

Induction of Long-Term H-Y-specific Tolerance in Female Mice Given Male Lymphoid Cells While Transiently Depleted of CD4⁺ or CD8⁺ T Cells

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Summary

Rejection of H-Y-bearing primary skin grafts and generation of H-Y-specific cytolytic T cells by female mice requires the participation of both CD4⁺ and CD8⁺ T lymphocytes. Studies were conducted to investigate long-term tolerance of H-Y antigen induced in female mice by transiently depleting them of CD4⁺ and/or CD8⁺ T cells and, at the same time, giving them an injection of male lymphoid cells. We confirmed that after recovery of CD4⁺ to normal levels, female mice that had been transiently depleted of CD4⁺ cells and concurrently given an injection of male spleen cells were unable to generate H-Y-specific cytolytic T cells. Tolerance was also manifest by greatly extended survival (probably permanent in most cases) of male skin grafts. Further investigations revealed that female mice transiently depleted of CD8⁺ cells, and concurrently given an injection of male spleen cells, were similarly tolerant of H-Y antigen later when numbers of CD8⁺ T cells returned to normal. Moreover, small numbers of male cells were detectable in spleen and lymph nodes of tolerant females long after they had been given an injection of male cells and depleted of either CD4⁺ or CD8⁺ T cells, whereas no male cells were detected in (nontolerant) females given male cells and control antibodies. These findings show that tolerance of the relatively weak transplantation antigen, H-Y, can be achieved simply by giving male antigen-bearing spleen cells to the host while it is transiently depleted of a type of cell it needs in order to reject those cells, thus allowing the male cells to persist in the host. Furthermore, depletion of helper cells is not obligatory to achieve tolerance. It has been hypothesized that tolerance of H-Y antigen in females given male lymphoid cells while temporarily depleted of CD4⁺ lymphocytes results from unresponsiveness (anergy) induced in H-Y-specific CD8⁺ cells that are exposed to H-Y antigen in the absence of help from CD4⁺ cells. Interpretations of our findings are discussed in relation to this hypothesis.

Although it has been well established that tolerance of some self-antigens is due mainly to clonal deletion of self-reactive T cells in the thymus, it is unclear how tolerance is induced to cell-associated antigens not expressed in the thymus. Understanding how tolerance can be induced extrathymically has important implications for attempts to establish a nonresponsive state to allogeneic antigens borne on tissue grafts and for reestablishing self-tolerance in patients suffering from autoimmune diseases.

Bretscher and Cohn (1) have proposed a theory that can account for extrathymic tolerance induction. Although initially proposed as a model for cooperation between T and B cells in an immune response, the theory can be applied to systems involving only T cell responses (2). In essence, the theory says that in order for an antigen to be immunogenic when encountered by a potentially reactive T cell, antigen recognition must be accompanied by a costimulatory second signal, provided to potentially reactive CD4⁺ T cells by APCs, or to potentially reactive CD8⁺ T cells by CD4⁺

T helper cells. However, if antigen is encountered in the absence of a costimulus, the potentially reactive CD4⁺ or CD8⁺ T cell is rendered tolerant of the antigen, and fails to respond on subsequent encounters with it, even if accompanied by an appropriate costimulus.

In vitro studies lend credibility to this hypothesis (for a review see reference 3). It is also supported by results of experiments performed in mice involving the Qa-1 antigen (2, 4). Primary Qa-1-disparate skin grafts are not rejected by host mice unless the graft bears additional antigenic determinants, such as H-Y antigen, which are rejectable on their own. In such cases, the host can reject grafts later on that bear the Qa-1 disparity alone. In contrast, if the initial graft bears only the Q-1 disparity, then the host is unable to reject another graft of this type later on, even if it is accompanied by H-Y antigen. These results have been interpreted to mean that H-Y antigen stimulates helper cells, whereas the Qa-1 antigen by itself does not. The help provided by H-Y-stimulated cells provides a costimulus to potential anti-Qa-1 effectors.

In the absence of H-Y-induced help, however, potential anti-Qa-1 effector cells become tolerized upon encounter with the Qa-1 antigen, and are unable to respond later on, even if help is provided.

