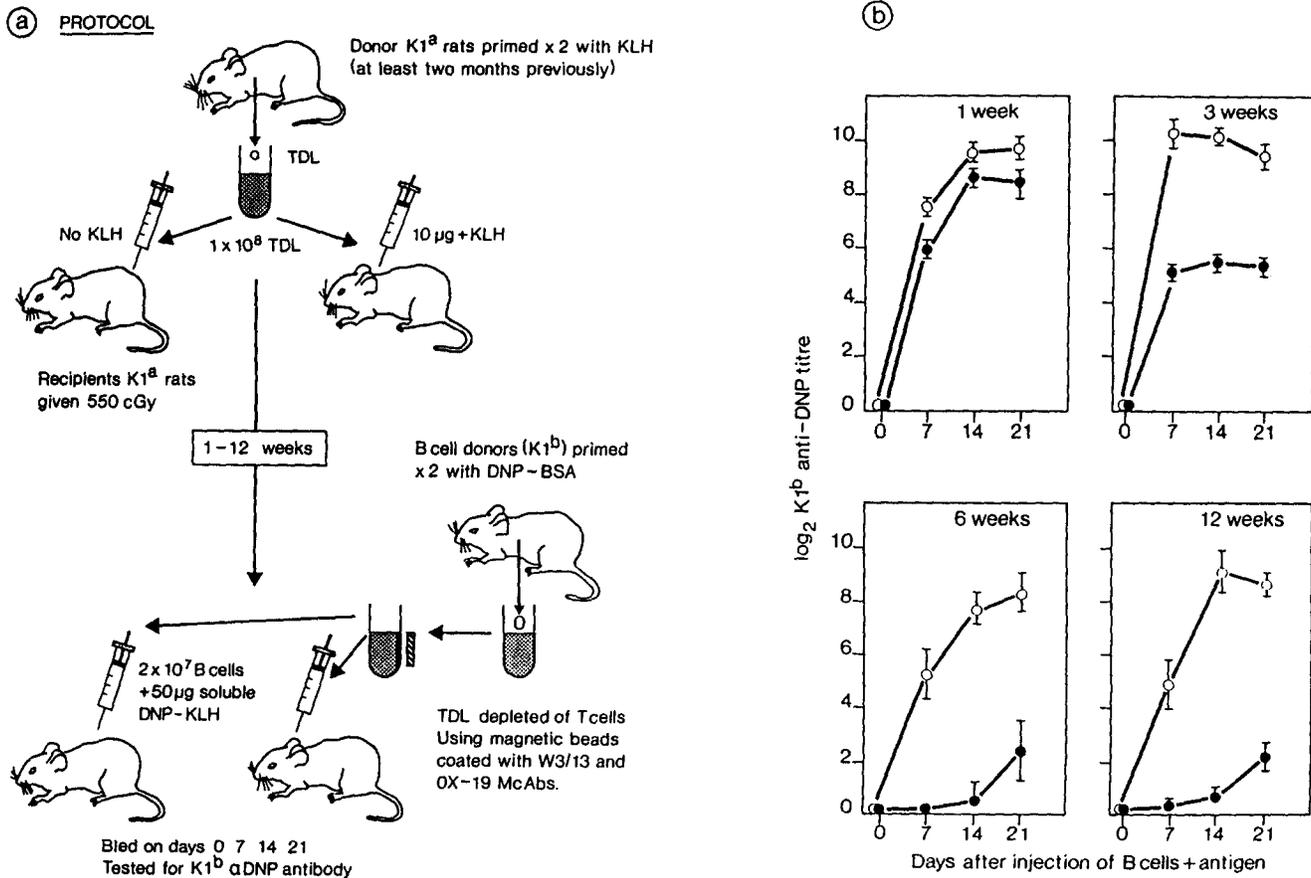


Figure 1. Memory to KLH wanes in the absence of antigen. (a) A schematic representation of the experimental protocol used to investigate the antigen dependence of memory T helper cells. (b) The ability of KLH-specific T helper cells to support a DNP-specific B cell response 1, 3, 6, or 12 wk after transfer either with or without antigen, into adoptive hosts. As a measure of T helper cell activity in vivo, we follow the kinetics of anti-DNP antibody production from 2×10^7 K-allo type ($K1^b$) distinct B cells after co-injection into these rats with $50 \mu\text{g}$ soluble DNP-KLH. Open circles represent the responses in rats that received KLH with T cells, and the closed circles represent those that did not. Mean and SDs of 6–10 animals are plotted.

