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

By Lori A. Jones,* L. Thomas Chin,* George R. Merriam,† Lawrence M. Nelson,‡ and Ada M. Kruisbeck*

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 Vβ11+ T cells in BALB/c mice and Vβ5+ and Vβ6+ 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-Vβ 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,
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 neoIx 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-2d strains; Y3P [reference 20] for H-2b 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 (10⁵/100 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 Immunocytometry, 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 × 10⁵ T cell–enriched spleen cells in 200 μl complete DMEM (10% FCS, 5 × 10⁻² 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 [3H]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 × 10⁵ irradiated (2,000 rad) spleen cells in 96-well flat-bottom microtiter plates. After 3 d, cultures were pulsed with 1 μCi of [3H]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 × 10⁵ CTLL-2 cells in flat-bottomed microtiter plates. Cultures were incubated for 36 h and then pulsed with 1 μCi of [3H]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, 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 of 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 Vβ11⁺ cells is significantly increased in thymectomized BALB/c mice as compared to sham-thymectomized littermates. In addition, Vβ6⁺ T cells also escape deletion in neoIx DBA/2 mice but remain unaffected in the neoIx 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 Vβ5⁺ T cells in the neoIx 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 Vβ11⁺ 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 days after birth. Peripheral T cells were analyzed when mice were 2-3 mo old. Percentages for TCR expression are given in Table 1.

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-2a), 90% of female mice develop ophthalmitis, and 30% of BALB/c mice (H-2d) develop gastritis. In sharp contrast, thymectomized DBA/2 mice (also H-2d) 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 deleterional 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 H-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.

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 Vβ11+ T cells in the thymus were tested as well. It can be seen that the Vβ11+ T cells from this non-deleting 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 cell 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 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 ophophritis (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.

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.
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

| Vβ  | 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).

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
We cannot rule out that thymic cells are crucial for tolerance but dressed, but the present studies indicate it is not: after thymic clonal anergy is thymus dependent was not previously and outside the thymus. Whether peripheral 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 nude 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 nude 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 three-fold 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.

We thank Drs. Richard J. Hodes, Louis A. Matis, Dan L. Longo, Juan C. Zúñiga-Pflücker, and Jeffery K. Taubenberger for their critical review of the manuscript and helpful comments, and Fran N. Haussman and David A. Stephany of the National Institute of Allergy and Infectious Diseases Flow Cytometry Laboratory for their expert FCM analysis.

L. Thomas Chin is a Howard Hughes Medical Research/National Institutes of Health Research Scholar.

Address correspondence to Dr. Lori A. Jones, Biological Response Modifiers Program, National Cancer Institute, National Institutes of Health, Building 10, Room 12N226, Bethesda, MD 20892.

Received for publication 30 May 1990 and in revised form 2 August 1990.

References

1. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. Cell. 49:273.
2. Kappler, J.W., U. Stenerz, J. White, and P. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature (Lond.). 332:35.
3. MacDonald, H.R., R. Schneider, R.L. Lees, R.C. Howe, H. Acha-Orbea, H. Festenstien, R.M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor V6 use predicts reactivity and tolerance to Mls-encoded antigens. Nature (Lond.). 332:40.
4. Bill, J., O. Kanagawa, D.L. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of V61-bearing T cells. J. Exp. Med. 169:1405.
5. Woodland, D., M.P. Happ, J. Bill, and E. Palmer. 1990. Requirement for cotolerogenic gene products in the clonal deletion of I-E reactive T cells. Science (Wash. DC). 247:964.
6. Kiestlow, P., H. Blüthmann, U.D. Stenerz, M. Steinmetz, and H. von Bohemer. 1988. Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4+CD8+ thymocytes. Nature (Lond.). 333:742.
7. Qin, S., S. Cobbold, R. Benhamin, and H. Waldmann. 1989. Induction of classical transplantation tolerance in the adult. J. Exp. Med. 169:779.
8. Rammensee, H., R. Kroschewski, and B. Frangoulis. 1989. Clonal anergy induced in mature V66+ T lymphocytes with Mls-1a antigen. Nature (Lond.). 339:541.
9. Ramsdell, F., T. Lantz, and B.J. Fowlkes. 1989. A nondele-
tional mechanism of thymic self tolerance. Science (Wash. DC). 246:1038.
10. Roberts, J.L., S.O. Sharrow, and A. Singer. 1990. Clonal deletion and clonal anergy in the thymus induced by cellu-
lar elements with different radiation sensitivities. J. Exp. Med. 171:935.
11. Lo, D., L.C. Burky, R.A. Flavell, R.D. Paltimer, and R.L. Brinster. 1989. Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells. J. Exp. Med. 170:87.
12. Hodes, R.J., S.O. Sharrow, and A. Solomon. 1989. Failure of T cell receptor V6 negative selection in an athymic environment. Science (Wash. DC). 246:1041.
13. Fry, A.M., L.A. Jones, A.M. Kruisbeek, and L.A. Matis. 1989. Thymic requirement for clonal deletion during T cell development. Science (Wash. DC). 246:1044.
14. Smith, H., M.M. