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Anergy and Exhaustion Are Independent Mechanisms of Peripheral T Cell Tolerance

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Summary

We studied the interactions of male-specific T cell receptor (TCR)-α/β-transgenic (TG) cells with different concentrations of male antigen in vivo. We constructed mouse chimeras expressing different amounts of male antigen by injecting thymectomized, lethally irradiated mice with various ratios of male (immunoglobulin [Ig] H^a) and female (IgH^b) bone marrow. These chimeras were injected with male-specific TCR-α/β-transgenic cells. These experiments allowed us to monitor antigen persistence and characterize antigen-specific T cells in terms of their frequency, reactivity, and effector functions (as tested by elimination of male B cells in vivo). In the absence of antigen, virgin TG cells persisted but did not expand. Transient exposure to antigen resulted in cell expansion, followed by the persistence of increased numbers of antigen-reactive T cells. In contrast, antigen persistence was followed by two independent mechanisms of tolerance induction: anergy (at high antigen concentrations), where T cells did not differentiate into effector functions but persisted in vivo as unresponsive T cells, and exhaustion (at lower antigen concentrations), where differentiation into effector functions (B cell elimination) occurred but was followed by the disappearance of antigen-specific T cells.

The mechanisms underlying the generation and maintenance of secondary ("memory") T cell responses are unclear. T cell memory may result from the presence of an increased frequency of antigen-specific cells (1). It is also possible that antigen stimulation may induce changes in "virgin" T cells and generate a memory T lymphocyte, of poorly characterized properties (2–4). Classically, in vivo antigen immunization should induce cell division, but eventually a fraction of antigen-specific T cells would revert to the resting state and persist by increased cell survival. Thus, virgin T cells would be short-lived and memory cells long-lived, the latter forming the basis of long-term memory responses (5).

These concepts were challenged by recent in vivo studies. First, it was shown that virgin T lymphocytes can persist as nondividing cells (6–9), whereas immunization occasionally led to the rapid disappearance of antigen-reactive T cells (10–12). It has also been suggested that activated T cells cannot survive in the absence of antigen. In these circumstances, long-term memory, rather than resulting from the generation of long-lived cells, might be due to antigen persistence and continued cell division (13–15). Other experimental results suggested, however, that survival of memory cells might take place in the absence of antigen (16, 17).

In experimental systems where accurate follow-up of antigen-specific cells is possible, confrontation with antigen usually leads to tolerance rather than to secondary responses. Studies of TCR-transgenic (TG)[1] mice (6, 7, 18) and immune reactivity to superantigens (19, 20) have shown that immunization, besides inducing T cell activation and expansion, can render antigen-specific cells unresponsive (anergic) to further antigen stimulation (6, 7, 19), and/or lead to the disappearance of antigen-specific cells (18). Anergy and the disappearance of antigen-specific cells were both interpreted as being part of the same process and a consequence of extensive cell division (18). Tolerance induction in these and other systems was attributed to inadequate helper activity (10, 21) and to differences in antigen dose (18) or presentation (22). It is difficult to extrapolate most of these results, since not all the parameters of the immune response were evaluated simultaneously.

We have constructed thymectomized male/female mouse bone marrow (BM) chimeras (repopulated with different numbers of male BM cells) and used them to study interactions of TG TCR-α/β male-specific cells with different amounts of male antigen in vivo. Using this approach, we were able to induce either tolerance or immune responses, study antigen persistence, and follow the frequency, func-

[1] *Abbreviations used in this paper:* BM, bone marrow; FSC, forward scatter; MöTG, T cell populations monoclonal for the TCR-TG receptor; SIg, serum Ig; TG, transgenic; TX, thymectomized.

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tional reactivity, and effector functions of antigen-specific T lymphocytes.

Materials and Methods

Mice. C57Bl/6 Thy1.1 or Thy1.2 mice TG for a TCR-α/β specific for the male antigen have been described elsewhere (23, 24). In some experiments, TG RAG2−/− mutant mice were also used. These and syngeneic C57BL/6-IgH-C^b mice were obtained from Centre de Service des Animaux de Laboratoire (CSAL) (Orléans, France) C57BL/6-IgH-C^a mice congenic for the IgM heavy chain locus were obtained from the breeding colonies of the Institut Pasteur.

Mouse Chimeras. 8-wk-old thymectomized (TX) B6 mice were irradiated (850 rads) and reconstituted with 5 × 10^6 T cell–depleted BM cells containing different percentages of male B6.IgH-C^a and female B6.IgH-C^b cells. Where noted, these mice were injected with Ig^- lymph node cells from normal, TG, or TG RAG2−/− mice 24 h after BM transfer.

Cell Labeling. We used the following mAbs for cell surface staining (6): F23.2 (anti-TCR-β TG chain β_T) (23) and T3.70 (anti-TcR-α TG chain α_T) (24); H35-17-2 (anti-CD8-β chain) (25); 19XE5 (anti-Thy1.1) (26); 30H12 (anti-Thy1.2); anti-B220 (PharMingen, San Diego, CA) (27); RS3.1 (anti-μ^a) (28); MB8.6 (anti-μ^b) (29); 331 (anti-IgM); 1M781 (anti-CD44) (10). These antibodies were used directly coupled to PE, FITC, or biotin (revealed with streptavidin-tricolor) or -PE [CALTAG Labs, South San Francisco, CA]). Cells were analyzed on a FACScan by use of the Lysis II (Becton Dickinson & Co., Mountain View, CA) program.

Identification of TG Populations in Recipient Mice. In these studies, we identified $CD8^+$ populations from female TG mice by use of an anti-CD8 mAb that recognizes the β chain of the CD8 molecule (25). This antibody was used (rather than an anti-CD8α chain antibody) since its expression is restricted to CD8 populations generated in the thymus. Expression of CD8α can be induced in multiple cell types by activation events at the periphery.

The absolute number of male-specific TG T cells was evaluated as described elsewhere (6). Briefly, the total number of TG $CD8^+$ cells was calculated from the percentage of $CD8^+$ TG lymphocytes and the total number of cells recovered from the spleen. This number, plus the percentage of CD8-expressing α_T, was used to calculate numbers of male-specific lymphocytes in the spleen. To determine the percentage of $CD8^+$ cells expressing α_T, cell suspensions were first depleted of B and $CD4^+$ lymphocytes by magnetic sorting with coated Dynabeads (Dynal, A.S., Oslo, Norway), and stained with anti-TCR-α_T mAb T3.70 and anti-CD8β mAb. Since these were kinetic experiments (in which the same injected cell suspension was studied at different time points after transfer), all recipient mice could not be analyzed simultaneously, and slight fluctuations of labeling intensity between different experiments were unavoidable. To overcome this handicap, T cell populations recovered after transfer were compared in each experiment with those from one female TG mouse analyzed simultaneously. CD8$\alpha_T{}^+$ can be readily visualized in female TG $CD8^+$ cells (24). CD8$\alpha_T{}^+$ cells in recipient mouse chimeras were identified as those expressing similar levels of TCR-α_T.