primary response, most likely due to direct priming by the DNP-KLH challenge of new T cells produced by the regenerating host, showed that the host environment had not become hostile to introduction of new indicator B cells. These rats simply no longer contained memory T cells able to help for a secondary response. In contrast, animals given KLH at the time of original transfer maintained the ability to help for at least 12 wk. Thus, by a functional test, the presence of antigen seems to be vitally important for maintaining T cell help.

Maintenance of Cytotoxic T Cell Memory. To test memory in CTL, we chose the in vitro response of female B6 mice to the male-specific antigen, H-Y, a situation where the difference between primary and secondary responses is virtually absolute; normal, unprimed female mice are unable to generate any CTL specific for H-Y in in vitro culture, even with the addition of soluble helper factors, whereas mice primed by a single injection of male cells can generate excellent in vitro responses for the rest of their lives. The experimental protocol was essentially the same as above, except that spleen and lymph node cells were used as responders and athymic nude mice as recipients. We purified T cells from B6.PLThy-

1.1 mice that had been primed 2 mo previously with a single injection of Mitomycin-treated syngeneic male cells, transferred them with or without additional Mitomycin-treated male cells into B6 *nu/nu* recipients, and waited for various lengths of time. We then boosted the mice again in order to expand any residual memory cells and tested them 2 wk later for their ability to generate CTL responses in vitro. Fig. 2 shows a time course for the decay of the anti-H-Y response. At 6 wk, there was little difference between mice that did or did not receive H-Y at the time of transfer, but by 16 wk, the response of the antigen-free group had waned to a barely detectable level. The group that received antigen continued to respond well for at least 7 mo. It should be noted that all mice, irrespective of whether they were given male cells upon transfer, respond to allogeneic cells, demonstrating the maintenance of functional T cells in these mice. One mouse from the antigen-free group responded at 7 mo (mouse 7; 28 wk). While we believe that this is due to the carry over of a small amount of antigen and the subsequent expansion of a dominant clone, we cannot exclude that a very small number of memory cells are truly antigen independent. For

