

Thymus Epithelium Induces Tissue-specific Tolerance

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Summary

Most current models of T cell development include a positive selection step in the thymus that occurs when T cells interact with thymic epithelium and a negative selection step after encounters with bone marrow-derived cells. We show here that developing T cells are tolerized when they recognize antigens expressed by thymic epithelium, that the tolerance is tissue specific, and that it can occur by deletion of the reactive T cells.

It is generally believed that the primary role of the thymic epithelium (TE)¹ is to serve as an environment in which T cell precursors differentiate into mature T cells, as well as to selectively promote maturation of those T cells deemed to be "useful" by virtue of their affinity for self-MHC molecules. Although the question of whether cells of the thymic stroma also play a role in the induction of tolerance has been asked several times, the results have been difficult to fit into any clear model. Using several different approaches and a variety of antigens, some groups have reported that TE does not generate tolerance (1), whereas others have found that it does. Among the latter, some have found that T cells specific for antigens presented by the TE are deleted (2, 3), while some report only partial deletion (4–6). Others have found anergy (7) or a form of split tolerance in which the T cells react *in vitro* but not *in vivo* (8–10).

Several hypotheses have been put forward to explain these results. It has been suggested that TE induces deletion of high affinity but not low affinity T cells (3, 11), that it induces a form of partial anergy (7), or that it establishes an immunological network that maintains tolerance *in vivo* but is disrupted *in vitro* (8). To incorporate positive as well as negative selection, it has also been suggested that the TE is composed of two sorts of tissues that express different antigens, one that positively selects and one that induces tolerance (5).

Here we have tested another possibility. Starting with the assumption that the response against MHC molecules is actually directed against a panoply of MHC-peptide complexes (12, 13), that the peptides complexed with MHC molecules are derived from normal cellular proteins (14–16), and that different tissues, because of their different functions, might well express different arrays of proteins, we concluded that each tissue should display its own unique pattern of MHC-peptide complexes, which we call a MAP (MHC antigen

profile). We reasoned that if the TE induces tolerance, it would naturally tolerize only for the MHC-peptide complexes included in its own surface MAP, sparing T cells capable of responding to the MAPs of other tissues. Since the strength of the response against any particular tissue would depend on how its MAP overlapped with the MAP of the TE, many of the reported cases of split tolerance induced by TE might actually be unrecognized cases of tissue-specific tolerance.

To test this view, we reexamined the tolerogenic capability of TE using two sorts of transplantation antigens: allogeneic MHC molecules and the minor transplantation antigen H-Y. We chose these two antigens because: (a) both are intrinsically expressed by thymic epithelium; (b) both elicit responses from CD4⁺ as well as CD8⁺ T cells; (c) reactivity to both can be assessed by *in vivo* as well as *in vitro* tests; and (d) male bone marrow-derived cells are able to tolerize for male skin (17). We thought it likely therefore that H-Y would consist of one or at most a small set of peptide-MHC complexes shared by the surface MAPs of many tissues, whereas allogeneic MHC would consist of a large set of MHC-peptide complexes that vary from tissue to tissue. We found that the concept of tissue-specific tolerance does indeed account for the previously reported variety in the tolerogenic behavior of TE.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, nudes of both strains, as well as heterozygous litter mates, AKR, and timed pregnant mice were purchased from the Frederick Animal Facility (National Cancer Institute, National Institutes of Health, Frederick, MD). H-Y TCR transgenic mice (18) were bred at our colony (Bioqual, Gaithersburg, MD) onto the B10 and B10.A backgrounds.

Thymic Stroma (TS)

Thymuses from fetal (14 d, with day of plug = 0) male or female B6 or BALB/c mice were cultured for 5–7 d in transwell culture plates (Costar, Cambridge, MA) with 1.35 mM deoxyguano-

¹ Abbreviations used in this paper: MAP, MHC antigen profile; TE, thymic epithelium; Tg, transgenic; TS, thymic stroma.

sine (19) (Sigma Chemical Co., St. Louis, MO). They were then washed and transplanted into recipient mice. The thymic rudiment that remains after deoxyguanosine treatment contains TE and a small number of macrophages (20). These macrophages are not competent professional APC in that they do not initiate rejection of the TE in fully allogeneic recipients (21), nor do they appear to induce tolerance to the MHC they express (1). Evidence that the treatment effectively removed professional APC comes from Figs. 3 and 6, where we found that B6 male or female deoxy-treated thymuses transplanted into BALB/c nude mice did not impart tolerance to B6 APC, whether the tests were done in vitro by MLR or in vivo by the rejection of B6 splenic fragments. We will refer to these grafted thymic rudiments as TS.

Thymus-transplanted Chimeras

All chimeras were created by transplanting TS (four to five thymuses) under the left kidney capsule of adult animals as previously described (1). For the experiments depicted in Fig. 1, B6 male or female TS was grafted into female B6 nude mice. For Figs. 2 and 3, B6 male or female TS was grafted into B10.A (H-2^b) transgenic (Tg) recipients carrying the anti-H-Y TCR. For Figs. 4–9, B6 or BALB/c TS was grafted into female BALB/c nude mice. The chimeras were used after a minimum of 6 (transgenic recipients) or 8 wk (nude recipients).

In Vitro Assays

Medium and Supplements. All in vitro cultures were set up in IMDM containing 10% FCS, 5×10^{-5} M β -mercaptoethanol, and 50 μ g/ml gentamycin.

Mixed Leukocyte Reaction (MLR). Splenic responder cells were panned on anti-IgM- (10 μ g/ml; Southern Biotechnologies Associates, Birmingham, AL) and anti-Ia- (14.4.4, purified in our laboratory; 10 μ g/ml) coated plates to remove B- and Ia-positive cells and plated at the indicated concentrations in 96-well, flat-bottomed plates (Costar, Cambridge, MA) with 4×10^5 γ -irradiated (2,000 rad) stimulator spleen cells/well. After 4 d, cultures were pulsed with 1 μ Ci/well [³H]thymidine, harvested 18 hours later on a 96 well harvester (Brandell, Gaithersburg, MD), and counted on a β counter (β plate, 1205; Pharmacia, LKB, Gaithersburg, MD). Data are shown as Δ cpm.

Cytotoxic Assays (CTL). 4×10^6 spleen responder cells per 2-ml well were cultured for 5 d with 2×10^6 splenic irradiated stimulator cells in 24-well plates (Costar), after which they were harvested and tested for CTL activity using the JAM Test (22), an assay that measures CTL activity by DNA degradation rather than membrane integrity. Briefly, the targets were Con A (2.0 μ g/ml; Sigma Chemical Co.) blasts, pulsed after 36 h of culture for 8 h with [³H]TdR. The blasts were washed, plated at 10^4 target cells/well in round-bottomed 96-well plates (Costar) that contained titrated numbers of the responder population. After 3 h, the plates were harvested as described for the MLRs. Percent specific killing was calculated as: $100 \times$ spontaneous DNA retention (cpm) – experimental DNA retention (cpm)/spontaneous DNA retention (cpm); where spontaneous retention is the [³H]TdR-labeled DNA obtained from targets incubated without killers, and experimental retention is the [³H]TdR obtained in the cultures containing both targets and responder cells.

