Failure of Clonal Deletion in Neonatally Thymectomized Mice: Tolerance Is Preserved through Clonal Anergy

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Summary

Self-tolerance is achieved in part through intrathymic deletion of self-reactive T cells. The necessity of the thymus for this process is suggested by the development of autoimmune diseases in neonatally thymectomized (neoTx) mice and by the failure of clonal deletion in nude mice. Indeed, the present study demonstrates that neonatal thymectomy on day 3 after birth results in the failure of clonal deletion of $V\beta11^+$ T cells in BALB/c mice and $V\beta5^+$ and $V\beta6^+$ T cells in DBA/2 mice. However, these potentially autoreactive cells are nonfunctional as measured by proliferation and lymphokine production after stimulation with appropriate anti-$V\beta$ mAbs or stimulator cells. It appears that this induction of nonresponsiveness may have occurred extrathymically: the early neonatal thymus (presumably the source of the peripheral T cells observed in neoTx mice) also contains T cells with self-reactive receptors, but these cells are fully functional. Therefore, neonatal thymectomy aborts deletion of self-reactive T cells, but self-tolerance is maintained through functional inactivation of potentially self-reactive clones.

The immune system must be prepared to mount a response against foreign antigens while at the same time fail to generate destructive responses against self-antigens. Three theories by which T cell tolerance to self-antigens may be maintained have been postulated: clonal deletion, direct clonal inactivation (anergy), and indirect clonal inactivation via regulatory (suppressor) cells. Acquisition of T cell tolerance by clonal deletion has in fact been demonstrated. T cells with specificity for certain MHC-encoded antigens plus unidentified peptides, minor lymphocyte stimulatory (Mls)[1] antigens recognized in the context of class II MHC, and male H-Y antigens in the context of class I MHC are deleted during intrathymic maturation (1–6). Therefore, these potentially self-reactive T cells never reach the periphery. Under conditions in which clonal deletion is inefficient, a second mechanism is utilized to maintain T cell tolerance: self-reactive T cell clones specific for class II or class II–associated Mls antigens can be rendered nonresponsive, although the mechanism(s) by which this anergy is achieved is not fully understood (7–11). Such potentially self-reactive cells will therefore not respond to self-antigens, despite escaping deletion in the thymus. The final mechanism by which T cell tolerance could theoretically be achieved is by active suppression of the function of autoreactive cells through suppressor or regulatory cells; to what extent this third alternative operates to impede harmful self-reactivity is not clear.

It was demonstrated recently that functional lack or removal of a thymus can, in two different systems, result in impaired clonal deletion. Athymic nude mice, which lack a functional thymus, fail to delete T cell clones with potentially self-reactive TCRs (12, 13). Despite the presence of these "forbidden" clones in the periphery, athymic mice remain relatively healthy and do not develop autoimmune diseases. In neonatally thymectomized (neoTx) mice, T cell clones with potentially self-reactive antigenic specificity can also be seen to escape to the periphery (14). Unlike nude mice, however, many strains of neoTx mice develop a multitude of autoimmune diseases, suggesting a causative link between the escaped self-reactive T cell clones and the observed autoimmune phenomenon (15, 16).

We tested this hypothesis by examining the effect of neonatal thymectomy on the development of a spectrum of T cell specificities in several mouse strains and by analyzing the functional reactivity of potentially autoreactive cells that escaped clonal deletion. We demonstrate that thymectomy can indeed result in a failure to delete certain "forbidden" T cell clones. More importantly, this failure to delete T cells with possible autoreactive TCRs occurs not only in mouse strains that develop autoimmune diseases upon neonatal thymectomy,

[1] *Abbreviations used in this paper:* Mls, minor lymphocyte stimulatory; neoTx, neonatally thymectomized.

but also in strains of mice not susceptible to autoimmune diseases, thus questioning the relationship between the failed clonal deletion and autoimmune disease. Finally, the "forbidden" T cell clones that did escape to the periphery in neoTx mice do not appear to be functional. T cell tolerance appears to be maintained by clonal anergy through a mechanism that cannot be restored by the addition of IL-2. Taken together, these findings demonstrate that, while clonal deletion may be dependent on the presence of a thymus, maintenance of clonal anergy is not. When the absence of a thymus results in a failure of the clonal deletion process, tolerance is nevertheless induced by a mechanism of clonal anergy.

Materials and Methods

Mice. Timed pregnant BALB/c, DBA/2, and C57Bl/6 mice were obtained from the National Cancer Institute (Frederick, MD).

Thymectomy. 1–8-d-old pups were anesthetized by cooling and thymectomized according to the method of Sjodin et al. (17).

Enrichment for T Lymphocytes. LNs and spleens were removed from mice and dissociated into single cell suspensions. RBC were removed from the spleen cell suspensions by lysis with ammonium chloride followed by washing with PBS. Cells were incubated with the appropriate anti-class II antibody (MKD6 [reference 18] and 14-4-4 [reference 19] for H-2^d strains; Y3P [reference 20] for H-2^b strains) for 30 min on ice, washed, resuspended in complement (Accurate Chemical & Scientific Corp., Westbury, NY), and incubated at 37°C for 30 min. Dead cells were removed by centrifugation on Lympholyte M (Cedarlane Laboratories).