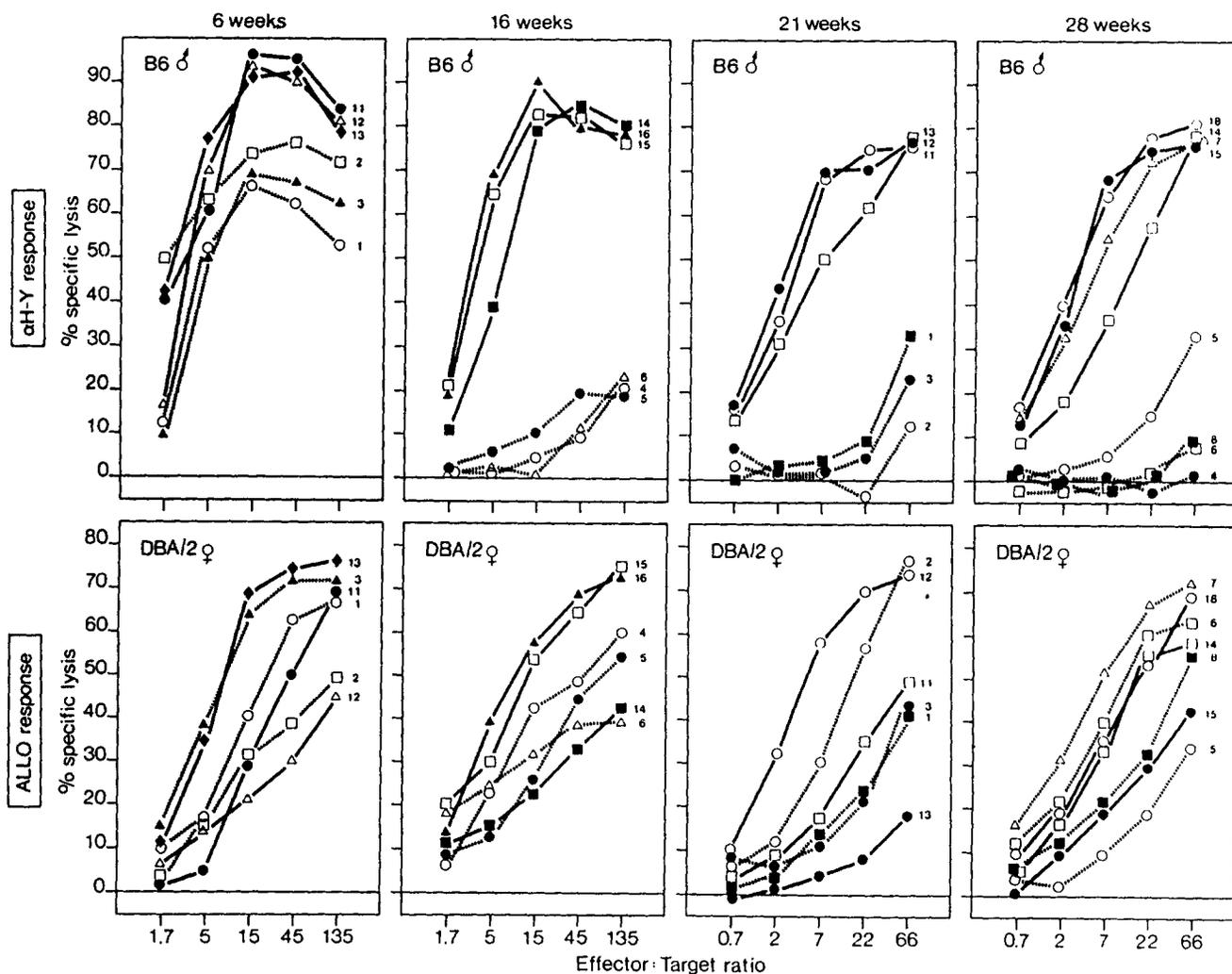


Figure 2. Memory to H-Y wanes in the absence of antigen. H-Y-primed T cells, transferred into nude recipients with or without a simultaneous injection of H-Y-bearing cells, were boosted with male cells at the times indicated over each panel, cultured 2 wk later in standard 5-d MLC, and tested for cytotoxicity against both male and allogeneic target cells. Solid lines represent responses from recipients that received H-Y at the time of transfer; broken lines represent responses of recipients that received primed T cells but no additional antigen. Responses of individual mice are plotted. We point out that the fact that the mice at 6 and 21 wk have the same numbers has no meaning. This occurred as the chimeras were set up on different days.

the most part, despite an expansionary stimulus given *in vivo* before testing for responses *in vitro*, we saw no significant long-term memory to H-Y.

Memory CTL Are Not Revealed by Addition of H-Y-specific Helper T Cells. Our failure to detect an anti-H-Y CTL response may only reflect lack of specific T cell help during the *in vitro* restimulation. To check this, we added a helper T cell clone at the start of the 5-d culture. The T helper clone, Marilyn, which recognizes H-Y in the context of A^b, is able to replace the help needed *in vitro* by primed normal CTL from which the CD4 T helper cells have been depleted (Fig. 3 A). Addition of Marilyn enhances the *in vitro* response of cells from a recipient (mouse 2) that exhibits residual anti-H-Y activity (Fig. 3 B) but will not restore activity to a mouse (mouse 1) in which anti-H-Y memory has waned completely

(Fig. 3 B). In this experiment, we did not inject the H-Y-specific helper T cell clone *in vivo* before testing for memory CTL, and so we cannot rule out the possibility that the loss of CTL response is a reflection of a lack of specific T cell help *in vivo*. Thus, while *in vivo*, the long-term maintenance CTL memory may depend upon T helper memory; in functional terms, memory loss in the absence of antigen is also a characteristic of CTL.