In Vivo Assays

Skin Grafts. Animals were grafted on the dorsal thorax with tail skin. The grafts were sutured to keep them in place until complete healing was achieved. They were protected with a surgical

bandage that was removed after 6 d, and inspected then and every 2 d thereafter. Primary and secondary skin grafts were performed similarly.

Spleen Grafts. 3–5-mm fragments of the indicated spleens were obtained by compressing the capsule and cutting at the compression line. These small “pillows” of spleen were then grafted under the kidney capsule of the recipients. They were examined after 21 d.

Untreated Thymus Grafts. Four thymus lobes from 15-d-old B6 fetuses were grafted under the right kidney capsule of each host and analyzed at various times later by visual inspection, cell counts, and FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA).

Cytofluorimetry. Cells from grafted thymuses were counted, washed, and stained with the following mAbs: either anti-D^d or K^b-FITC (Pharmingen, San Diego, CA), plus CD8-red 613 (Gibco BRL, Gaithersburg, MD) and CD4-PE (Pharmingen), or T3.70-biotin (23), CD8-red 613 and CD4-FITC (Pharmingen), followed by a wash and streptavidin-PE (Pharmingen). They were analyzed on a FACScan[®] flowcytometer using FACScan[®] Research software (Becton Dickinson & Co.).

Results

Testing the Tolerogenic Capacity of TS in Nude Recipient Mice. To see whether H-Y presented by TS would induce tolerance to other male tissues, we treated male and female C57BL/6 (B6) fetal thymuses with deoxyguanosine to remove the bone marrow-derived cells, grafted the resulting TS into B6 female nudes, and tested these chimeric animals in vivo and in vitro for tolerance to B6 male and female tissues.

2 mo after thymus grafting, the chimeras were immunized intraperitoneally with 2×10^6 B6 male spleen cells. They were grafted 2 wk later with B6 male, B6 female, and allogeneic AKR skin, and tested for CTL activity 30 d after rejection of the AKR skin. Fig. 1 shows that nudes grafted with male TS did not generate CTL specific for male targets or reject male skin, whereas nudes grafted with female TS generally responded as well as control B6 females in both of these tests, and all the animals responded to third-party AKR. Thus, by both in vivo and in vitro tests, we found that male TS induced tolerance to other male tissues.

Although this finding was suggestive of the tolerogenic capacity of deoxy-treated TS, it was not definitive. Since both the TS and the recipients were H-2^b, the intact H-Y molecule might have been transferred to host dendritic cells, which would then have processed the molecule and presented it to tolerize immature T cells in the grafted thymus (24). We therefore retested the tolerizing capacity of TS in two situations designed to circumvent the activity of bone marrow-derived APC.

Testing the Tolerogenic Capacity of TS in Transgenic Mice. Our first approach was to graft B6 male or female TS into H-2^b mice transgenic for an anti-H-Y TCR. This TCR, distinguished by the mAb T3.70, was originally cloned from a CD8⁺ T cell clone isolated from a B6 female mouse. It uses the MHC D^b molecule as a restriction element and cannot recognize H-Y complexed with H-2^a (25). Under normal conditions in H-2^b female thymuses (such as B6), T3.70⁺ T cells develop nicely, forming plump thymuses that contain a large proportion of T3.70⁺ CD8⁺ single-positive cells (23, 25). However, in male H-2^b mice the T3.70⁺ T cells are

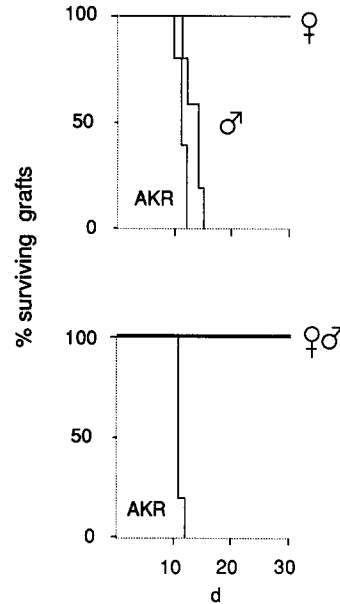
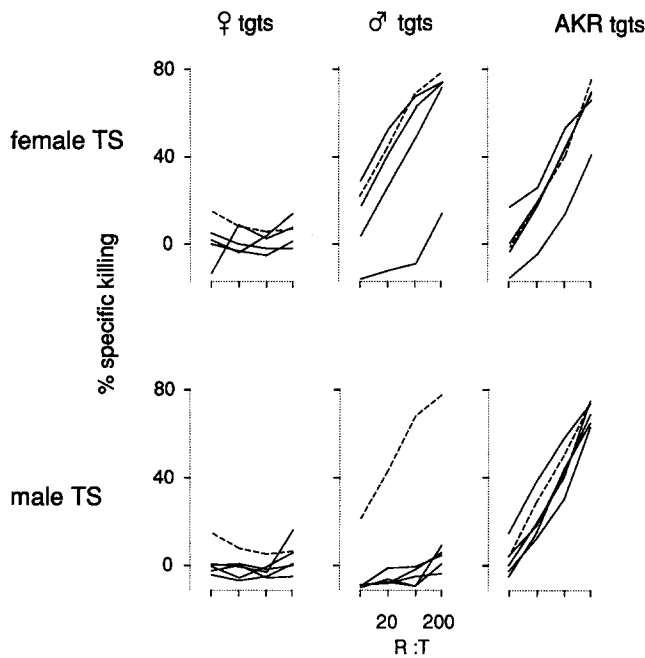


Figure 1. Chimeras reconstituted with male TS are tolerant to H-Y in both in vivo and in vitro tests. Individual B6 female nude mice were grafted with B6 female (*top*) or male TS (*bottom*), primed to H-Y after 8 wk and tested 14 d later for skin graft rejection (*right*) and 30 d later for CTL (*left*) responses against B6 male, B6 female, and allogeneic AKR targets and skin. The dashed lines in the CTL tests represent the CTL activity of a normal B6 female mouse primed at the same time as the chimeras.

deleted at an early stage of development (26). These severely depleted thymuses contain an overabundance of double-negative T cells, and few CD8⁺ single-positive T cells carrying T3.70. In contrast, H-2^a mice (such as B10.A) express no H-Y/D^b deleting or selecting elements recognizable by the T3.70 TCR. Therefore, the transgenic T cells do not develop and the thymuses produce and export T cells bearing endogenous receptors. In the B6 TS → B10.A Tg chimeras, the only tissue able to present H-Y with D^b is the grafted H-2^b stroma. Thus, even if H-2^a dendritic cells from the B10.A hosts were able to capture the H-Y antigen from the male B6 TS and represent it (24), T3.70⁺ T cells would not recognize the H-Y/H-2^a complexes. Therefore, any positive or negative selection events in these thymuses must be due to recognition of H-Y/D^b complexes on the transplanted TS itself.