PCR Amplification of Y Chromosome–specific Genes. Spleen and BM cells (5 × 10^6) from individual mouse chimeras were pelleted and incubated in lysis buffer (0.5% Tween 20, 50 mM KCl, 2.5 mM $MgCl_2$, 600 μg/ml proteinase K in 10 mM Tris-HCL, pH 7.5) for 1 h at 55°C, followed by 10 min at 100°C. The lysate was spun for 5 min at 10,000 rpm, and the DNA in the supernatant was extracted with phenol-chloroform and ethanol precipitated. For PCR analysis, 10 μl of DNA dilutions corresponding to different cell numbers was added to a total 50-μl reaction mixture containing 200 μM each dNTP, 10 mM Tris-HCL, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 2 U of Taq polymerase (Bioprobe International, Inc., Tustin, CA) and 500 ng of each nucleotide primer. Amplification consisted of 35 cycles at 94°C for 10 s, 65°C for 30 s, and 72°C for 30 s in a thermocycler (model 9600; Cetus Corp., Berkeley, CA). A 15–20 μl aliquot was electrophoresed on a 4% Nusieve agarose (FMC BioProducts, Rockland, ME) TBE gel. Primers for the Y chromosome *Zfy-1* gene were (5'-3'): CCTATTGCATGGACTGCAGCTTATG and GACTAGACATGTCTTAACATCTGTCC (30). DNA from male cells was titrated and, in the conditions used, we were able to amplify the *Zfy-1* gene from the DNA corresponding to a single male cell.

In Vitro Proliferation. 5 × 10^4 $CD4^-$ SIg^- spleen cells were cultured with 5 × 10^5 irradiated (1,800 rad) spleen cells from male or female nude mice. 3 d later, cultures were supplemented with 10 U/ml of rIL-2 and expanded for a further 2 d. At the end of this period, cell growth was evaluated by [^{3}H]thymidine incorporation (1 mCi/culture) (7).

Results

Experimental Protocol

In these experiments, we produced mice expressing different amounts of male antigen. These mice were injected with TG T cells expressing a male-specific ($\alpha_T\beta_T$) TG receptor and studied at different time points after cell transfer.

Host Mice Expressing Different Amounts of Male Antigen. TX lethally irradiated C57BL/6 (B6) mice were injected with a total of 5 × 10^6 T cell–depleted BM cells containing female (B6.IgH-C^b) and 10, 50, or 90% male (B6.IgH-C^a) congenic BM cells. These BM donor mice differ in their IgM allotype, a property used to identify B cells and secreted IgM of male (μ^{a+}) or female (μ^{b+}) origin in the host mice. 1–2 mo after BM injection, we studied the distribution of female and male B cells in the BM, spleen, and lymph nodes, as well as the presence of the female or male IgM allotype in the serum of host mice. The peripheral B cell compartment was fully reconstituted. The percentage of μ^{a+} male B cells in the B cell pools and that of the μ^{a+} allotype in the serum of these mice was identical to the percentage of male BM cells injected in the BM inoculum, that is, these mice contained 0, 10, 50, or 90% of male B cells. Although the precise quantitation of any antigen is not possible in vivo (because of unknown variables related to the rates of antigen processing and presentation, etc.), the 10–90% range of male BM cells injected ensured significant variations in the total amount of HY antigen present in the different mouse chimeras.

T Cell Populations Expressing a TG TCR-α/β Specific for the Male Antigen. T lymphocytes injected into host chimeras were TG for an TCR-α/β receptor ($\alpha_T\beta_T$) specific for the male antigen. Recognition of the male antigen by TG cells requires coexpression of high surface levels of TCR-$\alpha_T\beta_T$ and CD8. In male TG mice, male-reactive T cells are eliminated in the thymus. Peripheral T cells that escape deletion are tolerant of the male antigen and express little or no CD8 ($\alpha_T\beta_T CD8^{lo}$). Female TG mice contain a population of

male-reactive T cells ($\alpha_T\beta_T CD8^{hi}$) that is positively selected in the thymus and represents 25–50% of the $CD8^+$ peripheral T cell pool. Both female and male mice also have T cells ($CD4^+$ or $CD8^+$) expressing endogenous TCR-α chains ($\alpha_E\beta_T$) that are not specific for the male antigen (23, 24). In some experiments, T cell populations injected were from female TG RAG2−/− mutant mice. These mice do not rearrange endogenous T cell receptors, and all T cells injected exclusively expressed the TG TCR-$\alpha_T\beta_T$.

In Vivo Elimination of Male Cells by TG-TCR Male-specific T Cells

Injection of tolerant T cells from male TG mice had no effect on the number of male B lymphocytes recovered from chimeric mice (Fig. 1 *A*). In contrast, injection of T cells from female TG mice (that contained a male-reactive $\alpha_T\beta_T CD8^{hi}$ population) was able to induce the disappearance of μ^{a+} male B cells (Fig. 1 *B*). Elimination of B cells was dependent on their expression of the male antigen, as the female TG population never affected female B cells: the loss of the μ^{a+} male population was readily compensated for by the increase in μ^{b+} female B cells, and both the total number of B cells and serum IgM levels were similar in all chimeras studied.

T cells from female TG mice injected into male/female chimeras contain male-reactive $\alpha_T\beta_T CD8^{hi}$ as well as cells expressing endogenous T cell receptors. To verify if elimination of male B cells in these chimeras was due to the effect of the male-reactive $\alpha_T\beta_T CD8^{hi}$ cells, we performed two types of experiments. First, male/female chimeras were injected with similar numbers of T lymphocytes from female B6 mice, but these T cells were found to have a minimal effect on male B cells (Fig. 1 *D*). Second, the same chimeras were injected with a monoclonal $\alpha_T\beta_T CD8^{hi}$ population, obtained from female TG RAG2−/− mice. As shown in Fig. 1 *E*, these monoclonal T cells eliminated male B cells in vivo and, on a per cell basis, they were actually far more efficient than T populations from TG polyclonal mice. These findings show that male-reactive $\alpha_T\beta_T CD8^{hi}$ T cells can reject target hematopoiesis-derived cells expressing the male antigen in vivo.

We cannot determine the precise mechanism by which male B cells are eliminated in vivo. As $\alpha_T\beta_T CD8^{hi}$ T cells are unable to induce polyclonal B cell activation and thus to induce terminal B cell differentiation (von Boehmer, H., personal communication), it is likely that B cell elimination in vivo results from direct cytotoxicity of this $CD8^+$ TG population for male B cells and/or their immature BM precursors. We conclude that $\alpha_T\beta_T CD8^{hi}$ T cells are able to mediate effector functions and reject hematopoiesis-derived cells expressing the male antigen in vivo.

These eliminated male cells may be either BM precursors or mature B cells. The present protocol thus mimics the confrontation of T cells with replicating antigen during infection or BM transplantation. Depending on how the immune system deals with the initial cohort of antigen, the antigen load may gradually increase or diminish.