Others have performed experiments in which the H-Y antigen itself was the target for tolerance induction (2, 5). Female mice were temporarily depleted of CD4⁺ cells and concurrently given an injection of male lymphoid cells. Weeks later, when the number of CD4⁺ cells had returned to normal, the females were challenged with male cells to determine whether they could be primed to generate H-Y-specific CTL. They were unable to, however, unlike control mice that had not received male cells. The authors concluded that exposure of potentially H-Y-reactive female CD8⁺ T cells to male antigen-bearing cells at a time when CD4⁺ cells were not available to provide help rendered the CD8⁺ cells tolerant and unable to respond later on after repopulation of CD4⁺ cells. It should be noted that generation of H-Y-specific cytolytic T cells by female mice requires the participation of both CD4⁺ and CD8⁺ T cells, as does rejection of primary male skin grafts (6).

Two points about this experiment require comment. Tolerance was not assessed using skin grafts. This is relevant because in some circumstances female mice can reject H-Y-incompatible skin grafts even though they cannot be primed to generate H-Y-specific CTL (7-9). Also, it was not determined whether it was the CD8⁺ T cells, rather than the CD4⁺ cells which repopulated the hosts, that were actually tolerized. The Bretscher and Cohn hypothesis predicts tolerization of the CD8⁺ population in this experiment.

The following experiments were undertaken to investigate in more detail the process of tolerance induction by the method just outlined. First, we wanted to determine whether the mice were tolerant of H-Y by the criterion of acceptance of male skin grafts. Furthermore, we wished to determine whether it is obligatory to deplete helper cells in order to establish tolerance, and whether we could detect persistent male cells in tolerant female hosts.

Materials and Methods

Mice. C57BL/6J (abbreviated B6), B6.C-H-2^d/aBy (Hw19), B6.PL-Thy-1^a/Cy (B6-Thy-1.1), and BALB/cBy × B6)F₁ (CB6F₁) mice were bred in a barrier-sustained facility at the Trudeau Institute from founder stocks obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were age matched in each experiment and maintained under controlled temperature and light/dark conditions. They received commercial laboratory chow and acidified water ad libitum. Serum samples were taken regularly from sentinel mice and analyzed (Research Animal Diagnostic and Investigative Laboratory, University of Missouri, Columbia, MO) to verify the absence of microbial pathogens that might adversely affect immunological functions.

mAbs. mAbs GK1.5 (ratIgG2b, American Type Culture Collection [ATCC, Rockville, MD] TIB 207) and 2.43 (rat IgG2b ATCC TIB 210) were used to deplete CD4⁺ and CD8⁺ cells, respectively. mAb-containing ascites grown in CB6F₁ mice were purified by 45% ammonium sulfate precipitation and DEAE ion-

exchange chromatography. Eluted mAbs were dialyzed against PBS, sterile filtered, and frozen at -70°C until used. FITC-conjugated F(ab')₂ fragments of GK1.5, 2.43, or the anti-Thy-1.2 mAb 30H12 (ATCC TIB 107) were also used for flow cytometric analyses. In some experiments, culture supernatant of the anti-Ia^{b/d} hybridoma 28-16-8S (ATCC HB35) was used, with rabbit complement, to enrich numbers of Thy-1⁺ cells in spleen and lymph node cell preparations by eliminating MHC class II⁺ cells.

Depletion of T Cell Subsets In Vivo. Mice were depleted of CD4⁺ or CD8⁺ cells essentially as described by Guerder and Matzinger (2). Briefly, 5-7-wk-old females were given two intraperitoneal injections containing 1 mg each of the appropriate antibody 24 h apart. Control mice received equal amounts of normal rat IgG (ICN Biomedicals, Inc., Costa Mesa, CA). Flow cytometric analysis (FACSscan[®], Becton Dickinson & Co., Mountain View, CA) confirmed that depletion of CD4⁺ or CD8⁺ spleen cells was >99% 3 d after the last injection. Weekly flow cytometric analysis of spleen cells from antibody-treated mice also showed that repopulation of depleted cells began about 2 wk after the last antibody injection and stabilized at near normal levels by 9 wk.