We used the two well-established criteria, thymus cell number and T cell phenotype, to assess the effect of male and female B6 TS on the development of the Tg T3.70⁺ T cells in male and female H-2^a hosts. Fig. 2 shows the numbers of thymocytes present in the grafts 6 wk after grafting. Female TS grafts were well repopulated, ranging from 1 to 5 × 10⁷ cells, and contained on average 200-fold more T cells than male TS grafts, which ranged from 0.2 to 20 × 10⁴ cells/graft. Female grafts transplanted into male recipients (an excellent source of potentially reprocessable H-Y) were slightly smaller than those grafted into female bodies, indicating that the cells of the TS may be able to pick up and present a certain amount of H-Y to induce a small amount of deletion. However, the massive deletion that is seen in “normal” B6 male transgenic mice was only seen in chimeras in which the TS itself intrinsically expressed the H-Y antigen.

Using the mAb T3.70 to identify the cells expressing the transgenic TCR, we analyzed the specificity of the depletion induced by male TS. Fig. 3 illustrates the CD4 and CD8

staining patterns of thymocytes expressing T3.70 in “normal” transgenic (Tg) (H-2^a × H-2^b)F₁ mice and in B6 TS → B10.A transgenic chimeras. In the F₁ transgenics, where both selecting and nonselecting MHC types are present, the thymuses of female mice (Fig. 3 *a*) allow the differentiation of large numbers of CD8⁺ cells expressing high levels of the transgenic TCR. In contrast, there are virtually no TCR⁺ CD8⁺ cells among the few cells remaining in a typical F₁ male transgenic (Fig. 3 *b*). Fig. 3, *c* and *d*, represent the profiles of two mice from Fig. 2, showing that cells from the female or male H-2^b TS grafted into male H-2^a mice are virtually indistinguishable from those of the control transgenics. B6

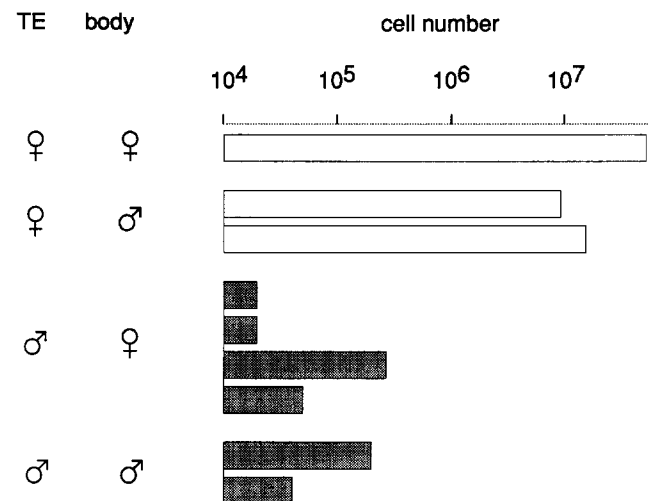


Figure 2. Male TS induces severe depletion of thymocyte populations in H-2^a anti-H-Y transgenic mice. Male or female B6 TS grafts were transplanted into intact H-2^a male or female anti-H-Y transgenic mice. 6 wk after grafting, the total number of lymphoid cells in each transplanted thymus was counted.

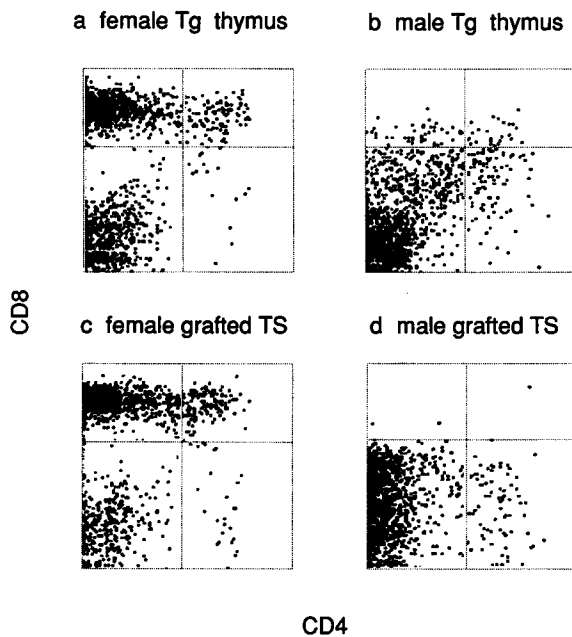


Figure 3. Male TS induces deletion of H-Y-specific transgenic T cells. Comparison of the CD4 and CD8 staining patterns of H-2^a TCR T3.70^{hi} thymocytes found in B6 female (c) and male (d) TS grafted into male B10.A transgenic recipients with those of T3.70^{hi} thymocytes found in normal (H-2^a × H-2^b)F₁ female (a) and male (b) transgenic thymus.

female TS (Fig. 3 c) allows the differentiation of large numbers of CD8⁺ TCR⁺ cells, whereas the very few surviving T cells in B6 male TS (Fig. 3 d) are mostly double negative. Thus, B6 male TS induces deletion of T3.70⁺ T cells, whereas female TS does not, even in an environment where H-Y is expressed by the bone marrow-derived cells migrating into the thymus.

We concluded that TS has the ability to present its own antigens to tolerize immature CD8 T cells and that, at least in the case of H-Y, it does so by inducing deletion of the reactive cells.

Testing the Tolerogenic Capacity of Male B6 TS in BALB/c Nude Recipients. The transgenic mice had allowed us to assess tolerance by visual phenotyping of cells bearing a single TCR. To look at functional, whole-body tolerance, we grafted male or female B6 TS into female BALB/c nude recipients and grafted them with male and female B6 skin. Here again the reprocessing of H-Y by the H-2^d host APC should not affect T cells able to recognize H-Y with H-2^b class I or class II molecules, and any tolerance induced to B6 male tissues should be due only to the TS itself. Based on published reports showing that TS-grafted chimeras accept skin of the thymic type (8, 9), we predicted that B6 female TS would tolerize only for female B6 skin and that B6 male TS should tolerize for both female and male B6 skin. The results were surprising.