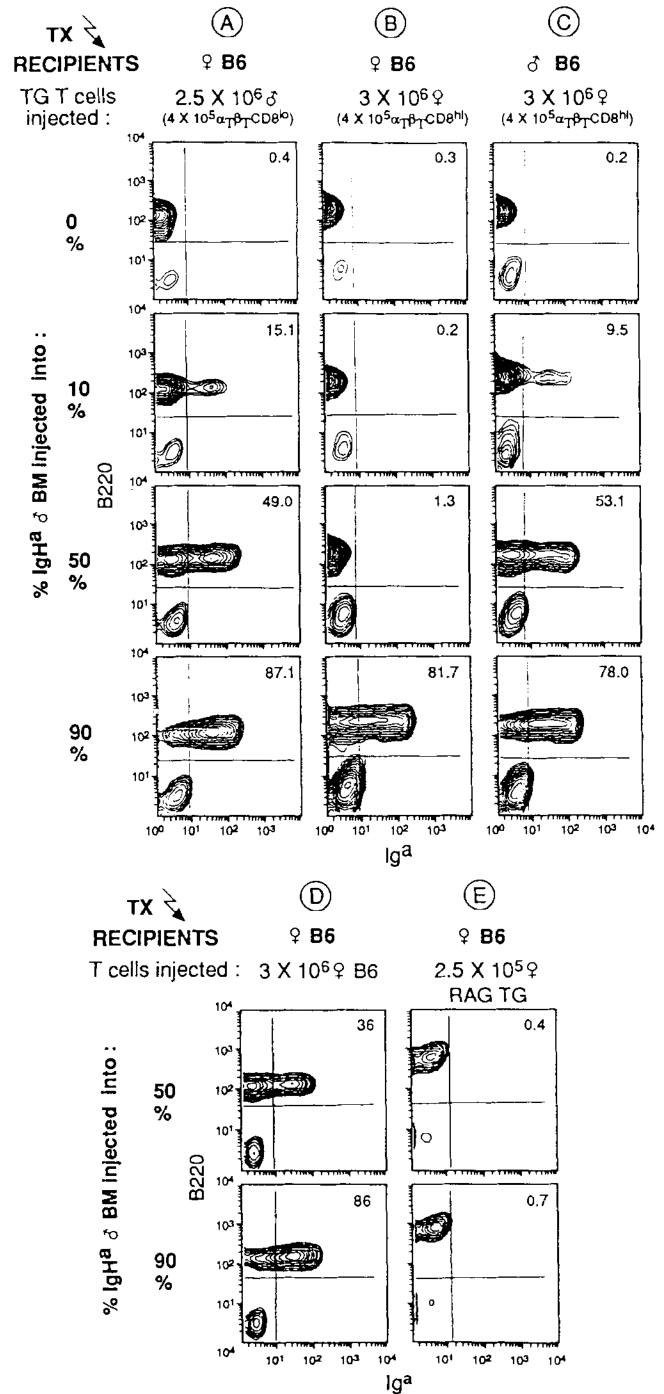

Figure 1. Frequency of male (μ^a) B cells in the spleen of chimeric mice 1 mo after BM reconstitution. Female (*A*, *B*, *D*, and *E*) and male (*C*) TX irradiated mice, reconstituted with 0, 10, 50, and 90% IgH^a male BM cells, were injected i.v. with (*A*) 2.5 × 10^6 T cells from male TG mice (containing 4 × 10^5 $\alpha_T\beta_R CD8^{lo}$ T cells), (*B* and *C*) 3 × 10^6 T cells from female TG mice (containing 4 × 10^5 $\alpha_T\beta_T CD8^{hi}$ T cells); (*D*) 3 × 10^6 T cells from female B6 mice, or (*E*) 2.5 × 10^5 $\alpha_T\beta_T CD8^{hi}$ monoclonal T cells from female TG RAG2-deficient mice and studied 1 mo (*A–C*) and 6 wk (*D* and *E*) later. Data are for one of three mice studied at these time points with similar results.

The Amount of Male Antigen Determined the Fate of TG T Cells, As Well As Their Capacity to Eliminate Male B Cells

Female TX Mice Reconstituted With 100% Female BM Cells. T cells from female TG mice transferred into these chimeras behaved in the same way as after adoptive transfer into female nude mice (6–8, 31). In the absence of the male antigen, male-specific $\alpha_T\beta_T$CD8^{hi} cells never expressed CD44 (Fig. 2 *B*) and did not expand (Fig. 3): the number of $\alpha_T\beta_T$CD8^{hi} cells recovered did not vary with time after injection and corresponded to the fraction of donor T cells expected to home to lymphoid organs after intravenous injection (32). Because TG cells expressing other TCR specificities expanded in these mice, $\alpha_T\beta_T$CD8^{hi} cells became a minority population after adoptive transfer. They could, however, be detected by FACS® analysis (Fig. 2 *B*) as well as by their capacity to proliferate to the male antigen in vitro, a property that persisted for at least 4 mo after transfer (Table 1). These results suggest that T cells never exposed to nominal antigen (virgin T cells) can persist in vivo for long periods (6–9, 31).

In the presence of male antigen, that is, in the male/female chimeras, all $\alpha_T\beta_T$CD8^{hi} cells expressed CD44 (Fig. 2 *C*) and their numbers increased. The kinetics of cell expansion differed from what we previously described after transfer into nude mice (6, 7), since it started by 2-3 wk after transfer and was maximal after 1 mo (Fig. 3). The fate of these cells, as well as their effect on male B cells, varied in the different experimental groups.

Female TX Hosts Reconstituted With 10% Male BM. In these chimeras, injection of $\alpha_T\beta_T$CD8^{hi} TG cells resulted in the elimination of μ^{a+} male B cells, which were rare up to 3 wk after BM injection and undetectable thereafter (Fig. 1 *B*). No μ^a Ig was then found in the serum of these mice. The $\alpha_T\beta_T$CD8^{hi} expanded population persisted (Figs. 3 and 4), maintaining the capacity to respond to the male antigen for up to 5 mo after transfer (Table 1). As we could not detect male B cells or serum μ^a later in these chimeras, whereas the male-reactive expanded population persisted, these results indicate that previously activated T cells might have survived in the absence of antigen.

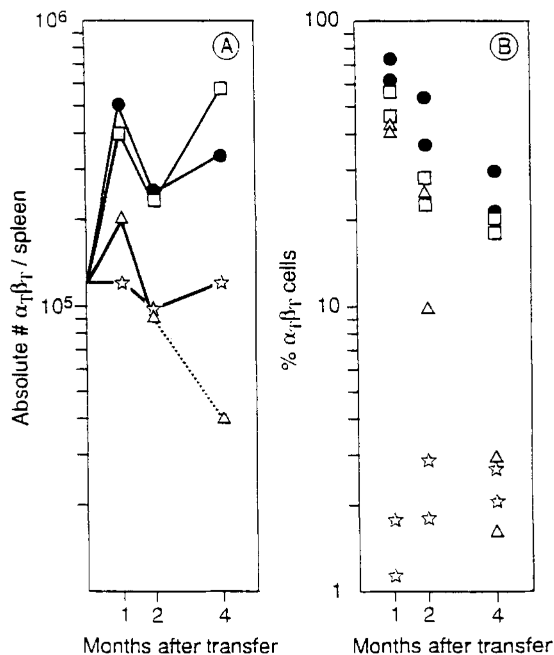

Figure 3. Fate of $\alpha_T\beta_T$CD8^{hi} T cells after transfer into female TX recipients reconstituted with 0 (☆–☆), 10 (□–□), 50 (Δ–Δ), or 90% (●–●) male BM. Results are from one experiment in which mice were studied from 2 wk to 5 mo after BM transfer. In total, 97 mice were studied in three independent experiments, with 2–3 mice per experimental group and time point. (*A*) Number of $\alpha_T\beta_T$CD8$^+$ T cells in the spleen (mean of 2 mice) evaluated as described in Materials and Methods. All mice had similar numbers of TG CD8$^+$ lymphocytes in the spleen, but the percentage of $\alpha_T\beta_T$ cells varied. In mice reconstituted with 50% male BM, we could not identify $\alpha_T\beta_T$CD8$^+$ cells as distinct populations in the spleen 4 mo after BM injection: the proportion of $\alpha_T\beta_T$ within CD8$^+$ cells was <0.8%. (*B*) Percentage of $\alpha_T\beta_T$CD8$^+$ cells in the BM of the same mice. We did not evaluate absolute numbers of male-specific cells in BM, since we found exhaustive BM suspensions difficult to obtain and quantification of cell yields inaccurate. We found variations in $\alpha_T\beta_T$ expression in mice reconstituted with 50% male BM. Thus, data for individual mice are shown rather than means.