Spleen Cell Preparation. Cell suspensions were made by gentle mechanical disruption of B6 male or female spleens in ice-cold Hepes-buffered HBSS. After washing in HBSS, cells were suspended at 10⁸/ml for primary injections, or at 2 × 10⁷/ml for CTL priming. Primary injections of 5 × 10⁷ cells were given intraperitoneally 6 h after the last mAb injection. Cells for CTL priming (10⁷ per host) were injected 9 wk after the primary cell injections.

CTL Assays. Between 2 and 4 wk after mice were given cells for CTL priming, cultures of spleen cells from treated B6 females were initiated to generate H-Y-specific CTL by a slight modification of previously described methods (9). Briefly, ~5 × 10⁷ B6 female spleen cells were incubated with a similar number of irradiated (2,000 rad) B6 male stimulator cells or control Hw19 female stimulator cells for 5 d. Approximately 10⁷ B6 male (H-Y antigen), B6 female (negative control), or Hw19 female (MHC alloantigen positive control) spleen cells depleted of RBC by hypoosmotic shock were cultured in medium containing 2.5 μg/ml Con A (Sigma Chemical Co., St. Louis, MO) or 10 μg/ml *Salmonella enteritidis* LPS (Difco Laboratories, Inc., Detroit, MI) for 48 h to obtain lymphoblasts for use as targets. Labelling of target blasts and assay of cytolytic activity were carried out as described (9).

Skin Grafting. Tolerance of H-Y antigen by treated females was assessed in vivo by survival of orthotopic male tailskin grafts (10, 11). Skin graft survival times in different groups were compared using the one-tailed Wilcoxon two sample rank-sum test (12).

Persistence of Injected Male Lymphocytes. Cells (2 × 10⁷/ml) obtained from B6 (Thy-1.2) male donor spleens and lymph nodes (axial, brachial, and inguinal) were incubated for 45 min on ice with culture supernatant from an anti-Ia^{b/d} hybridoma, washed, and incubated with rabbit complement for 60 min at 37°C to reduce numbers of macrophages and Ia⁺ B lymphocytes. Cells were washed, counted, and injected intraperitoneally at 5 × 10⁷ per host into female B6-Thy-1.1 mice that were treated with mAbs, to deplete CD4⁺ or CD8⁺ cells, or with control rat IgG. FACS[®] analysis (Becton Dickinson & Co.) revealed that about 80% of the injected cells were positive for the Thy-1.2 marker.

At 1, 5, and 9 wk after injection of Thy-1.2⁺ male lymphoid cells into B6-Thy-1.1 females, their spleens and lymph nodes were removed, depleted of Ia⁺ cells as previously described, and analyzed by flow cytometry for Thy-1.2⁺ male donor cells. There was no detectable staining of control Thy-1.1⁺ cells by the anti-Thy-1.2-specific antibody.

Results

Induction of Tolerance of Skin Grafts. Preliminary experiments were performed to confirm the findings of others (2) regarding tolerance induction detectable by CTL assays. Female B6 mice depleted of CD4⁺ cells, or given control rat IgG, were given an injection of male spleen cells as described and allowed to recover for 9 wk. At that time, CD4⁺ spleen cells had returned to about 90% of the number found in non-depleted age-matched controls. Mice in various groups were challenged with an intraperitoneal injection of male spleen

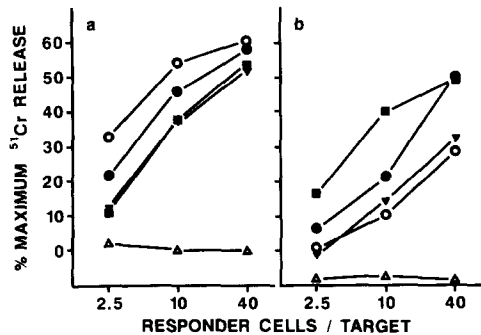


Figure 1. Percent ⁵¹Cr-release from labeled B6 male LPS (a) or Con A (b) target blasts in CTL assays. Effector cells generated in vitro from spleen cells from females treated initially with depleting mAb and B6 male spleen cells (Δ), depleting mAb and B6 female spleen cells (▼), control rat IgG, and male spleen cells (O), or control rat IgG and female spleen cells (●). mAbs used for depletion were anti-CD4 (a) or anti-CD8 (b). (■) Label release from Hw19 targets by effectors from females treated initially with depleting mAb and male spleen cells. Label release from female targets was negative.