Clearly, in the absence of antigen, T cells lose their ability to mount characteristic secondary responses; the responses revert to primaries in regenerating hosts, (Fig. 1) or are lost altogether in nonregenerating hosts (Fig. 2). Thus, memory, the immune system's ability to mount a faster, more effective response the second time it is challenged by an antigen, requires the persistence of the original antigen. We do not know

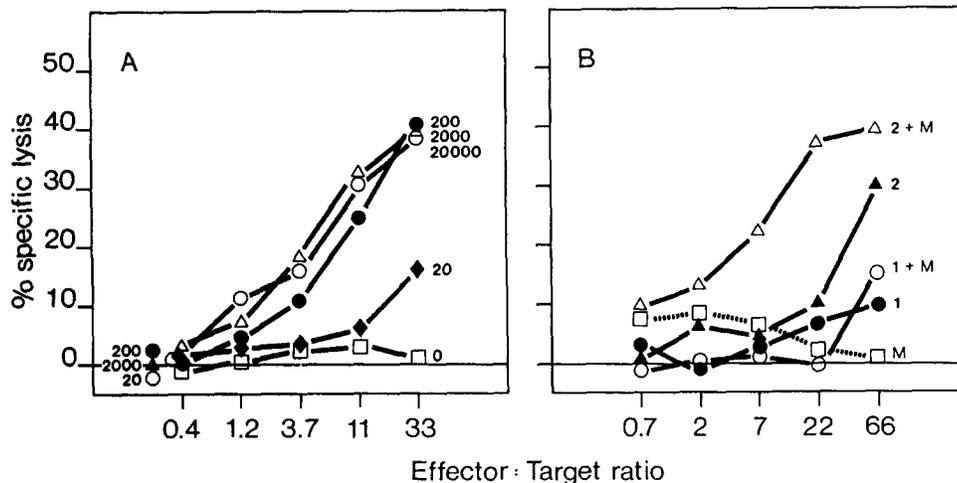


Figure 3. T cell help added in vitro does not restore CTL memory loss. (A) The potency of the H-Y-specific T helper clone, Marilyn. At the beginning of a 5-d MLR, graded numbers of this clone were added to H-Y-primed spleen cells from which the CD4 T helper cells had been removed. Symbols represent 20,000 cells (○), 2,000 cells (△), 200 cells (●), 20 cells (◆), or no Marilyn cells (□) added. The level of killing activity generated by Marilyn when stimulated alone against B6 males is indicated (●, ▲, ○). (B) Marilyn's inability to restore activity to spleen cells from long-term "parked" recipients. We added 2,000 Marilyn cells to the cultures at time 0. At day 5, the cultures were tested for lysis. Symbols represent mouse 1 cells alone (●), mouse 1 cells + Marilyn (○), mouse 2 cells alone (▲), mouse 2 cells + Marilyn (△), and Marilyn alone (□). It should be noted that mouse 1 gives no H-Y-specific CTL response either with or without additional T cell help in vitro, while mouse 2 exhibits a poor but measurable response in vitro that is enhanced upon addition of the helper clone, Marilyn.

whether each new encounter of a memory cell with persisting antigen leads to cell division. The signal may simply allow survival. However, taken together with the recent evidence that "memory" cells and newly activated cells express the same surface markers (4, 17-21), our data suggest that the memory state is probably marked by a certain level of continuous activation rather than by a vastly lengthened life span. Indeed, experiments have shown that massive proliferation seen immediately after antigen exposure settles down with time to a constant low-level turnover of memory cells (22). We should point out two things about these experiments; first, the rate of decay that we observe after adoptive transfer cannot be used as an accurate measure of the half-life. Second, these experiments, performed in clean animal facilities, stress the importance of persisting antigen, however, in a "wild" population, re-exposure to the original or a cross-reacting

antigen may also play a role. We also feel that it would be unfair to associate the name of Celada with the concept of long-lived memory cells, as the notion of memory loss in the absence of antigen was suggested by him 20 yr ago (1).

Finally, there is the question of how a nonreplicating antigen can persist. At present, the only known repositories are on the surface of follicular dendritic cells, specialized dendritic cells found only in B cell follicles, which can maintain native antigens as antigen-antibody complexes for months if not years (9, 10). Should no other depots be found, an inescapable conclusion is that the survival of T cell memory must be dependent on the production of antibody and on antigen presentation by memory B cells (23). This has strong implications for the design of peptide vaccines, even to antigens that (like H-Y) have previously been thought to elicit no antibody.

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