In Chimeras Reconstituted With 50% of Male BM, Elimination of Male B Cells Was Never Complete. Maximal depletion was seen 1–2 mo after BM reconstitution, and even then 1–3% of μ^{a+} male B cells remained (Fig. 1 *B*). The initial expansion of the $\alpha_T\beta_T$CD8^{hi} population was followed by a rapid decrease: 2 mo after transfer, male-specific cells were rare in the spleen and diminished in the BM; they were absent from both organs later on (Figs. 3 and 4). At these later times, T cells from these mice were unreactive to the male antigen (Table 1), confirming that $\alpha_T\beta_T$CD8^{hi} cells were absent. It should be noted that disappearance of male-specific T cells from these chimeras was not preceded by downregulation of the CD8 molecule at the cell surface (Fig. 4). In contrast, during anergy induction, downregulation of CD8

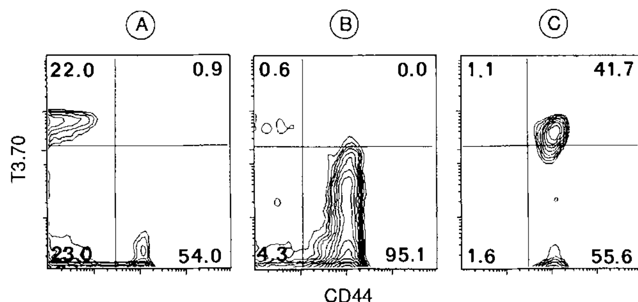

Figure 2. CD44 expression by TG CD8$^+$ T lymphocytes recovered from a female TG mouse (*A*), TX irradiated mice reconstituted with 100% female BM cells (*B*), or 10% male BM cells (*C*) 5 mo after lethal irradiation and BM transfer. All mice were studied simultaneously. Spleen cell suspensions were depleted of SIg$^+$ and CD4$^+$ T cells and triple-labeled with the mAbs T3.70, anti-CD8, and anti-CD44. The figure shows CD44 and T3.70 expression by gated CD8$^+$ T cells. Male-specific cells expressing high levels of TG TCR-α chain (T3.70^{hi}) can be readily visualized in female TG mice (*A*). $\alpha_T\beta_T$CD8^{hi} T cells in chimeric mice were those expressing similar levels of the TG TCR-α chain.

Table 1. *In Vitro Response of Spleen Cells from Mouse Chimeras in the Presence of Male Antigen*

Percent male BM injected	Time after transfer					
	1 mo		4 mo		5 mo	
	♀ *nu/nu*	♂ *nu/nu*	♀ *nu/nu*	♂ *nu/nu*	♀ *nu/nu*	♂ *nu/nu*
0	3,688	20,645	5,840	23,520	ND	ND
10	5,725	45,979	1,346	18,146	4,472	73,779
50	4,827	9,761	1,713	1,588	1,079	512
90	7,540	17,989	2,788	3,184	5,095	7,429

Female TX irradiated hosts were injected with 5 × 10^6 T cell–depleted BM containing 0, 10, 50, or 90% cells from IgH^a male donors; 24 h later, these chimeras received 4 × 10^5 $\alpha_T\beta_T CD8^{hi}$ TG T cells. At different times (mo) after transfer, 4 × 10^4 $CD4^-SIg^-$ spleen cells from each individual chimera were cultured with 5 × 10^5 irradiated (1,800 rad) spleen cells from either male or female (*nu/nu*) B6 mice, in the presence of rIL-2 (7). Values represent the mean [^{3}H]thymidine uptake of triplicate cultures. Similar results were obtained in all mice studied in two different experiments.

expression preceded partial deletion of male-specific TG cells (6; see also Fig. 4 and below).

The disappearance of $\alpha_T\beta_T CD8^{hi}$ cells from these chimeras was not due to the absence of antigen: (*a*) male B cells were always detected (Fig. 1 *B* and below); (*b*) the amount of male antigen was sufficient to induce the expansion of a new set of virgin T cells (see below); and (*c*) in some mice, a substantial fraction of male hematopoietic precursors must have survived elimination by effector T cells since, after the disappearance of $\alpha_T\beta_T CD8^{hi}$ cells, the frequency of male B cells in some mice increased again (Fig. 5). These results demonstrated that the continuous presence of the male antigen was not sufficient for the survival of a male-reactive TCR TG population.

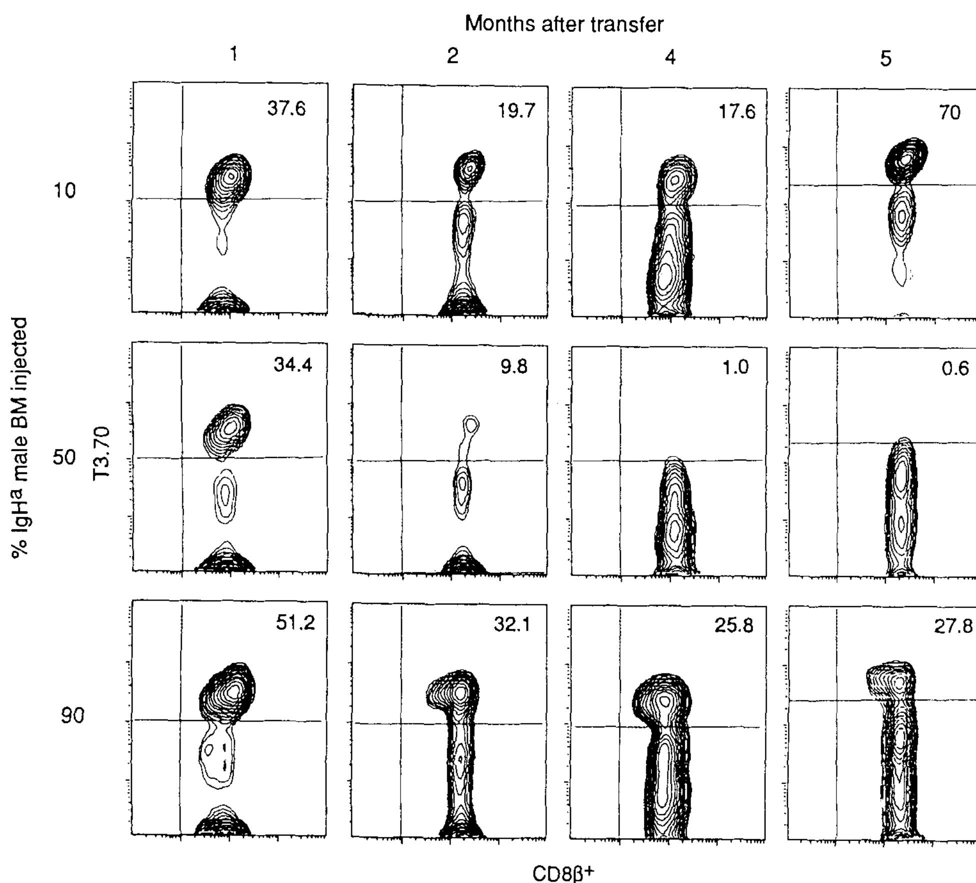

Figure 4. CD8 populations in male/female chimeras at different time points after transfer. The $CD8^+$ T cells shown are from mice represented in Table 1, and not from the same kinetics experiment shown in Fig. 3. At each time point, mice from individual groups were studied simultaneously. This was a kinetics experiment (the same transferred population was studied at different time points after injection), and recipient mice at different time points were not studied on the same day. Slight variations of labeling intensity between individual assays were unavoidable. Therefore, in each experiment, a TG female mouse was simultaneously studied as a control. Male-specific cells expressing high levels of TG TCR-α chain ($T3.70^{hi}$) can be readily visualized in female TG mice (Fig. 2 *A*). $\alpha_T\beta_T$ $CD8^{hi}$ T cells in recipient mice were those expressing similar levels of TG α chain. This strategy was used in all experiments.

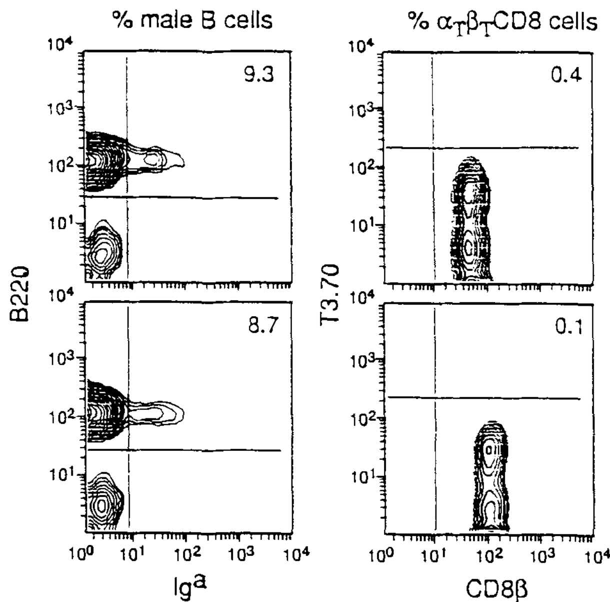

Figure 5. Frequency of male (μ^a) B cells (*right*) and $\alpha_T\beta_T$CD8^{hi} T cells (*left*) in the spleen of two chimeric mice injected with 50% male BM and TG T cells 4 mo after BM reconstitution. We tested 25 such chimeras. In the 15 chimeras studied 1 or 2 mo after BM transfer, $\alpha_T\beta_T$CD8^{hi} cells were present, and male B cells represented up to 3% of the B cell pool. In the 10 chimeric mice studied from 3 to 6 mo after BM injection, $\alpha_T\beta_T$CD8^{hi} cells were absent. Half these mice maintained a low percentage (1–3%) of male B cells. In the other mice, the percentage of male B cells increased to 8–25% of the B cell pool. Results for two of these latter mice (not studied on the same day) are shown.

In TX Female Hosts Reconstituted With 90% of Male BM, $\alpha_T\beta_T$CD8^{hi} TG Cells Induced a Small and Transient Decrease in the Number of Male B Cells 1 mo After Transfer (Fig. 1 B). The fraction of male B cells increased thereafter, to represent ~90% of the peripheral B cell pool 2–5 mo after reconstitution (not shown). In male TX hosts, $\alpha_T\beta_T$CD8^{hi} TG cells had no substantial effect on male B cells, even when they only constituted a small fraction of the B cell pool (Fig. 1 C). In all these chimeras, $\alpha_T\beta_T$CD8^{hi} cells expanded for 1 mo, slowly decreasing thereafter; they were still present in substantial numbers 4–5 mo after transfer (Figs. 3 and 4). Persisting male-specific cells downregulated surface levels of CD8 (Fig. 4) and were unresponsive to the male antigen (Table 1), that is, they became "anergic." We have described similar phenomena when $\alpha_T\beta_T$CD8^{hi} TG lymphocytes were transferred to male nude recipients (6, 7). These anergic T cells were devoid of suppressor activity: the fraction of μ^a-secreting cells and μ^a serum levels were comparable to those found in similar chimeras that had not received male-specific cells (not shown). It should be noted that CD8 downregulation and anergy induction were also observed when $\alpha_T\beta_T$CD8^{hi} TG lymphocytes were transferred to TX male mice reconstituted with 100% female B cells (not shown). Thus, male B cells appeared unnecessary for anergy induction.

Persistence of Virgin T Cells in the Absence of Antigen

Our previous results suggested that long-term survival of virgin mature T lymphocytes did not require antigen recognition: when we transferred $\alpha_T\beta_T$CD8^{hi} cells to female mice

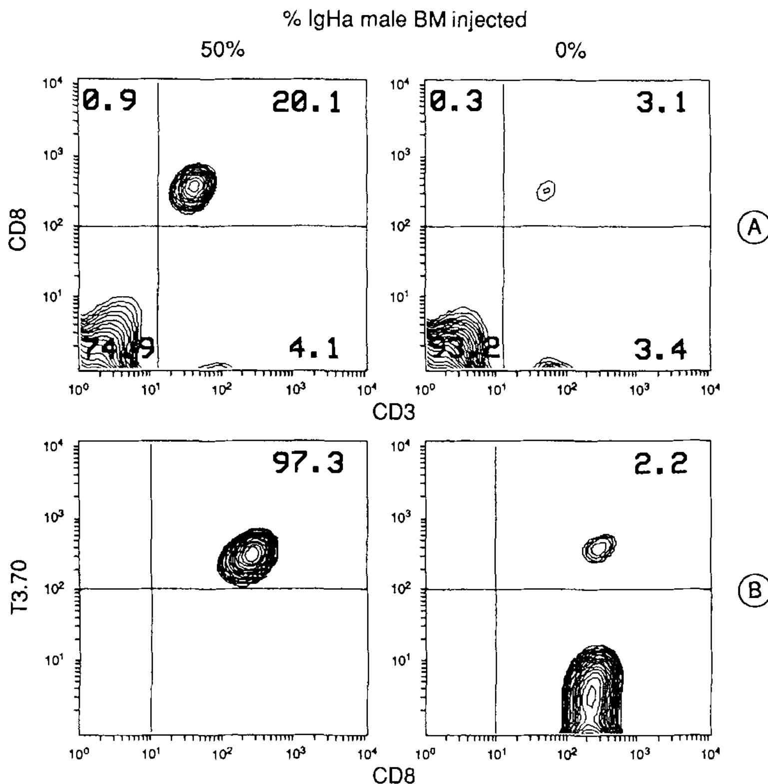

Figure 6. Persistence of virgin T cells in the absence of antigen. TX irradiated mice reconstituted with 50 (*left*) or 0% male BM (100% female, *right*) were injected 24 h later with 2.5 × 10^5 monoclonal $\alpha_T\beta_T$CD8^{hi} cells from female TG RAG2−/− mice and studied 6 wk after transfer. (*Top*) CD8 expression in total spleen cells. (*Bottom*) T3.70 expression by CD8$^+$ T cells. Spleens were depleted of SIg$^+$ cells before this study. Data are for one of three mice studied at this time point with similar results.

reconstituted with 100% female BM cells, these T cells did not expand but persisted for long periods (Figs. 2 and 3 and Table 1). In these experiments, however, we could not exclude the possibility that persisting $\alpha_T\beta_T CD8^{hi}$ cells coexpressed low levels of endogenous TCR-α chains at their surface, and thus had multiple specificities. In such circumstances, cell survival could be due to the recognition of other antigens. To verify this hypothesis, we injected female TX irradiated mice reconstituted with 100% female BM with mature T cells from female TG RAG2−/− mice. These donor mice cannot rearrange endogenous TCR, and all T cell populations are monoclonal for the TCR-TG receptor (MóTG). TG RAG2−/− mice have very few mature T cells in the peripheral pools (Rocha, B., manuscript in preparation), and so we injected our chimeras with a few T cells (2.5×10^5 i.v.). In these experiments, and in the absence of TG cell expansion or cell death, about 5×10^4 TG cells should be recovered from the spleen of host mice (only 20% of a T cell cohort settles in the spleen after i.v. injection; 32). The results of one of these experiments are shown in Fig. 6.