cells to see whether they could be primed for generation of H-Y-specific CTL in vitro (Fig. 1 a). H-Y-specific CTL activity was generated in cultures of spleen cells taken from females treated at the outset with control rat IgG and either male or female spleen cells, or from females treated with antibodies that depleted CD4⁺ cells and given female cells. In contrast, no H-Y-specific CTL activity was obtained from females treated initially with anti-CD4 antibodies and given male spleen cells, although activity was generated against MHC-disparate Hw19 targets (control), indicating that the tolerance was H-Y specific.

To evaluate tolerance wholly in vivo, female mice receiving the same initial treatments with antibodies and male or female spleen cell injections were given grafts of B6 male skin 9 wk later, rather than being challenged for CTL priming. The results (Table 1) closely paralleled those of the CTL studies. Almost all of the mice depleted of CD4⁺ and given an injection of male spleen cells at the outset were unable to reject male skin grafts, whereas male skin grafts were rejected by females in the other groups. Survival of male skin grafts on rat IgG-treated females given male spleen cells was somewhat extended, a finding consistent with a previously published report (13).

Tolerance in Females Depleted of CD8⁺ Cells. In experiments similar to those involving females depleted of CD4⁺ cells, female B6 mice were treated with anti-CD8 mAb or control rat IgG and given injections of either male or female spleen cells. Treated mice were challenged with male spleen cells at 9 wk for CTL priming or were given grafts of male skin. Similar to findings in mice depleted of CD4⁺ cells, females depleted of CD8⁺ cells and given male spleen cells

Table 1. Survival of Male Tail Skin Grafts on B6 Females Temporarily Depleted of CD4⁺ T Cells

Group	mAb treatment	Injected cells	Skin graft survival
Experiment 1			<i>d</i>
A	Control rat IgG	B6 female*	15, 24, 24, 24, 28†
B	Control rat IgG	B6 male	28, 35, 52, 70, 109§
C	anti-CD4	B6 female	21, 24, 28, 28, 28
D	anti-CD4	B6 male	39, >190 (× 4)§
Experiment 2			
A	Control rat IgG	B6 female	16, 22, 26, 26, 26
B	Control rat IgG	B6 male	26, 33, 37, 42, 63§
C	Anti-CD4	B6 female	19, 22, 26, 26, 28
D	Anti-CD4	B6 male	42, >151 (× 4)§

* 5×10^7 spleen cells intraperitoneally.

† B6 male skin grafts applied 63 d after cells were injected. Five female hosts per treatment group.

§ Significantly ($p < .01$) prolonged survival compared with group A.

|| Significantly ($p < .05$) prolonged survival compared with group B.

were tolerant of H-Y by either the criterion of failure of CTL priming (Fig. 1 *b*) or skin graft survival (Table 2), whereas mice in the other treatment groups were not tolerant. Thus, tolerance can be achieved by transient depletion of either the potential helper (CD4⁺) or effector (CD8⁺) population.

Persistence of Male Cells in Lymphodepleted Females. We wished to determine directly whether persisting male cells could be found in females that had been depleted of CD4⁺ or CD8⁺ cells at the time of injection of the male cells. Using essentially the same protocol as described earlier for tolerance induction, equivalent depleting injections of anti-CD4 or anti-CD8 mAbs, or control rat IgG, were given to B6-Thy-1.1 females which then received 5×10^7 B6 male (Thy-1.2) T cell-enriched spleen and lymph node cells.