Flow Cytometry Analysis. Cells were suspended in HBSS (without phenol red) containing 1% BSA and 0.1% sodium azide. Cells (10^6/100 μl buffer) were incubated on ice with 10 μl of the appropriate FITC-conjugated antibody. After washing, cells were stained with biotinylated mAb to CD4, and then allophycocyanin-labeled avidin (Caltag, So. San Francisco, CA). Control staining of cells with irrelevant antibody was used to obtain background fluorescence values. The samples were analyzed on a FACS 440 (B-D Automated Immunochemistry, Becton Dickinson & Co., Mountain View, CA) interfaced to a PDP 11/24 computer (Digital Electronics, Corp., Maynard, MA). Data were collected on 50,000 cells and are shown as contour diagrams, with a three-decade log scale of green fluorescence on the x-axis and a three-decade log scale of red fluorescence on the y-axis. Reagents used for direct staining were FITC- or biotin-conjugated anti-: pan TCR-α/β (21), Vβ3 (22), Vβ5.1, 5.2 (5), Vβ6 (23), Vβ8.1, 8.2 (24, 25), Vβ8.2 (26), Vβ11 (4), Vβ14 (27), and CD4 (28).

T Cell Proliferation Assays. For mAb-induced proliferation, the purified mAb was diluted to the indicated concentration in PBS and 30 μl was added per round-bottom microtiter well. Plates were incubated for 2–3 h at 37°C and then washed three times with PBS before use. 2×10^5 T cell–enriched spleen cells in 200 μl complete DMEM (10% FCS, 5×10^{-5} M 2-ME, 10 mM Hepes, 200 mM glutamine, 0.11 mg/ml sodium pyruvate, 10 U/ml penicillin, and 100 μg/ml streptomycin) were added per well. rIL-2 was added where indicated at a final concentration of 10 U/ml. After 48 h, cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 12–18 h later. Values represent the arithmetic mean of triplicate cultures. SEs were generally <10% of the mean.

For mixed lymphocyte cultures, the indicated number of T cell–enriched spleen cells was incubated with 5×10^5 irradiated (2,000 rad) spleen cells in 96-well flat-bottom microtiter plates. After 3 d, cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 12–18 h later. All determinations were performed in triplicate. Data are expressed as Δcpm (cpm values of experimental groups minus responder cells alone).

Lymphokine Assay. After 48 h of culture, 100 μl of supernatant harvested from mAb stimulation assays were incubated with 5×10^3 CTLL-2 cells in flat-bottomed microtiter plates. Cultures were incubated for 36 h and then pulsed with 1 μCi of [^{3}H]thymidine and harvested 8 h later. Data are expressed as Δcpm (cpm values of experimental supernatants minus cpm values of unstimulated supernatants). Values represent the arithmetic mean of triplicate cultures. SEs were <10% of the mean.

Results

Neonatal Thymectomy Results in Escape of Some T Cells from Deletion. Development of mAbs with specificity for the Vβ chain of the T cell receptor has enhanced the ability to analyze objectively the development of the T cell repertoire, in that it replaced complex functional procedures to determine TCR specificities with flow cytometry analysis. In the BALB/c strain of mice, the repertoire is negatively affected by the expression of I-E^d, which results in intrathymic deletion of Vβ5 and (if other so-called "co-tolerogens" are present as well [reference 5]) Vβ11-expressing T cells, and by the expression of Mls-2a, which removes T cells with Vβ3 from the repertoire. The DBA/2 strain is also affected by expression of Mls-1a, which results in deletion of Vβ6- and Vβ8.1-expressing T cells, in addition to the deletions found in BALB/c.

To examine whether clonal deletion of these particular T cell clones is dependent on the presence of a thymus, we thymectomized mice on day 3 after birth and analyzed the peripheral T cell repertoire 2–4 mo later. Two-color flow cytometry analysis of Vβ expression vs. CD4 was performed on T cell–enriched lymph node cells (Fig. 1) as well as spleen cells (data not shown). The resulting data were normalized for the number of TCR-α/β^+ T cells (Table 1). As shown in Table 1, the percentage of $V\beta11^+$ cells is significantly increased in thymectomized BALB/c mice as compared to sham-thymectomized littermates. In addition, $V\beta6^+$ T cells also escape deletion in neoTx DBA/2 mice but remain unaffected in the neoTx BALB/c mice. This effect is also seen when the results are converted to absolute numbers: although thymectomized mice generally have two- to threefold lower numbers of T cells, the increases in percentages of certain Vβ-expressing T cells with self-reactive receptors are at least as high and often higher (data not shown). While the increases are always small, they were consistently observed in >100 thymectomized mice investigated over a period of 1 yr. In contrast, Vβ3-bearing T cells, which are deleted in normal mice of both strains because of Mls-2a expression, remain undetected in thymectomized mice of either strain. The rescue of deletion of Vβ5 and Vβ11 appeared to be strain dependent. There was no significant increase in the percentage of $V\beta5^+$ T cells in the neoTx BALB/c mice, whereas the same treatment of DBA/2 mice always significantly increased the number of Vβ5-expressing T cells. Conversely, the number of $V\beta11^+$ T cells was always enhanced in BALB/c mice, but not affected in DBA/2 mice. As an additional control, mice were also