3 mo after grafting the TS, we immunized the chimeras with B6 male spleen cells and 2 wk later grafted them with B6 male, B6 female, and BALB/c skin. To our great surprise (Fig. 4), both male and female B6 skin grafts were rapidly rejected, regardless of the sex of the grafted thymus. It seemed

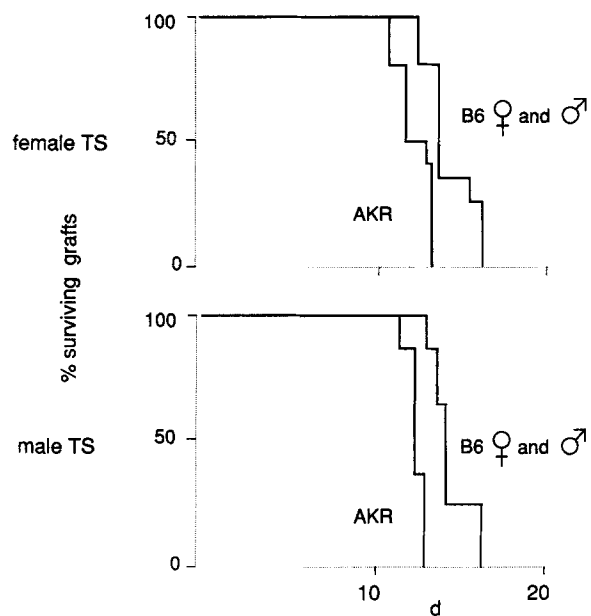


Figure 4. Neither B6 female nor B6 male TS tolerizes BALB/c nude recipients for acceptance of B6 skin grafts. Eight B6 female and eight B6 male B6 TS → BALB/c nude chimeric animals were primed with 2×10^6 B6 male spleen cells. 3 wk later they were grafted with B6 female, B6 male, and AKR tail skin and examined 1 wk later and every 2 d thereafter.

that neither the female nor the male B6 thymuses had induced tolerance.

In the earlier reports, the TS-grafted recipients that had accepted thymic type skin had not been immunized before skin grafting (8, 9). We therefore repeated our tests with a second set of B6 TS → BALB/c nude chimeras that were not primed before grafting. Fig. 5 a shows that the primary skin grafts were accepted. However, using the standards set by Billingham et al. (27), we saw that they were not absolutely healthy, being slightly swollen and not covered with luxurious tufts of hair. When regrafted, the same animals rapidly rejected the second grafts (Fig. 5 b). Thus, it seemed that the chimeras had achieved a certain level of tolerance to B6 skin antigens, enough to allow tentative survival of the primary grafts but not enough to allow graft acceptance in primed animals.

B6 TS Does Not Tolerize T Cells Specific for B6 Spleen, whether Tested In Vivo or In Vitro. The pattern of skin graft rejection seen in the B6 TS → BALB/c nude chimeras is similar to that sometimes seen when recipient and graft differ by certain weak minor histocompatibility antigens, and is consistent with the notion that the surface MAPs of skin and TS are strikingly alike but that enough differences remain to serve as targets for a primed immune system. Nevertheless, many groups have reported discrepancies between in vivo and in vitro assays of the tolerant state in thymus grafted chimeras (3, 9–11), and it has been suggested that the tolerant state induced by TE may be “fragile” and easily perturbed, e.g., by culturing the cells in vitro (8, 10) or perhaps by immunization of low affinity cells (3). We therefore tested a

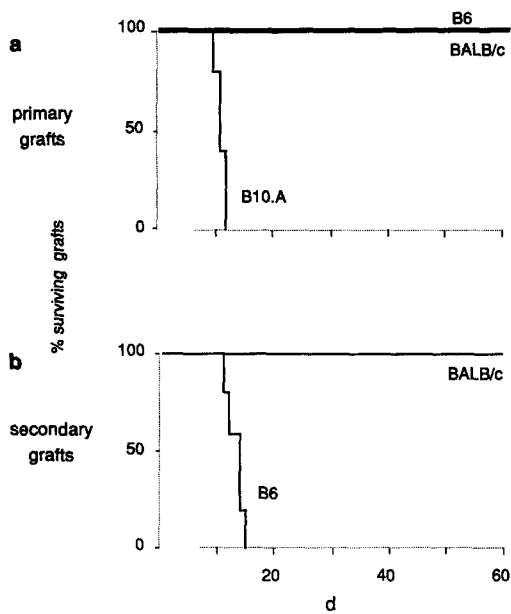


Figure 5. B6 TS \rightarrow BALB/c nude chimeras accept primary but not secondary B6 skin grafts. (a) 16 unprimed chimeric animals were grafted on the dorsal thorax with B6, BALB/c, and B10.A tail skin and examined 1 wk later and every 2 d thereafter. (b) 55 d after the primary graft they were regrafted with B6 and BALB/c skin and examined as for the primary grafts. The primary BALB/c grafts remained intact for >120 d. The primary B6 grafts went through a rejection crisis at the same time as the secondary grafts and emerged from this reduced in size.

set of unprimed B6 TS \rightarrow BALB/c nude chimeras against other tissue antigens, using both in vivo and in vitro assays.

As was previously reported, we found that unprimed B6 TS \rightarrow BALB/c nude chimeras generated both CTL and MLR

responses to B6 spleen stimulators (Fig. 6). The chimeras were tolerant of BALB/c, and responded 3–30-fold less well to B6 than to third-party AKR cells. Thus, by titrated in vitro tests, it appeared that B6 TS induced a substantial reduction in the response to B6 spleen cells though it did not produce a state of complete tolerance.

To compare the in vivo and in vitro responses to the same tissue, we grafted small “pillows” of intact spleen from B6 nude or BALB/c nude mice (to avoid a GVH reaction) under the kidney capsules of our experimental chimeras and, as a control, also into nude chimeras that had been reconstituted with BALB/c TS. Fig. 7 shows that B6 TS \rightarrow BALB/c chimeras were as responsive to B6 spleen antigens in vivo as they were in vitro. By macroscopic examination (Fig. 7 A), we found that both the control (*left pair*) and experimental (*right pair*) mice rejected B6 spleen grafts within 3 wk and retained syngeneic BALB/c spleen grafts in perfect condition for at least 2 mo. Examination of tissue sections showed that the rejection of B6 spleen by experimental B6 TS \rightarrow BALB/c chimeras was slightly less vigorous than by control BALB/c TS \rightarrow BALB/c mice. Fig. 7 B shows the typical structure of a B6 spleen graft being rejected by a control chimera. By 3 wk iron deposits abound, the follicular structure has disappeared, and much of it has been replaced by fibrotic tissue. In contrast, a syngeneic BALB/c graft accepted by an experimental B6 TS \rightarrow BALB/c chimera (Fig. 7 C) maintains the general architecture of a normal spleen with easily distinguishable red and white pulp. Fig. 7 D shows that the B6 spleen graft in the same chimera, though not yet completely destroyed, shows signs of rejection. Iron deposits are visible, and the follicular structure is completely disrupted.