Host females were killed 1, 5, and 9 wk later, and their spleen and lymph node cells analyzed separately for persistent Thy-1.2⁺ male cells by FACS[®]. At 1 wk, Thy-1.2⁺ cells were easily detectable in mice in all three antibody treatment groups (data not shown). At the 5- and 9-wk points, Thy-1.2⁺ cells were found in spleen and lymph node preparations from females depleted initially of either CD4⁺ or CD8⁺ cells, but not in females given control IgG. Percentages of Thy-1.2⁺ cells in the T cell-enriched populations that were analyzed at 9 wk are shown in Fig. 2. From knowledge of the numbers of cells in the spleen and lymph nodes of the mice, and the extent of T cell enrichment by anti-Ia treatment, we calculate that in CD4-depleted mice, 0.28% of the lymph node cells, and 0.99% of the spleen cells were Thy-1.2⁺ male cells at 9 wk (Fig. 2, *a* and *d*). In CD8-depleted mice, 0.1% of the lymph node cells and 0.06% of the spleen cells were male cells (Fig. 2, *b* and *e*). Fewer than 1 in 10^4 of the T cell-enriched spleen and lymph node populations from control IgG-treated mice were Thy-1.2⁺ (Fig. 2, *c* and *f*).

The key finding is that male cells were detectable, albeit in small numbers, only in females given treatments that successfully induced tolerance, and were apparently eradicated in females shown to be nonintolerant.

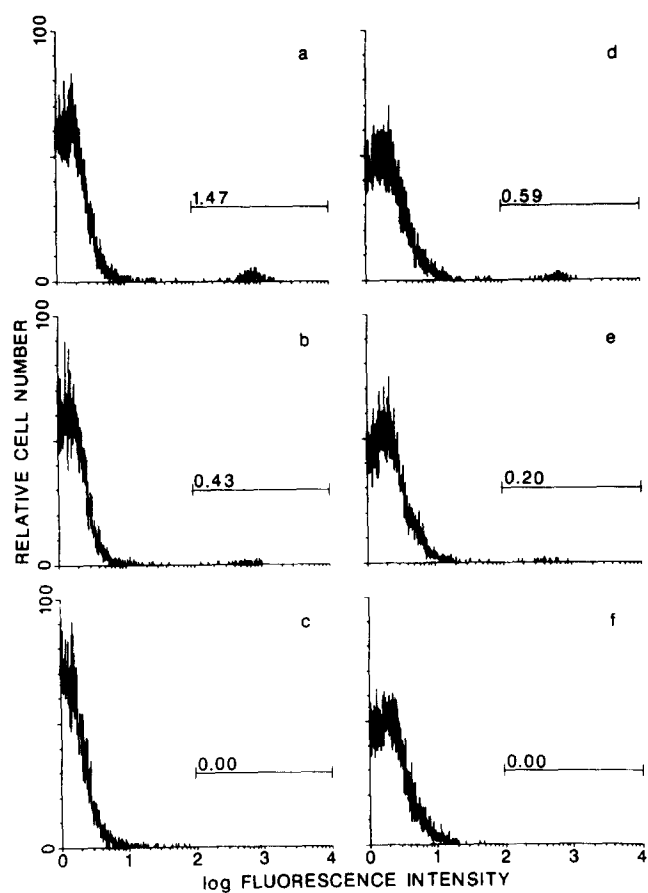


Figure 2. Thy-1.2⁺ B6 male lymphocytes remaining in lymph nodes (*a-c*) or spleen (*d-f*) of B6-Thy-1.1 females that were treated 9 wk earlier with anti-CD4 (*a* and *d*), anti-CD8 (*b* and *e*), or control rat IgG (*c* and *f*) and given an injection of T cell-enriched male cells spleen and lymph node cells. Numbers indicate percentage of Thy-1.2⁺ cells detected by FACS[®] analysis in Ia⁺ cell-depleted spleen and lymph node populations. See text for percentages of male cells per organ.

Table 2. Survival of Male Skin Grafts on Females Temporarily Depleted of CD4⁺ T Cells

Group	mAb treatment	Injected cells	Skin graft survival
A	Control rat IgG	B6 female*	16, 22, 26, 26, 26 [†]
B	Control rat IgG	B6 male	26, 33, 37, 42, 63 [§]
C	anti-CD8	B6 female	26, 26, 26, 26, 26
D	anti-CD8	B6 male	>190 ($\times 5$)

* 5×10^7 spleen cells intraperitoneally.