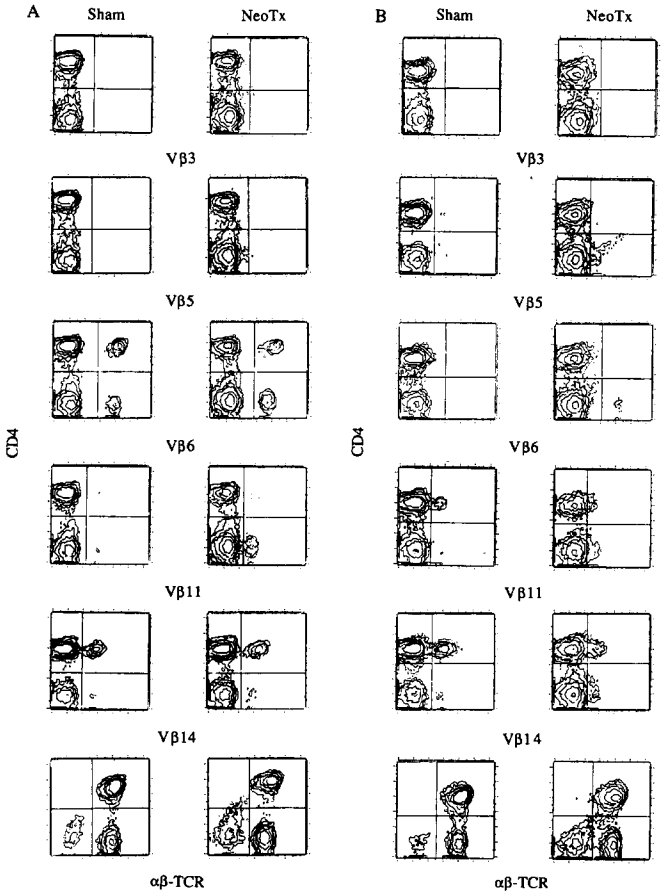

Figure 1. Two-parameter flow cytometry analysis of cell surface expression of the indicated Vβ vs. CD4 on T cell–enriched LN cells from sham or thymectomized mice in BALB/c (*A*) or DBA/2 (*B*) mice. Mice were thymectomized 3 d after birth. Peripheral T cells were analyzed when mice were 2–3 mo old. Percentages for TCR expression are given in Table 1.

analyzed for Vβ14 expression. While expression of T cells with a Vβ14 TCR is variable and dependent on the presence of certain positive selecting elements (27), no self-antigen has been defined that results in elimination of Vβ14$^+$ cells.

Table 1. *Vβ Expression in neoTx Mice*

	BALB/c		DBA/2	
	Sham	neoTx	Sham	neoTx
Vβ3	0.1 ± 0.03	0.7 ± 0.19	0.2 ± 0.04	0.2 ± 0.21
Vβ5	0.1 ± 0.03	0.3 ± 0.12	0.8 ± 0.12	2.6 ± 1.14
Vβ6	11.8 ± 0.08	11.9 ± 0.19	0.1 ± 0.05	2.1 ± 0.28
Vβ11	1.0 ± 0.11	3.7 ± 0.54	2.1 ± 0.24	2.7 ± 1.85
Vβ14	8.2 ± 0.09	8.4 ± 0.59	10.2 ± 0.52	7.6 ± 1.34

Vβ expression was determined by immunofluorescent staining with the anti-Vβ mAbs described in Material and Methods. Analysis was performed on T cell–enriched LN cells from 2–3-mo-old sham or thymectomized mice. Values indicated are percentage of TCR-α/β$^+$ LN cells and are reported as the mean ± SE from three separate experiments.

Clearly, the number of Vβ14-expressing T cells in the periphery was not enhanced as a result of thymectomy. The decrease in Vβ14$^+$ T cells sometimes observed in neoTx DBA/2 mice is possibly a consequence of the increase in T cells with self-reactive receptors. It is interesting to note that both in the BALB/c and DBA/2 strains, the majority of T cells that escape deletion are in the CD8$^+$ subset (Fig. 1). In conclusion, the absence of a thymus results in the appearance of low but significant T cells with autoreactive receptors in thymectomized mice, which is consistent with earlier reports in nude mice (12, 13).

T Cells That Escape Clonal Deletion Are Not Functional. neoTx mice develop organ-localized autoimmune diseases, provided the thymectomy is performed between days 1 and 4 after birth; yet, not all strains are equally susceptible to this phenomenon (16). In the A/J mouse strain (H-2^a), 90% of female mice develop oöphoritis, and 30% of BALB/c mice (H-2^d) develop gastritis. In sharp contrast, thymectomized DBA/2 mice (also H-2^d) do not develop any known autoimmune disorders. Since in both DBA/2 and BALB/c mice thymectomy results in a failure of clonal deletion (see above), it appears that factors other than MHC determine whether such potentially autoreactive cells are indeed harmful. We therefore tested the hypothesis that autoimmune diseases are related to the presence of the "forbidden" T cell clones that had escaped deletion in the thymus by analyzing peripheral Vβ6$^+$ and Vβ11$^+$ T cells from thymectomized mice for their functional capabilities. This analysis is particularly relevant in view of the recent finding that, even when Vβ6- and Vβ11-expressing T cells are of the CD8$^+$ phenotype, they may still display Mls-1a (references 29 and 30, for Vβ6 cells) or I-E (reference 31, for Vβ11 cells) reactivity.

We first tested whether neoTx mice are capable of generating normal T cell responses to antireceptor antibodies. In thymectomized DBA/2 mice, stimulation by anti-Vβ8.2 TCR mAb was used as a control, since Vβ8.2$^+$ T cells are not deleted in DBA/2 mice. Indeed, T cells from normal and thymectomized mice responded equally well to anti-Vβ8.2 mAb stimulation, demonstrating that T cells from neoTx mice are functional (Fig. 2 *A*). For the BALB/c strain, stimulation with anti-Vβ6 mAb was used as a control, since no known deletional elements affecting the level of Vβ6$^+$ T cells are expressed in BALB/c. The results demonstrate that also in this control, responsiveness was unaffected by thymectomy (Fig. 3 *B*). As an additional control, T cells from DBA/2 and BALB/c mice were stimulated with anti-pan TCR-α/β mAb (21); this mAb reacts with all TCR-α/β–expressing T cells, regardless of the type of β chain used. Again, T cells isolated from thymectomized mice responded equally well as T cells from normal mice (Fig. 3 *A* for BALB/c; data not shown for DBA/2). Taken together, these findings establish that neonatal thymectomy does not result in a generalized defect in T cell function.