MóTG cells expanded after transfer into male/female chimeras, and numerous $CD8^+$ T cells were seen in the spleen (Fig. 6, *upper left*) of these mice. These $CD8^+$ T cells were virtually all MóTG (Fig. 6, *lower left*). In 100% female BM chimeras injected with MóTG cells, >90% of spleen cells were B lymphocytes, and few T cells were present (Fig. 6, *upper right*). These minority $CD8^+$ populations could be analyzed after depletion of SIg^+ cells. Most of these $CD8^+$ cells were non-TG (probably residual host cells surviving irradiation), but an MóTG population was clearly identified (Fig. 6, *lower left*). These results demonstrate that virgin mature T cells can survive in vivo in the absence of their nominal antigen.

The number of MóTG cells present in this mouse spleen can be calculated. Since spleen cell recovery was 74×10^6, the number of $CD8^+$ T cells (3.1% of spleen cells) was 2.3×10^6, and the number of MóTG (2.2% of $CD8^+$) was 4.82×10^4. This number was very similar to the cohort of MóTG cells expected to home to the spleen after i.v. injection (5×10^4 TG cells). We obtained similar results in three other experiments. These results indicate that $\alpha_T\beta_T CD8^{hi}$ virgin cells persisted but did not expand after transfer.

Presence of the Male Antigen in Different Chimeric Mice 5–6 Mo After T Cell Transfer

In the above experiments, we identified the male antigen in male/female chimeras by scoring μ^{a+} male B cells or μ^{a+} SIg. It should be noted, however, that the absence of both these parameters does not rule out the presence of the HY antigen in chimeric mice. First, male BM-derived cells other than B cells may be present in these mice. Second, we cannot exclude presentation by female APC (although the HY antigen is presented by class I MHC molecules). Since the HY antigen, like most minor transplantation antigens, has not yet been isolated, and as elution and identification of small amounts of specific peptides present in vivo may not be possible, we tested for the presence of male antigen indirectly in two independent sets of experiments.

First we investigated if the quantity of antigen present in these chimeras was sufficient to induce the expansion of a new cohort of virgin TG cells. For this purpose, chimeric mice were reconstituted with 0, 10, 50, or 90% male BM and injected with $Thy1.2^+$ $\alpha_T\beta_T CD8^{hi}$ T cells; they were then transferred, 5 mo later, with a new set of virgin $Thy1.1^+$ $\alpha_T\beta_T CD8^{hi}$ T cells and studied 1 mo later.

As shown in Fig. 7 (*top*), most of the $CD8^+$ T lymphocytes present in these chimeric mice were $Thy1.2^+$, that is, they belonged to the first cohort of TG lymphocytes injected. These results were expected, since $Thy1.2^+$ TG cells were injected into lethally irradiated (and thus T cell–deficient) mice, whereas $Thy1.1^+$ TG cells were injected into T cell–reconstituted mice. We have previously observed that T lymphocytes expand readily after transfer into T cell–deficient hosts, whereas little expansion is observed when T cells are injected into T cell–reconstituted mice (26). As described above, in $Thy1.2^+$ $CD8^+$ cells, a male-specific $\alpha_T\beta_T CD8^{hi}$ TG population was readily detected in chimeras reconstituted with 10 or 90% male BM, but was absent from mice reconstituted with 50% male BM. In contrast, $Thy1.2^-$ $\alpha_T\beta_T CD8^{hi}$ TG cells (probably $Thy1.1^+$) were more abundant in chimeras reconstituted with 50 and 90% male BM than in mice reconstituted with 10% BM. To study this second set of virgin $Thy1.1^+$ TG cells, $CD8^+$ populations from recipient mice were further depleted of $Thy1.2^+$ cells. As shown in Fig. 7 (*middle* and *bottom*), $\alpha_T\beta_T CD8^{hi}$ $Thy1.1^+$ cells parked for 1 mo in 0 or 10% male BM–reconstituted mice persisted as small resting cells and did not expand. After transfer into other chimeras (initially reconstituted with 50 or 90% male BM, in which we detected male B cells), this second set of virgin T cells was activated (as shown by increased size) and expanded. We also noted lower Thy1 expression in these latter populations, as well as in "anergic" $Thy1.2^+$ cells (recovered in mice reconstituted with 90% male BM). These results demonstrated that, 5–6 mo after BM transfer, the amount of male antigen present in 10% of male BM chimeras was not sufficient to induce the expansion of virgin TG populations. In contrast, in 50 or 90% of male BM chimeras, male antigen persisted in sufficient amounts to stimulate a newly transferred set of virgin TG cells.

In a second set of experiments, we tested for male DNA by PCR amplification of the Y chromosome–specific *Zfy-1* gene (30). Fig. 8 *A* shows the sensitivity of the method, that is, amplification of DNA in two male cells, but bands of similar intensity were observed when one cell, rather than two cells, was studied. As expected in the limiting conditions of one cell per well per PCR, negative and positive results were obtained. This method thus identified BM-derived male cells other than B cells and also increased the threshold of detection of male cells 10,000-fold relative to FACS® analysis, since we were able to amplify this gene in the DNA from one or two male cells.

We tested for the presence of the *Zfy-1* gene in 20 mice (5 mice from each group) 5–6 mo after BM injection. In the 10 chimeras reconstituted with either 90 or 50% male BM, the *Zfy-1* gene was identified even when only 10^3 spleen cells were studied. As expected, the 180-bp–specific band was ab-