Thus, we saw no discrepancy in the results of the different

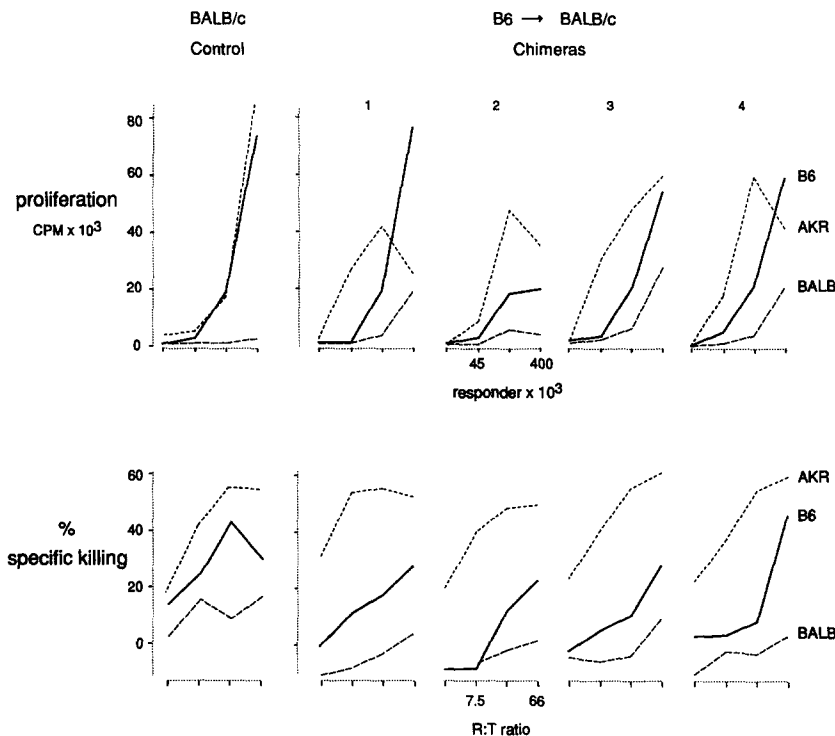


Figure 6. Chimeras reconstituted with B6 TS respond to B6 spleen cells in vitro. Represented are the mixed lymphocyte (*top*) and cytotoxic reactions (*bottom*) of four chimeric animals and a control BALB/c mouse to B6 (*solid line*), BALB/c (*small dashes*), and third-party AKR (*large dashes*) cells. Data are shown as Δ cpm over backgrounds that varied from 5 to 20% of the specific response. CTL assays were done by the JAM Test.

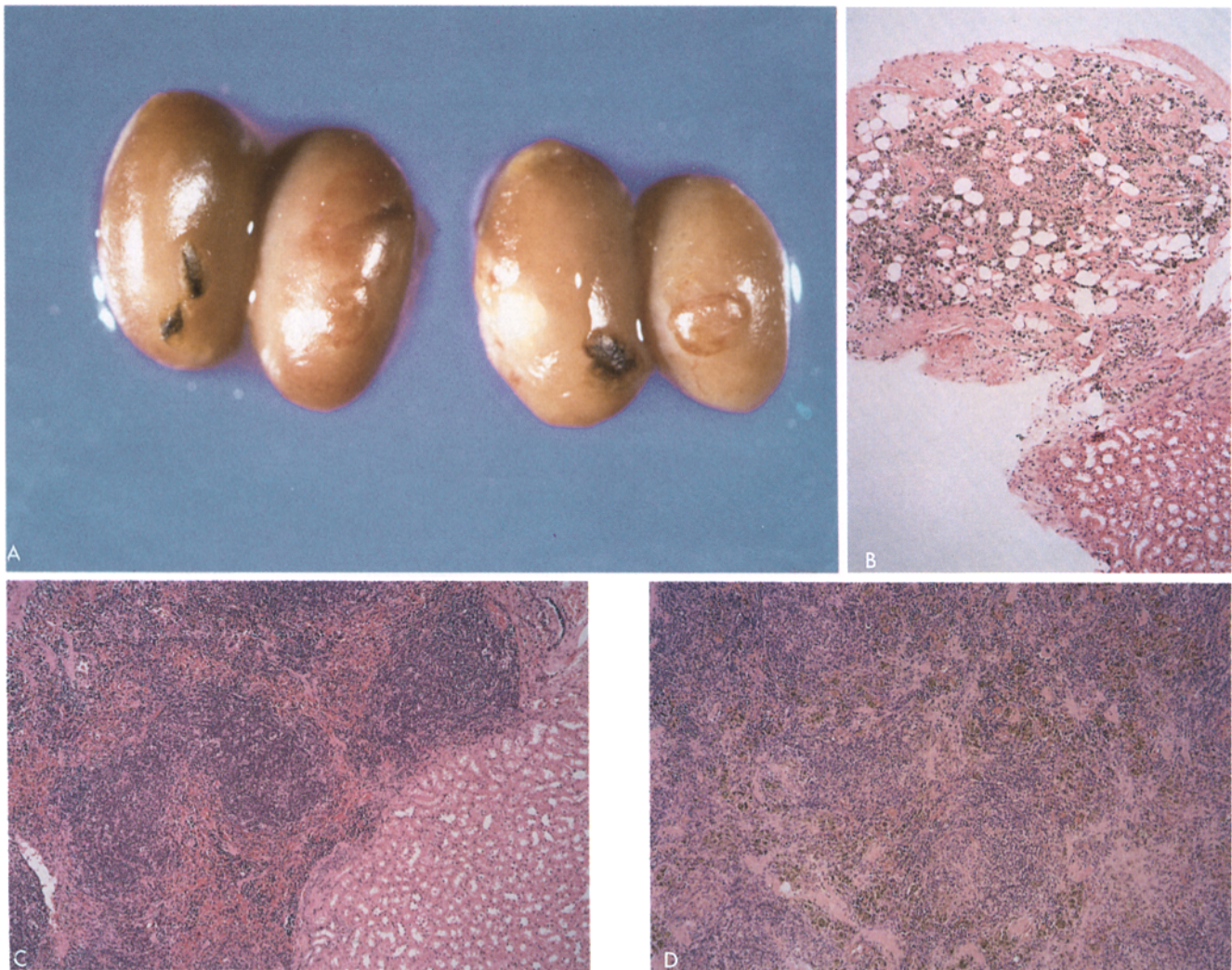


Figure 7. Chimeras reconstituted with B6 TS reject B6 spleen grafts in vivo. (A) The state of B6 nude and BALB/c nude spleen fragments grafted under the kidney capsules of chimeric mice 21 d after grafting. Hosts were B6 TS → BALB/c nude chimeras (*right two kidneys*) and BALB/c TS → BALB/c nude chimera controls (*left two kidneys*). Note that the B6 spleens have the same necrotic appearance in both control and experimental chimeras, while the BALB/c grafts remain intact. (B–D) Thin sections of the grafts stained with H & E. (B) Positive control: B6 spleen grafted into a BALB/c TS → BALB/c nude chimera. (C) Negative control: BALB/c spleen grafted into BALB/c TS → BALB/c nude chimera. (D) Test graft: B6 spleen grafted into a B6 TS → BALB/c nude chimera.

assays. Whether we tested against skin or spleen, in vivo or in vitro, we found that BALB/c T cells that matured in B6 TS were not tolerant of other B6 tissues, through their responses were less vigorous than those of normal BALB/c mice.

B6 TS Tolerizes T Cells Specific for B6 TS. Two explanations existed for our results and those of others. Having found tolerance by in vivo tests and reactivity in vitro, Sprent and his colleagues (3, 4) proposed that TS induces an incomplete state of tolerance, deleting only the highest affinity T cells, and that the in vitro MLR can be mediated by cells with lower affinity than can GVH or skin graft rejection. Our finding, that unprimed chimeras reject spleen grafts while only primed mice reject skin, could also be assimilated into this picture if we postulated that memory T cells are more easily stimulated than virgin cells, and that tail skin, having few strong APC, is an inefficient initiator of primary im-

mune responses. A second possibility was that the tolerance induced by TS is no different from the profound tolerance induced by bone marrow-derived cells, but that it influences only those T cells specific for the TS surface MAP, leaving untouched any T cells specific for antigens that are unique to the MAPs of skin or spleen cells.