In contrast, although T cells expressing Vβ6 (in DBA/2 mice) and Vβ11 (in BALB/c) were found in the periphery of thymectomized mice, such cells were not responsive to stimulation over a wide range of specific mAb concentrations (Figs. 2 *B* and 3 *C*), even in the presence of IL-2. This defect

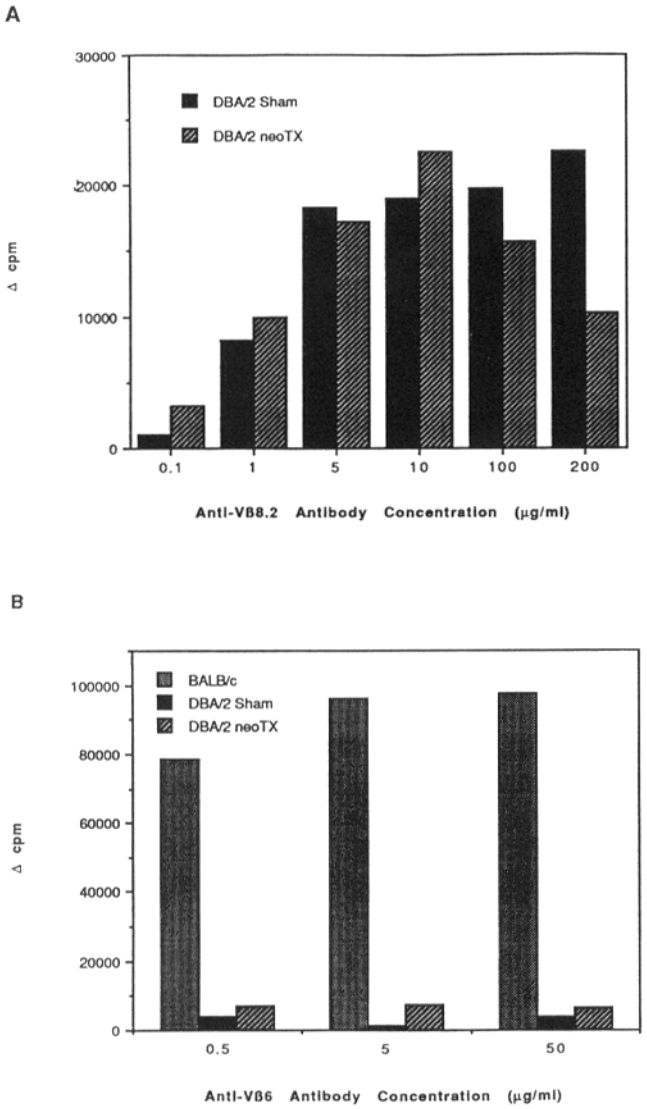

Figure 2. T cells that escape clonal deletion are not functional. T cell–enriched spleen cells from 2–3-mo-old sham or thymectomized DBA/2 mice were stimulated with anti-Vβ 8.2 (*A*) or anti-Vβ6 (*B*) mAb at the indicated concentration. rIL-2 was added at a final concentration of 10 U/ml.

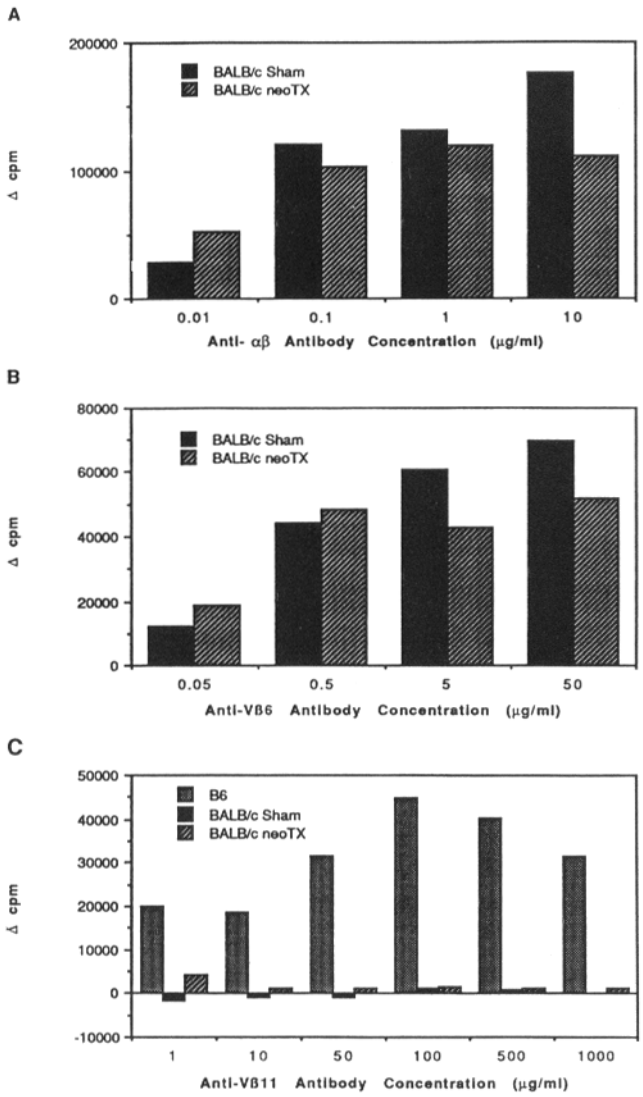

Figure 3. T cells that escape clonal deletion are not functional. T cell–enriched spleen cells from 2–3-mo-old sham or day 3 thymectomized BALB/c mice or control C57Bl/6 mice were stimulated with anti-α/β (*A*), anti-Vβ6 (*B*), or anti-Vβ11 (*C*) mAb at the indicated concentration. rIL-2 was added at a final concentration of 10 U/ml.