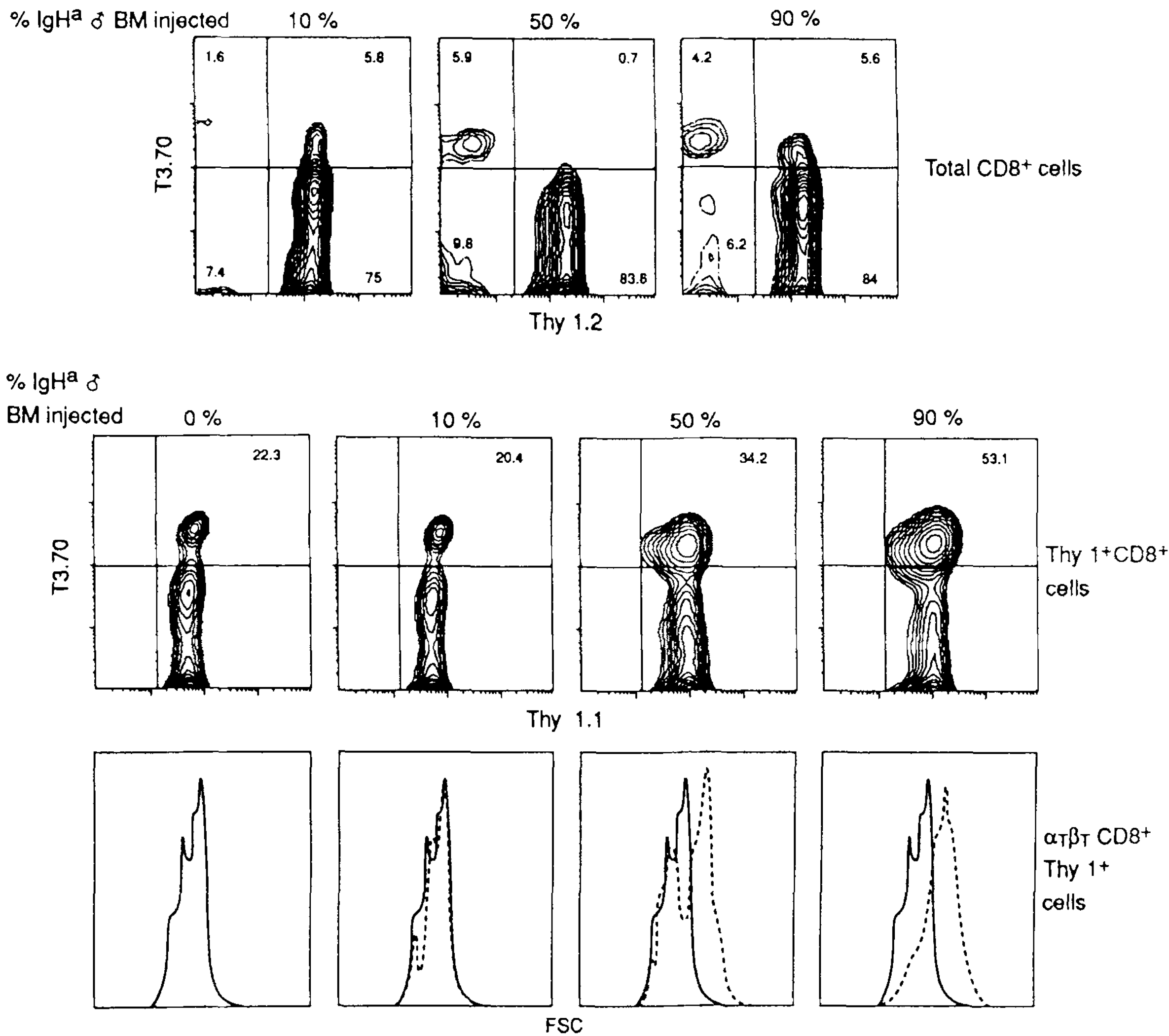

Figure 7. Expansion of a new cohort of male-specific virgin T cells in chimeric mice 5–6 mo after BM reconstitution and T cell injection. Female TX irradiated mice were injected with 0, 10, 50, or 90% male BM cells and 4 × 10^5 $\alpha_T\beta_T CD8^{hi}$Thy1.2$^+$ male-specific T cells. 5 mo later, all mice received 4 × 10^5 $\alpha_T\beta_T$CD86h^i Thy-1.1$^+$ male-specific cells, and they were studied 1 mo later. Data are for one of three mice tested in each experimental group, with similar results. (*Top*) Spleen cells depleted of Ig$^+$ and CD4$^+$ cells were triple-labeled with anti-Thy1.2, anti-CD8, and T3.70 mAbs. T3.70 and Thy1.2 expression in CD8$^+$ gated populations is shown. (*Middle*) Spleen cells depleted of Ig$^+$, CD4$^+$, and Thy1.2$^+$ T cells were triple-labeled with anti-Thy1.1, anti-CD8, and T3.70 mAbs. T3.70 and Thy-1.1 expression in CD8$^+$ gated populations is shown. (*Bottom*) Forward-scatter (FSC) profiles of CD8$^+\alpha_T\beta_T$ Thy1.1$^+$ cells in the same chimeric mice. The FSC profile of $\alpha_T\beta_T$ virgin Thy1.1$^+$ cells, parked for 1 mo in chimeras reconstituted with 100% female BM cells, is reproduced in all graphs (*solid lines*) and compared with the FSC profile of $\alpha_T\beta_T$ T cells in other chimeric mice (*dotted lines*). DNA from the spleen of the mouse reconstituted with 10% male BM shown in this figure was tested for the presence of the *Zfy-1* Y-specific gene and was found positive. The proportions of μ^a male B cells within B220 IgM$^+$ cells in these mice were (from left to right) 0.3, 0.2, 2, and 75%, respectively.

sent from mice that had received 100% female BM. Results varied in individual mice reconstituted with 10% male BM. In 3 out of 5 chimeras studied, we failed to detect the *Zfy-1* gene in either spleen or BM, indicating that male cells, if present, had a frequency $<10^{-6}$. In the remaining two mice, we could not detect this gene among BM cells, whereas in the spleen we only identified a specific band when the DNA from 10^6 cells was amplified, suggesting that the frequency of male cells in this organ was $<10^{-5}$ (Fig. 8).

To summarize, in 90 or 50% male BM chimeras studied 5–6 mo after reconstitution, male DNA was always present. In mice reconstituted with 10% male BM, male cells, if present, were at a frequency $<10^{-5}$ or 10^{-6}. This amount of male antigen was not sufficient to induce the expansion of newly transferred male-specific T cells (Fig. 7).

Discussion

We found that injection of T cells from female TG mice or monoclonal $\alpha_T\beta_T CD8^{hi}$ T cells (MoTG) from female TG RAG2−/− mice into male/female BM chimeric mice induced the rejection of male-derived hematopoietic cells. This demon-

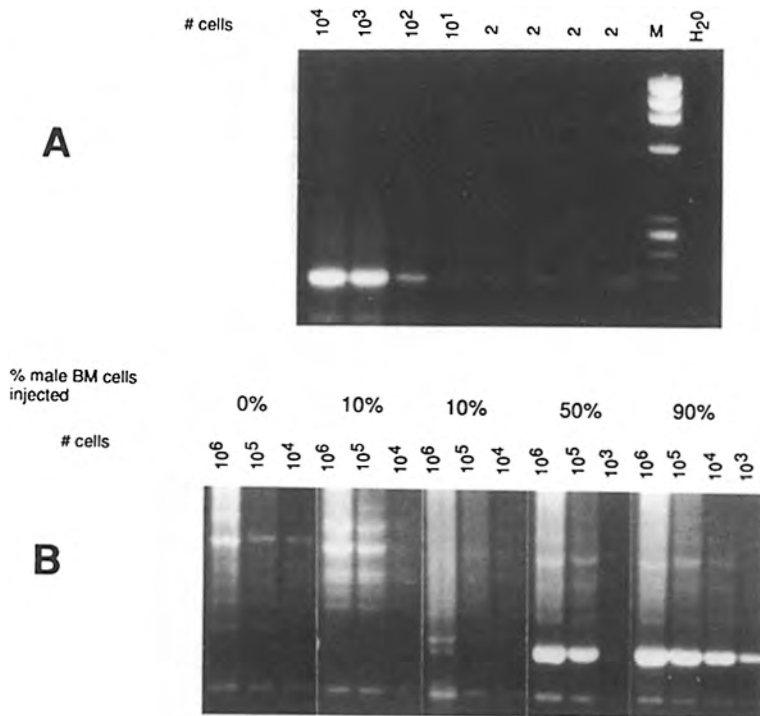

Figure 8. PCR amplification of the Y chromosome–specific *Zfy-1* gene (30) in the spleens of different mice. (*A*) Titration of male spleen cells. We tested DNA from different numbers of male spleen cells diluted in female spleen DNA (see Materials and Methods). We show the results of amplification of the DNA of two male cells, rather than one cell, since bands of similar intensity were seen in the two cases. *M*, marker bands (1,353, 1,078, 872, 603, 310, 281, 271, 234, and 194 base pairs). (*B*) DNA from different numbers of spleen cells from chimeric mice. TX irradiated mice were injected with 0, 10, 50, or 90% male BM and 4×10^5 $\alpha_T\beta_T$CD8^{hi} Thy1.2$^+$ T cells. A group of these mice was tested 5 mo after BM transfer. A second group was injected with CD8$^+\alpha_T\beta_T$ Thy1.1$^+$ virgin cells and studied 1 mo later. In both cases, the 180-bp–specific band, corresponding to the amplification product of the *Zfy* gene, was present in all mice reconstituted with 90 or 50% BM and absent from all mice reconstituted with 100% female BM. This band was present in two out of five tested mice reconstituted with 10% male BM. Results for a positive and a negative mouse are shown. PCR amplifications shown in *A* and *B* were done simultaneously.

strates that male-specific transgenic T cells ($\alpha_T\beta_T$CD8^{hi}) are able to mediate effector functions in vivo. It has previously been shown that anti-HY female TG mice, although containing large numbers of male-specific cells, show slow rejection of male skin grafts (32a). The anti-HY clone from which the TG TCR was isolated was selected after in vivo boosting with hematopoietic cells and long-term in vitro stimulation with male nude spleen cells (23). TG T cells may thus show some tissue specificity, being more prompt to reject male hematopoiesis-derived cells in vivo than, for example, male skin grafts.