To distinguish between these two possibilities, we grafted our chimeras with a fresh fetal (day 15) B6 thymus. This second graft was not treated with deoxyguanosine so that its resident APC population would act to stimulate a rejection response (21, 28). Because it had been suggested to us that a thymus may be poorly populated with APC and therefore, like tail skin, be insufficiently stimulatory to induce a rejection crisis in unprimed chimeras, we used primed recipients that had previously rejected either B6 skin or spleen grafts. As controls for acceptance and rejection, we also grafted in-

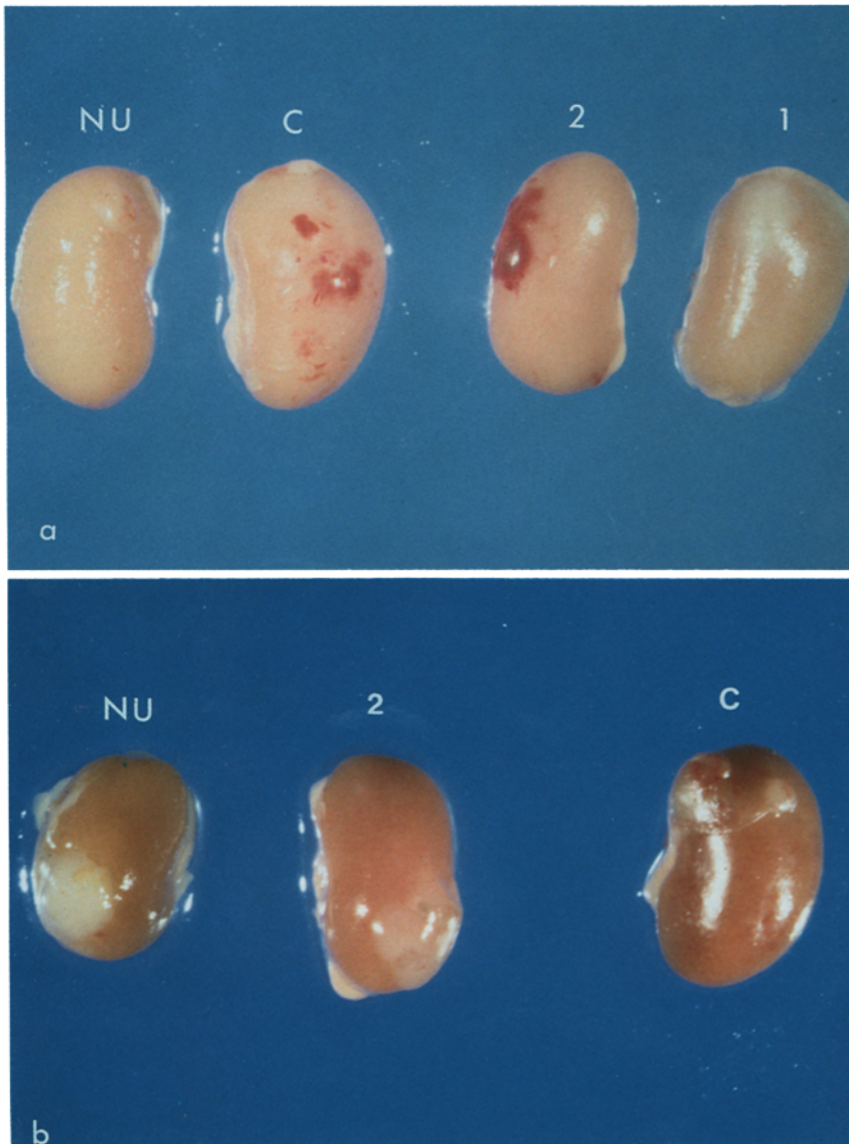


Figure 8. TS-transplanted chimeras accept B6 thymus grafts. 15-d untreated B6 fetal thymuses were transplanted into B6 TS → BALB/c nude chimeras that had previously been primed with B6 spleen cells and had rejected B6 skin or spleen grafts (labeled 2). As positive and negative controls, the thymuses were also grafted into normal immunocompetent BALB/c hosts (C) or into BALB/c nude mice (NU). Here we show the state of the grafted thymuses after 4 d (a) and 8 d (b). We also show the state of the original graft of B6 TS in the test recipient (1) 4 d after grafting the second thymus.

tact B6 thymuses into BALB/c nude and normal mice. At various times later we examined the thymuses macroscopically and their T cell subpopulations by FACS[®] analysis. We expected the highly primed chimeric recipients to reject the bone marrow components of the new thymus grafts. The question was, in the midst of this rejection crisis, would the epithelium itself be accepted? Figs. 8, a and b, and Fig. 9 show that it was.

By macroscopic inspection 4 d after grafting, the B6 thymus grafted into a chimera (Fig. 8 a, 2) resembled the one being rejected by a normal immunocompetent BALB/c mouse (Fig. 8 a, C). Both were bloody, and upon microscopic examination showed a disruption of the stromal architecture. This indicates that the intact thymus contained enough strong APC to initiate a rapid and violent rejection crisis. In contrast, the chimera's original TS graft (Fig. 8 a, 1) had the same white, plump, and healthy appearance as the unrejected thymus grafted into a nude mouse (Fig. 8 a, NU). By day 8 (Fig.

8 b, 2), the appearance of the test thymus had completely changed. Unlike the thymus grafted into a normal BALB/c (Fig. 8 b, C), it was now white, plump, and as healthy as the thymus grafted into a control nude mouse (Fig. 8 b, NU), suggesting that the rejection crisis had abated, leaving the thymic epithelium apparently intact. The chimeras rapidly rejected third-party AKR fetal thymus grafts (not shown), showing that their acceptance of the B6 epithelium was specific and not due to a generalized inability to reject thymic epithelium.