is not due to a failure of $CD8^+$ T cells to respond in such assays, as previous studies (26, 32) demonstrated that TCR occupancy and cross-linking are sufficient to induce proliferation in $CD8^+$ T cells, provided an exogenous source of lymphokines is added. To demonstrate that the anti-Vβ11 mAb could effectively activate T cells from normal mice, T cells from C57Bl/6 mice that lack expression of I-E and therefore do not delete $V\beta11^+$ T cells in the thymus were tested as well. It can be seen that the $V\beta11^+$ T cells from this nondeleting strain of mice responded in a dose-dependent fashion to specific stimulation (Fig. 3 *C*). Similarly, the anti-Vβ6 mAb's ability to activate T cells from normal mice was demonstrated for BALB/c mice (Fig. 3 *B*). Thus, neonatal thymectomy has resulted in a specific nonresponsiveness of those peripheral T cells (shown here for spleen cells; identical findings were obtained with LN cells, data not shown) which in normal mice are subject to intrathymic clonal deletion, while leaving responsiveness of other T cells subsets intact. With the caveat that the in vivo behavior of these cells might be different, it would therefore appear that the development of autoimmune diseases in neoTx mice is unlikely to be related to the failure of clonal deletion, since clonal unresponsiveness in the "forbidden" T cells would preclude any harmful effects.

Finally, we tested to which extent the observed unresponsiveness was due to a failure in lymphokine production. Under conditions of antigen presentation by defective APC, T cell clones can be made unresponsive to activation by their specific ligand (33). The lack of responsiveness is due to an inability

to produce IL-2 and can be overcome by the addition of the lymphokine to the cultures (33). In neoTx mice, however, the nonresponsiveness demonstrated by Vβ11$^+$ and Vβ6$^+$ in DBA/2 T cells could not be restored by the addition of either rIL-2 (Figs. 2 *B* and 3 *C*) or supernatants from Con A–activated T cells (data not shown) to the cultures. In addition, the dysfunctional T cells failed to produce detectable levels of lymphokines, whereas T cells that did respond to mAb stimulation by proliferation also produced lymphokines (Fig. 4).

DBA/2 Vβ6$^+$ T Cells from neoTx Mice Do Not Respond to Syngeneic Spleen Cells. Vβ6$^+$ T cells are deleted in mouse strains in which Mls-1a is expressed, and responding T cells in anti–Mls-1a MLCs predominantly utilize Vβ6$^+$ TCRs, indicating preferential reactivity of Vβ6 for the Mls-1a gene product (3). We therefore used syngeneic, Mls-1a$^+$ spleen cells as stimulator cells for examination of the functional status of the "forbidden" Vβ6$^+$ T cells derived from thymectomized DBA/2 mice. As shown in Fig. 5, T cells from control and thymectomized DBA/2 mice do not respond to syngeneic stimulator cells, while both populations responded to stimulator cells expressing allogeneic MHC antigens. The efficacy of the DBA/2 stimulator cells was demonstrated by their ability to generate proliferative responses in allogeneic C57Bl/6 responder cells as well as in Mls-1a–specific T cell hybridomas (data not shown). These results extend the mAb stimulation experiments, and demonstrate that the Vβ6$^+$ T cells remain tolerant to self-antigens expressed by syngeneic spleen cells.

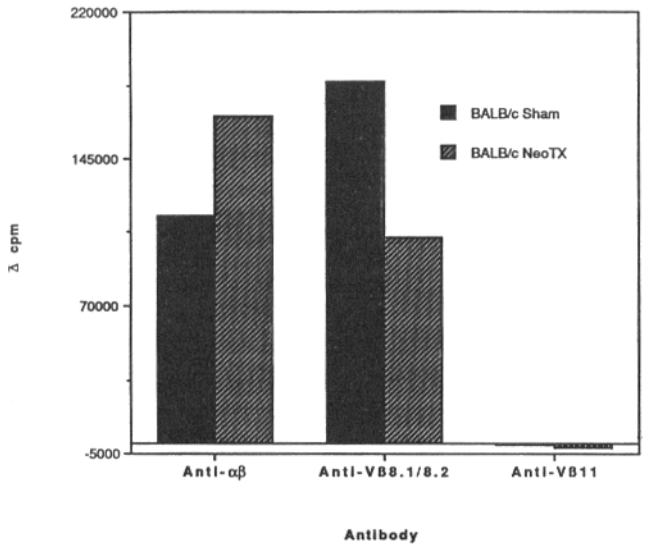

Figure 4. T cells that escape clonal deletion do not produce IL-2. T cell–enriched spleen cells from 2–3-mo-old sham or thymectomized BALB/c mice were stimulated for 48 h with anti-Vβ mAbs at doses of 0.5, 5, and 50 μg/ml. Supernatants were harvested from the cultures and assayed for lymphokine production by testing their ability to support proliferation of the CTLL cell line at different dilutions. Only data for the highest concentration of anti-Vβ (50 μg/ml) and supernatant (50%) are given; anti-Vβ11–induced activation did not generate responses at any of the doses tested.