† B6 male skin grafts applied 63 d after cells were injected. Five female hosts per treatment group.

§ Significantly ($p < .01$) prolonged survival compared with group A.

|| Significantly ($p < .01$) prolonged survival compared with group B.

Discussion

Our results show that injection of male lymphoid cells into female mice concurrently depleted of CD4⁺ or CD8⁺ T cells makes the mice tolerant of H-Y antigen later on, as evidenced by an inability to generate H-Y-specific CTL or to reject male skin grafts. Assessment of tolerance by skin grafting is pertinent because in some cases, female mice may be unable to generate H-Y-specific CTL, yet still able to reject male tissue grafts (7-9).

It was also found that females that became tolerant in these experiments harbored male cells in their spleens and lymph nodes, whereas females that were not tolerant, because they had not been temporarily depleted of CD4⁺ or CD8⁺ cells, did not possess male cells. In other words, tolerance was correlated with persistence of injected male cells in the females' lymphoid organs. A number of studies have suggested that a persistent source of antigen or cellular chimerism is needed to sustain tolerance (14-18). Our observations provide direct evidence of such a source.

Our results also show that it is not obligatory to deplete helper cells in order to achieve tolerance experimentally. Most other models of tolerance induction—including neonatal tolerance, or tolerance induced in adults that have been irradiated, given pharmacological agents, or treated with depleting antibodies—involve introduction of antigen into hosts that are physically or functionally depleted of potentially antigen-reactive cells. In almost all cases, this physical or functional depletion has not been specifically directed at helper cells versus other types of cells. Thus, it would not be apparent that depletion of helper cells may not be obligatory to achieve tolerance.

At an elementary level, the tolerance observed in our experiments can be explained on the basis of our interference, using depleting antibodies, with the ability of female mice to reject male lymphoid cells that we injected. This apparently allowed the injected cells to establish themselves in the females, and this somehow sufficed to create and maintain a state of tolerance. This explanation, consistent with published findings (19-21), implies that tolerance can be induced by any procedure that blocks rejection of introduced cells and allows the establishment of a state of persistent lymphoid chimerism in the host.

At a deeper level, however, our results do not answer the question of whether extrathymic tolerance occurs as a result of an encounter between antigen and a potentially responsive cell in the absence of a costimulus. It might seem that the tolerance induced in females depleted of CD8⁺ cells could not have occurred in this way. Potentially reactive CD8⁺ cells in these females could never have encountered H-Y antigen at a time when the hosts did not also have a full complement of CD4⁺ cells. However, speculative scenarios can be constructed for our findings in CD8⁺-depleted mice that accommodate the Bretscher and Cohn model (1). For example, the injected male cells may be immunogenic for only a brief period of time either because APCs deliver costimulatory signals for only a limited time after acquiring exogenous H-Y antigen, or because responding CD4⁺ cells provide help to CD8⁺ cells for only a limited time. In either case, costimulation or help would have waned long before there was repopulation of CD8⁺ cells, and despite the host possessing both antigen and CD4⁺ cells, the CD8⁺ cells would not receive costimulatory signals, and thus would be anergized. Alternatively, unrejected male cells in lymphodepleted female hosts would eventually become systemically dispersed (22), unlike freshly injected cells concentrated at an injection site or in draining lymph nodes. Thus, at times long after injection, the probability would be very low that a potentially responsive CD8⁺ cell encountering a male cell would simultaneously be near a source of help from a CD4⁺ cell encountering male antigen associated with MHC class II antigen on an APC. Incidentally, such a scenario could explain why intravenously injected male cells, which disperse rapidly, induce hyporesponsiveness to male skin grafts, even in female mice that are not temporarily depleted of CD4⁺ or CD8⁺ cells (13, 23).

Because of the innate attractiveness of the Bretscher and Cohn model in explaining tolerance (1), and its compatibility with a large body of experimental evidence, it is possible that further study will reveal that one or another of the scenarios just outlined is indeed correct.

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