To see if the epithelial components of the thymus grafts had been fully accepted, we examined their functional state by analyzing the T cell populations maturing within them. Fig. 9 shows the changes in total cell number and the relative proportion of various T cell subpopulations (shown by the partitions in the pie representing each data point) in each thymus at different times after grafting. Fig. 9, left, shows the kinetics of uninterrupted development. B6 fetal thymuses

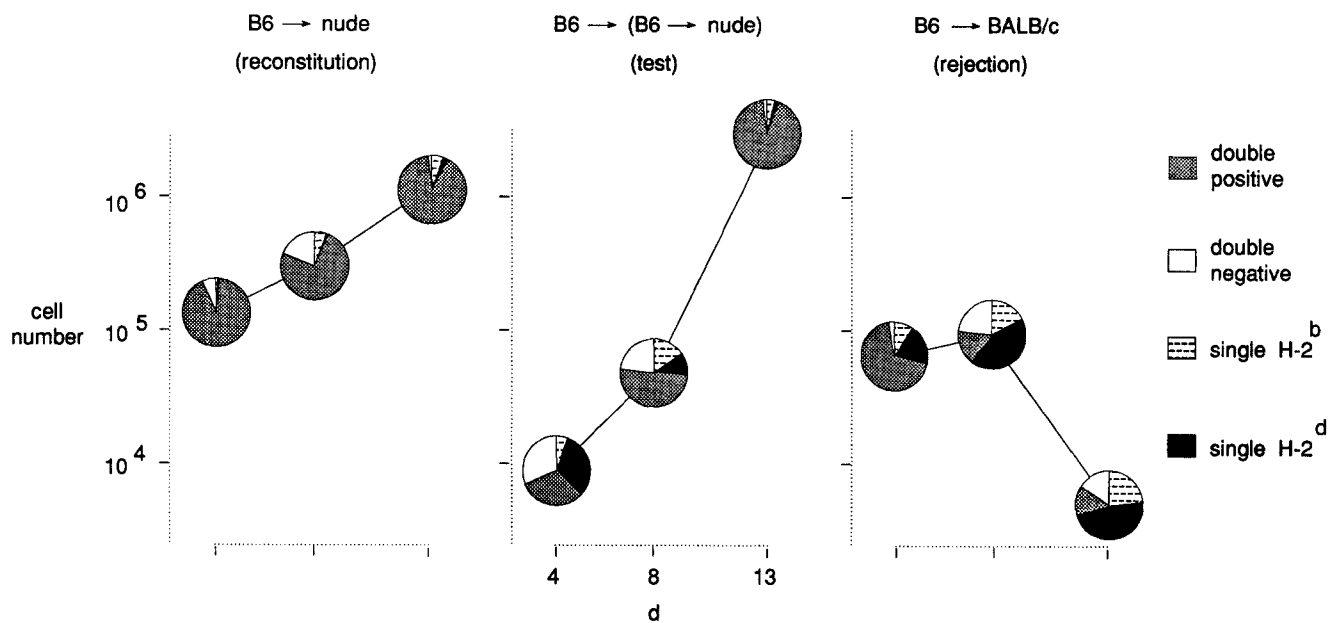


Figure 9. TS-transplanted chimeras reject the bone marrow but not the epithelial component of B6 thymuses. 15-d B6 fetal thymuses were transplanted into B6 TS → BALB/c nude chimeras, BALB/c nude mice, or normal immunocompetent BALB/c hosts as indicated, and analyzed on days 4, 8, and 13. The graph shows the number of cells (shown by the position of each pie graph on the y-axis) and the relative proportions of various T cell subpopulations (by the sizes of the slices within each pie) within each thymus. The cells from the grafted thymuses were counted, stained with directly labeled mAbs specific for CD4, CD8, and either D^d or K^b, and analyzed in three colors. The data include the chimeras shown in Fig. 8, and represent are of a total of 12 chimeras and an equivalent number of controls analyzed up to day 13. Another 10 were analyzed at various times up to day 100.

grafted into control BALB/c nude mice grew steadily over the next 13 d, reaching a size of $\sim 10^7$ cells, and continued to produce T cells. In contrast, Fig. 9, right, shows that B6 thymuses were quickly rejected by normal immunocompetent BALB/c mice. At first they expanded slightly with an inrush of mature host T cells, then declined in numbers as the rejection continued and had virtually disappeared by day 13. B6 thymuses (Fig. 9, middle) grafted into primed chimeras seemed at first to be rejected, as their constitution on day 4 resembled that of the rejected thymuses in BALB/c mice at day 8, yet they recovered by day 13 and were now on par, in both cell number and T cell subsets, with the unrejected thymuses in the nude recipients. Between days 35 and 100 these test thymuses remained functional, containing normal proportions of T cell subpopulations, which, when stained for H-2^d, resembled a normal BALB/c thymus (not shown). Thus, though the bone marrow component of the test thymus appeared to be rapidly rejected, the epithelium remained and continued to promote normal T cell development. From these results we concluded that the B6 TS → BALB/c chimeras, though clearly capable of responding to most B6 tissues, were tolerant of B6 thymic stromal cells.

Conclusion. Taken together, our results argue that: (a) thymic epithelium expresses a surface MHC-peptide MAP that overlaps with but is not identical to the MAPs of other tissues. (b) It induces tolerance to the antigens it expresses. The activity seen in vivo and in vitro against skin and bone marrow-derived cells is most likely due to T cells directed

against skin- and spleen-specific antigens not contained in the TS MAP. (c) It can induce tolerance by deletion.

Discussion

MAPs and the Concept of Tissue-specific Tolerance. It has been 15 yr since it was first proposed that reactivity against MHC antigens is actually directed against a plethora of MHC-antigen complexes (13) and, though the model has gained acceptance, its ramifications have not yet been widely applied. One of them, the concept of tissue-specific MAPs, is that each cell, because of its unique function, expresses its own particular set of proteins and consequently its own particular signature of surface MHC-peptide complexes. We find that the notions of tissue-specific MAPs and MAP-specific tolerance clear up a large set of otherwise confusing observations that have been accumulating for the last 40 yr.

In 1952, Billingham et al. (29) attempted to use reciprocal skin grafts to distinguish between identical and fraternal bovine twins but found the task to be impossible. Because bovine twins share placental circulation and become reciprocal chimeras (reported 10 yr before by Owen et al. [30]), both types of twins accepted each other's skin. Strangely though, some of the mutually tolerant calves reacted differently to maternal skin and this finding remained unexplained until 30 yr later, when Emery and McCullagh (31) repeated the experiments with flank rather than ear skin. With this more sensitive graft, they found that the twin calves were not fully

tolerant of each other's skin, though they hemopoietic chimeras. They concluded that tolerance of the MHC antigens of bone marrow does not extend completely to skin.

Billingham and Silvers (17) ran into the same problem in a meticulous study on H-Y in which they injected A/J male cells into neonatal B6 females. These mice later accepted B6 male skin grafts, showing that male A/J cells could tolerate for B6 male, but they rejected A/J skin. The authors wondered how this "split tolerance" to H-Y could exist in the absence of tolerance to A/J, since persistent chimerism should be necessary for both. 15 yr later, Boyse et al. (32) discovered Sk, a skin-specific antigen difference between A/J and B6. Thus, Billingham and Silver's split tolerance can be explained as tissue-specific tolerance. The B6 females were tolerant of A/J male hemopoietic cells but rejected A/J skin because of the Sk difference.