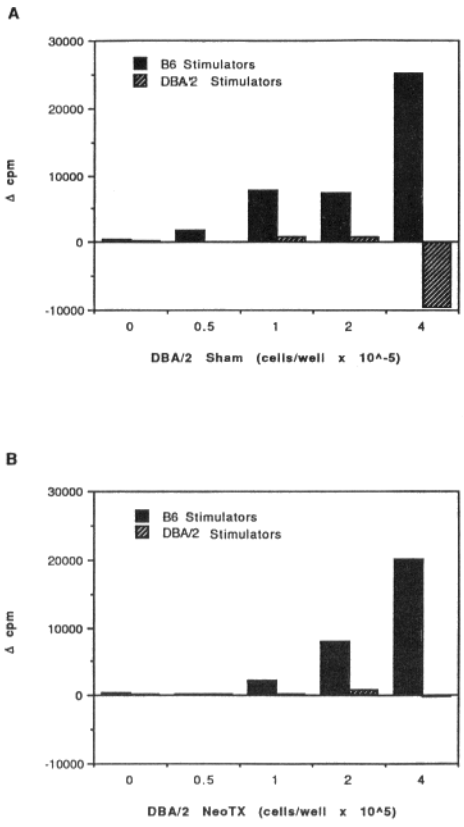

Figure 5. Vβ6$^+$ T cells from thymectomized DBA/2 mice do not respond to autologous spleen cells. T cell–enriched spleen cells from 2–3-mo-old sham (*A*) or thymectomized (*B*) DBA/2 mice were stimulated with DBA/2 or C57BL/6 irradiated spleen cells.

BALB/c Animals Thymectomized on Day 5 or 8 Also Have Increased Levels of Vβ11-expressing T Cells. As mentioned above, neonatal thymectomy may or may not result in the development of autoimmune diseases, dependent upon the strain of mice (16). Development of disease does not appear to be correlated with the failure of clonal deletion since both BALB/c mice, which do develop disease, and DBA/2 mice, which do not exhibit autoimmune diseases, fail to delete T cells with "forbidden" Vβs. This issue was additionally addressed by examining the T cell repertoire of mice thymectomized after day 4. It was previously reported that the timing of the thymectomy is critical in inducing autoimmune diseases. Mice thymectomized between days 1–4 may develop disease whereas those thymectomized later do not develop disease, even in strains extremely prone to neonatal thymectomy–induced autoimmune disease, such as A/J in which 90% develop oöphoritis (16). As shown in Table 2, the T cell repertoire in BALB/c mice thymectomized on day 5 or 8 is similar to that of mice thymectomized on day 3: the percentage of Vβ11$^+$ T cells is higher as compared with controls; the significance of the slightly higher Vβ11 values in the day 5/8-thymectomized vs. day 3-thymectomized mice cannot be evaluated, since much fewer mice were tested in the former group.

Together with the finding (see above) of unresponsiveness in anti-Vβ11 proliferation assays, the presence of Vβ11$^+$ T cells in mice that do not develop disease suggests that these "forbidden" clones are not involved in induction of autoimmunity.

The Early Postnatal Thymus Contains Functional T Cells with "Forbidden" Receptors. We next addressed the possible origin of the T cell clones that failed to be clonally deleted in the periphery of neoTx mice. Self-reactive T cells can persist in the neonatal thymus; in the DBA/2 strain of mice, mature T cells expressing Vβ6 can be detected in the thymus as late as 7–8 d after birth (reference 34; and our unpublished results). "Forbidden" T cell clones in neoTx mice might be derived from such early thymocytes with self-reactive receptors that may escape from the neonatal thymus before thymectomy is performed. We therefore examined the functional status of the mature Vβ6$^+$ thymocytes in newborn DBA/2 and (DBA/2 × C57Bl/6) F1 mice. Approximately 3% Vβ6$^+$ T cells can be detected by flow cytometry among the TCR-α/β-expressing single positive thymocytes in 4–5-d-old mice of these strains (reference 34; and data not shown); in contrast, <0.5% Vβ6$^+$ T cells can be detected in the thymus of adult mice of these strains. Surprisingly, these early postnatal thymocytes responded to stimulation by cross-linked anti-Vβ6 mAb both with and without the addition of rIL-2 to the cultures (Fig. 6 *A*; and data not shown). Also, anti-Vβ6 mAb induced lymphokine production, as measured in a CTLL proliferation assay, in neonatal but not in adult thymocytes (Fig. 6 *B*). The CTLL assay may be somewhat more sensitive than the proliferation assay, since neonatal thymocytes consistently generated lymphokine production (Fig. 6 *B*) but not proliferation (Fig. 6 *A*) at low anti-Vβ6 mAb doses. Thus, the "forbidden" T cells in the neonatal thymus have not yet been functionally inactivated. Since the T cell clones with autoreactive receptors in the periphery of neoTx mice are most likely derived from the neonatal thymus, anergy must have been induced extrathymically.