Rejection of male B cells was dependent on the amount of male antigen, confirming that differentiation into effector functions and elimination of target B cells by CD8$^+$ lymphocytes is dependent on the antigen load (18, 33). Similarly, the rate of elimination of infected or transformed cells by effector T cells in vivo may depend on the concentration of antigen.

In TX mice injected with different quantities of male BM, the fate of male-specific peripheral T cells, in the absence of thymic output, also appeared to be critically dependent on antigen load. In the absence of the nominal antigen, virgin functionally reactive T cells persisted in vivo but did not expand, and their frequencies were low. Low antigen concentrations induced expansion, effector functions (shown by the elimination of male B cells), and persistence of increased numbers of functionally reactive $\alpha_T\beta_T$CD8^{hi} lymphocytes. Effector functions and partial elimination of male B cells were observed when the amount of antigen increased, but antigen-specific TG cells disappeared at a later stage. Very high antigen concentrations induced anergy, after which antigen-specific cells did not mediate effector functions (i.e., did not reject male B cells) but persisted in vivo as CD8^{low} tolerant T cells. These results may be particularly relevant to the understanding of the conditions in which antigen-specific T cells persist in vivo, long-term T cell responses are maintained, and peripheral T cell tolerance is induced.

Our results confirm that virgin T cells (including MöTG populations) can survive in the absence of nominal antigen in a resting state (6, 9). It is possible that previously activated T cells survive in vivo, like virgin T cells, in the absence of cell division (8): as the expansion potential of T lymphocytes is limited (26), long-term T cell survival is unlikely in the presence of continuous division.

The maximal number of divisions a eukaryotic cell can undergo in vitro was estimated by Hayflick (34) to average 45. Similarly, the number of divisions that mature T cells are able to undergo in vivo is equivalent to the Hayflick number (26). Considering that the in vivo doubling time of $\alpha_T\beta_T$CD8^{hi} cells is 48 h (Rocha, B., unpublished observations), when cell division is continuous, the Hayflick number should be reached within 3 mo, and exhaustion of immune responses should then occur. Long-term T cell survival must require at least a temporary quiescent state. This may only be possible when antigen encounters are rare or absent. Indeed, in mice injected with 10% male BM, male cells rapidly disappeared, and high frequencies of male-reactive T cells were maintained in the apparent absence of antigen. The amount of male antigen present was not sufficient to induce activation or expansion of a second cohort of virgin T cells parked for 1 mo in these chimeras. Moreover, using a PCR technique that permitted identification of male cells at frequencies of 10^{-6}, we could not always detect the presence of male DNA in these chimeras. Although previous antigen exposure

may reduce the threshold required for cell stimulation, encounters with antigen in these chimeras must be rare. Activated T cells (like virgin T cells) may thus survive in the absence of continuous antigen stimulation and in the absence of extensive terminal differentiation (see below). It is possible that interactions with the same ligands that induced intrathymic positive selection, although of insufficient affinity to induce cell proliferation (35), permit the survival of peripheral T cells in the absence of nominal antigen.

Antigen persistence was clearly demonstrated in chimeras reconstituted with 90 or 50% male BM. We found that TG T cells became tolerant to the male antigen in these chimeras in two independent ways. In mice injected with 50% male BM, exhaustion was responsible for unresponsiveness: male-specific T cells were able to mediate effector functions and eliminate a large fraction of male cells, but eventually disappeared, allowing the late partial recovery of male B cells. In female mice reconstituted with 90% male BM, or after transfer into male hosts, male-specific cells initially proliferated, downregulated CD8 levels at the cell surface, became unable to mediate effector functions, and persisted in vivo as anergic T cells.

As to the mechanisms inducing these two types of tolerance, it is unlikely that continuous division alone was responsible for clonal exhaustion of $\alpha_T\beta_T$CD8^{hi} T cells in the 50% chimeras. Continuous division should generate a large clone (each cell may generate $\sim 3 \times 10^{13}$ cells [26]), but this was never the case in these mice, in which $\alpha_T\beta_T$CD8^{hi} T cell clones were smaller than those in other male/female chimeras. The gradual disappearance of antigen-specific cells was evident from 1 mo after transfer onwards, that is, well before expansion capacity should have been exhausted. It is likely that $\alpha_T\beta_T$CD8^{hi} T cells disappeared by terminal differentiation: the continuous presence of antigen in mice reconstituted with 50% male BM may have induced continuous differentiation into effector functions, and cytotoxic effector T cells may have a short life span (36). Conversely, in chimeras reconstituted with 90% male BM after anergy induction, both cell expansion (7) and effector functions become paralyzed; it is thus not surprising that anergic T cells may persist in vivo in these conditions.

Anergy was thought to emerge after extensive division of antigen-specific T cells and, therefore, to be similar to exhaustion (18). Our results show that anergy and exhaustion are different phenomena and independent mechanisms of tolerance. Induction of anergy required higher doses of antigen, which blocked proliferation (7) and effector functions, but antigen-specific T cells persisted. Exhaustion of the immune response was not preceded by CD8 downregulation or extensive division, permitted effector functions, and was probably caused by terminal differentiation of CD8$^+$ T cells. It occurred at antigen concentrations insufficient to induce anergy. It should be noted that in mice reconstituted with 50% male BM, most μ^{a+} cells were eliminated 1 mo after transfer, but the remaining 1–3% of male B cells were sufficient to delete all antigen-specific T cells 2–3 mo later. These differences may also be important, as we have shown that anergy is reversible (7), whereas exhaustion is an irreversible mechanism of peripheral T cell tolerance. Exhaustion may be particularly efficient in removing effector CD8 T cells recognizing self-antigens in vivo: if antigen persistence rather than antigen load is responsible for the exhaustion of the immune response, then this process may occur in response to self-antigens continuously produced at low concentrations in vivo (low-dose tolerance).

We found that persistence of antigen was not sufficient for the maintenance of functional reactive T cells. On the contrary, when antigen persistence could be demonstrated, that is, in chimeras reconstituted with 50 or 90% male BM, recipient mice became unresponsive to the male antigen (Table 1). If antigen persistence is associated with tolerance induction, it is likely that self-antigens, which are continuously present in vivo, induce tolerance rather than immune reactivity.

We also found that the presence of male-specific T cells favored peripheral repopulation by female μ^{b+} B cells. The total number of B cells remained constant in all chimeras studied, that is, the loss of the μ^{a+} male population was readily compensated for by the increase in μ^{b+} female B cells. Exploitation and mutualistic lymphocyte interactions may occur in an immune system in which the total number of cells is under strict control and each lymphocyte has to compete with other newly produced or resident lymphocytes for survival, proliferation, and/or differentiation (37).

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