Since then, >30 reports of tissue-specific antigens have appeared (33-37) and, with the concept of MAPs, may provide an explanation for three types of perplexing findings. The first is an experiment in which Gao et al. (3), to study the tolerizing capacity of TS, created twice-irradiated and twice bone marrow-reconstituted $P_1 \rightarrow (P_1 \times P_2)$ chimeras, reasoning that all host APC would be depleted by the irradiation and thus that P_2 MHC class II molecules would remain only on the TE. These animals did not react to P_2 in vivo (to cause GVH death) nor in vitro (as CTL), though they did generate MLRs. The authors found this split tolerance difficult to explain. They considered and discarded the notion of tissue-specific tolerance, arguing instead that the high affinity T cells had been deleted, leaving low affinity T cells that "for some reason" (3) could be activated only in vitro. Using the concept of MAPs, we suggest the alternative view that some of the in vivo tolerance they found was actually induced by tissues other than TE. For example, class II molecules are expressed by high endothelial venule (HEV) cells (38) and can be induced on many other tissues. Class I is expressed almost ubiquitously. The chimeric T cells were clearly unreactive to many radioresistant peripheral host tissues other than TS, and it is not surprising that they did not reject host type skin or cause GVH death in a second (also irradiated) host.

The view that peripheral tissues may elicit tolerance of their own MAPs can also be used to explain other cases in which TS seemed to tolerate for skin. In the *Xenopus* test (9), the graft of thymic epithelium consisted of an entire head with its surrounding skin, and in mouse chimeras made with 10-d fetal thymus (8, 39), the grafts consisted of pieces of embryonic somite, which contain the primordial cells for tracheal cartilage, thyroid, and parathyroid (40). In all of these studies the peripheral organs may have generated their own tolerance.

The second group of experiments includes transgenic mice expressing allogeneic MHC molecules controlled by tissue-specific promoters (41), or mice grafted with APC-depleted allogeneic tissues (42, 43), that do not reject the original MHC-bearing tissue but nevertheless respond when tested by in vitro assays. This form of split tolerance has been dis-

cussed in terms of anergy, suppression, networks, etc., but here also the concept of MAPs gives a clear picture in which T cell populations can be devoid of cells specific for a particular tissue (e.g., class II on pancreatic cells) and responsive to another (class II on spleen APC). In the case where pancreatic expression of an MHC molecule induced tolerance in the thymus (44), there may have been some thymic expression and, in the case (45, 46) where addition of massive amounts of lymphokines or other sources of help has "reversed" the tolerant state, we suggest that this allowed the activation of those few T cells that had not yet traveled through the tolerizing organ.

Tissue-specific MAPs may also explain why humans carrying foreign organ grafts may still respond to the donor's APC by MLR, why tolerance induced by blood transfusions reduces but often does not completely abolish reactivity to a kidney (47), and why some autoimmune diseases show a distinct pattern of multi-organ reactivity. In fact, in the majority of cases where a form of split tolerance has been explained by anergy or suppressive interactions, a simpler explanation might be that the tolerance is simply tissue specific.

Ontogeny of Tolerance. In the thymus, immature developing T cells can be rendered tolerant by antigens presented by TE, by double-negative thymocytes (48), by mature CD8 T cells (49), as well as B cells (50, 51), islets (52), and dendritic cells (53, 54). The dendritic cells need not be specialized thymic dendritic cells since dendritic cells from the spleen, which are the most efficient activators of mature T cells, are also the most efficient tolerizers of developing thymocytes (53). Thus it appears that a variety of tissues, including professional APC, can present antigen to induce tolerance in maturing thymocytes, supporting our earlier suggestion that the decision between deletion and activation is not imposed by the environment, but is an intrinsic property of the developing thymocyte itself (53).

Generalizing from these results, we postulate that tolerance induction can occur in three distinct phases. The first, in the thymus, is the most stringent. Here developing thymocytes pass through a stage where tolerance is their inherent response to antigens presented by any cell, regardless of that cell's normal function (53, 55). This might be a stage in which the TCR is connected to internal apoptotic pathways but not yet to activating pathways (56, 57), or in which the thymocytes do not yet express receptors for any form of APC- or helper cell-derived second signals (42, 58), leaving them acutely susceptible to TCR-mediated death. Such a susceptibility step will eliminate those cells specific for antigens found on TE, T cells, perhaps $CD5^+$ B cells, and dendritic cells (including antigens common to all cells, antigens specific to dendritic cells, and some peripheral antigens that have been picked up by dendritic cells). T cells that pass through this stage unscathed become mature and leave for the periphery. Here, as virgin cells, they enter a second phase in which they can be activated by professional APC but remain tolerizable to antigens presented by other types of cells, such as B cells (59, 60), keratinocytes (61), thyroid (28), normal or transgenic islets (41, 42), etc. Tolerance to the MAPs of peripheral tissues

can thus occur during this second phase of differentiation. There is also evidence for a third phase in which the need for signals from CD4 T helper cells creates a fail-safe mechanism (56) that maintains tolerance in B cells (62) and both virgin (63, 64) and memory CTL (64).

Positive vs. Negative Selection. If the tissue thought to be involved in positive selection also negatively selects, how do any T cells slip through the gauntlet? There are three possibilities. It has been suggested several times that there may be two different sorts of TS, one involved in positive selection, and the other, perhaps expressing different peptides (5, 12), in negative selection. The critical point is that the positively selecting cell must not negatively select. The data in Fig. 9 suggest that no such cell exists. If there were an epithelial tissue able to positively but not negatively select, the B6 TS → BALB/c nude chimeras would not be tolerant of it. When grafted with a second, intact B6 thymus, they would reject the positively selecting cell during the violent rejection crisis that occurs (Figs. 8, *c* and *d*, and 9), and the second thymus, having lost the positively selecting cells, would not be able to produce mature single-positive T cells. Yet we found that, when the second thymuses recovered from the rejection of their hemopoietic passengers, they were repopulated with new stem cells and went on to support T cell development in a manner indistinguishable from normal thymuses or those grafted into nude hosts. Thus they must have contained the cells responsible for positive selection.

Another proposal has been that the difference between posi-

tive and negative selection is based on affinity; the immature T cell is selected to live and continue its differentiation if it binds with low affinity to MHC molecules on TS, and is told to die if its affinity for these same molecules is high. There are several versions of this idea, none of them fully satisfying, some suggesting that the distinction is due to the adhesion molecules involved, some by the level of TCR, and others by the stage of differentiation of the T cell (reviewed in references 65 and 66).

A third possibility is that the TE may be involved in a positive selection step that is not based on the specificity of the TCR. We envisage a selection step in which T cells are screened, in the same manner as B cells, by an endogenous ligand somewhat like the postulated stromal element recognized by V pre-B (67, 68). The ligand would bind to conserved portions of the TCR β chain and also to MHC molecules on thymic stromal elements, allowing the coaggregation of MHC with the TCR. If the TCR is properly hooked up to CD3, CD4 or CD8, and internal signaling pathways, the crosslinking induced by the endogenous ligand generates the signal that allows the T cell to continue development. The ligand, which may be on the immature T cells themselves or on the stromal elements, thus probes each cell for the expression of a properly rearranged receptor that is properly connected. After this step, autoreactive cells would be negatively selected by both TS and bone marrow-derived cells, leaving a functional, mostly self-tolerant and widely heterogeneous T cell population to emigrate into the periphery (69).

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