Table 2. *Vβ Expression in neoTx BALB/c Mice*

	Sham	Day 5	Day 8
Vβ6	11.6 ± 0.2	11.5 ± 2.9	12.0, 10.7
Vβ11	1.6 ± 0.2	7.3 ± 2.5	6.8, 3.2

Vβ expression was determined by immunofluorescent staining with the anti-Vβ mAbs described in Material and Methods. Analysis was done on T cell–enriched LN cells from 2–3 mo-old BALB/c mice thymectomized on day 5 or 8 after birth. Control mice were littermates sham-thymectomized at the same time. Values indicated are percentages of TCR-α/β$^+$ LN cells and are reported as the mean ± SE of three separate experiments (for the sham- and day 5–thymectomized group) or as individual values from two separate experiments (for the day 8–thymectomized group).

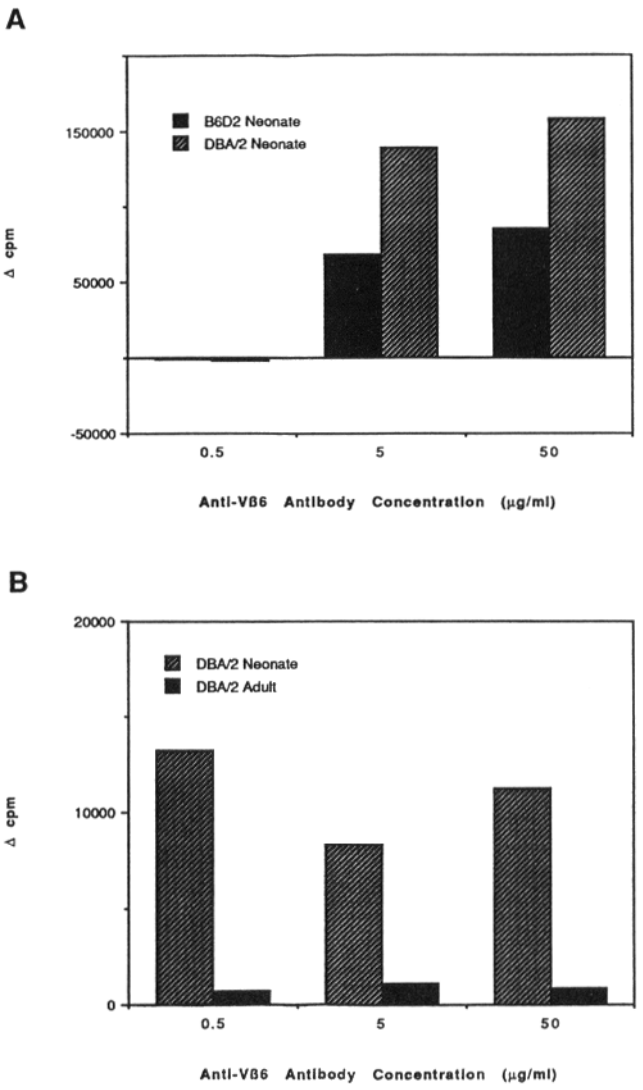

Figure 6. Vβ6$^+$ T cells from neonatal mice are functional. Mature single positive thymocytes from 4–5-d-old hydrocortisone-treated DBA/2 or (C57BL/6 × DBA/2)F1 mice and 6-wk-old adult hydrocortisone-treated DBA/2 mice were stimulated with anti-Vβ6 mAb at the indicated concentrations. Cells were analyzed for their ability to proliferate as measured by [^{3}H]TdR incorporation in the presence of rIL-2 at 10 U/ml (*A*). Culture supernatants were tested at 50, 25, 12.5, and 6.25% for their ability to support proliferation of CTLL cells (*B*). Only results for the highest concentration of supernatant are shown, since further dilution did not result in responsiveness of the adult DBA/2 thymocytes.

Discussion

Clonal deletion originating in the thymus represents a major mechanism for maintaining tolerance for self-antigens. At the same time, it appears that effective alternative modes of tolerance acquisition exist (7–11). The present study defines a system of apparent failed clonal deletion of potentially autoreactive cells: in neoTx mice, T cells with certain self-reactive receptors can be easily visualized; yet, thymectomy does not preclude clonal inactivation of these T cells. Since it is likely that also in normal physiological situations clonal deletion may often

fail, e.g., when the required interactions between developing T cells with self-reactive receptors and hematopoietic elements in the thymus do not occur, it is particularly relevant that nonreactivity to self-antigens is still ensured through induction of clonal nonresponsiveness in T cells with self-reactive receptors.

The present studies also reexamine the stage of development at which clonal deletion of T cells occurs. We observed the presence of T cells with self-reactive Vβ6 receptors in the thymus during the first week after birth, in agreement with earlier reports (34), and also found these cells to be functional by the criteria of receptor-induced proliferation and lymphokine production. Presumably, inefficient intrathymic Mls-1a presentation in the early neonatal thymus is in part responsible for the failed clonal deletion observed during this phase. Their disappearance from the thymus within the first 10 d after birth is consistent with migration (34), and may be additionally affected by clonal deletion at the single positive stage, consistent with recent results in TCR-transgenic mice (35). These findings demonstrate that single positive T cells with mature T cell characteristics, but self-reactive receptors, are generated in the thymus. Their presence in peripheral lymphoid organs of neoTx mice suggests such cells are allowed to migrate, despite their potentially harmful effects (reference 14; and present results). Together, these data expand on the initial clonal deletion studies which appeared to indicate that clonal deletion was completed at the double positive stage of T cell development in the thymus: elimination of T cells with autoreactive receptors was evident among all peripheral T cells and TCR-α/β-expressing single positive thymocytes, but only partially among the DP thymocytes (1–4), implying that deletion occurred before transition to the single positive stage. Further support for this concept has been obtained, including the observations that blocking of CD4 prevents deletion of Vβ17a and Vβ11 in the CD8 subset of thymocytes (36–38), and that in TCR-transgenic mice, CD4$^+$ CD8$^+$ thymocytes with self-reactive receptors are deleted (6, 39). It now appears that tolerance induction in fact occurs over a longer period of time during T cell development; the particular time of occurrence is likely to reflect differences in affinity of the receptor for the particular self-antigens and their site and level of expression, rather than receptor density and developmental stage of the differentiating thymocytes (35). Definition of the complexities of the clonal deletion phenomenon is particularly relevant if the molecular events accompanying this process are to be identified; rather than expecting deletion to be manifested at a discrete stage of T cell development, a much broader phase of development may need to be analyzed.

Clonal anergy was reported to represent an alternative pathway for acquisition of nonreactivity to self-antigens in several other systems (7–11), and shown to occur both inside and outside the thymus. Whether peripheral induction of clonal anergy is thymus dependent was not previously addressed, but the present studies indicate it is not: after thymectomy is performed on day 3 after birth, clonal anergy is nevertheless established in the peripheral T cell repertoire. We cannot rule out that thymic cells crucial for tolerance induction have migrated to the periphery before thymectomy, but would contend that such "escaped" thymic cells apparently occur in sufficient amounts to cause clonal anergy but not clonal deletion. It should be noted, however, that the ability to induce tolerance is not a property confined to thymic elements: allogeneic splenic dendritic cells, when added to fetal thymic organ cultures, can induce specific tolerance in the developing thymocytes (40), although the mechanism in this setting was not defined. Postthymectomy-induced tolerance induction may therefore indeed reflect a thymus-independent phenomenon. At face value, these results are at variance with our earlier report on specific enterotoxin responsiveness of T cells with potentially self-reactive receptors generated in nude mice (13), suggesting that clonal anergy is not generated in this model of failed clonal deletion. However, more recent studies show that also in nude mice, responsiveness of T cells with "forbidden" receptors to cross-linked anti-TCR antibodies cannot be obtained, while enterotoxin responsiveness may sometimes be intact (data not shown).

In the mouse strains tested here, neonatal thymectomy appears to affect T cells with certain self-reactive receptors more than others. This differential impact of thymectomy in different strains may well be related to the extent of deletion accomplished at the time of thymectomy: when intrathymic deletion of T cells expressing a particular Vβ is complete by 3 d after birth, thymectomy would not result in any appearance of "forbidden" clones in the periphery. Our results therefore predict that deletion of Vβ3$^+$ T cells, but not Vβ6$^+$ and Vβ11$^+$ T cells, is accomplished before day 3 of neonatal life. Consistent with this prediction, a recent study (41) reported the complete absence of Vβ3$^+$ T cells in the day 4 neonatal thymus of Mls-1a mice on a BALB background, while Vβ6$^+$ and Vβ11$^+$ T cells persisted in the thymus for 10–14 d after birth (41).

In view of the finding that peripheral T cells with potentially self-reactive receptors in neoTx mice are not functional by the criteria of in vitro activation, the question arises whether such T cells are involved in the pathogenesis of the autoimmune diseases observed in such mice (15, 16). Arguments against this concept can be raised on the basis of the observation that T cells with potentially self-reactive Vβ6 and Vβ11 receptors appear not only in mouse strains susceptible to autoimmune diseases following thymectomy (reference 14; present study), but also, as shown here, in non-autoimmune strains and in mice thymectomized at later times after birth (a condition not generating autoimmune diseases; see 15, 16). We therefore consider it unlikely that the autoimmune defects observed in thymectomized mice are due to direct pathological effects of T cells with Vβ6 and Vβ11 receptors. Several alternative hypotheses can be considered to explain the development of autoimmunity in certain mouse strains after thymectomy. First, a failure in clonal deletion may merely reflect only one of the consequences of neonatal thymectomy, and development of autoimmunity may be unrelated to this event. A second more conservative hypothesis relies on the presumption that failed clonal deletion is a primary event in the generation of autoimmunity: since the present analysis

is limited to those T cells whose receptor specificity can be identified by flow cytometry analysis, it is entirely possible that other self-reactive T cells, not detected with the present technology, also escape clonal deletion but fail to be inactivated for the pertinent antigens in certain mouse strains. The difference in susceptibility to development of autoimmunity in different mouse strains would then be related to the level and time of exposure to those self-antigens for which tolerance is to be acquired. This hypothesis is consistent with earlier observations (42–44) that autoimmunity can be transferred with peripheral T cells from thymectomized mice, and that the efficacy of such transfers is dependent on the strain and age of the recipients. Third, since suppressor cells that can prevent such transfers of autoimmunity have been postulated (42–44), the strain-independent outcome of the consequences of thymectomy may reflect the extent to which such regulatory cells are generated and then operate to prevent the harmful effects of T cells with self-reactive receptors.

Finally, the present study also raises questions about the fate of T cells with self-reactive receptors in normal mice with an intact thymus: if the thymus exports such cells during the first days after birth, why are they generally undetectable in normal mice? In part, visualization of such cells will be harder in normal mice, since postnatal expansion of other T cells is more pronounced in normal mice. However, dilution effects alone cannot be responsible for this phenomenon: although thymectomized mice on average had two- to threefold fewer T cells than normal littermates, the relative increase in T cells with self-reactive receptors was at least as high and often higher. The inevitable conclusion presents itself that thymus-independent mechanisms determine the fate of T cells with self-reactive receptors that were exported from the thymus, and future studies will address the nature of these mechanisms.

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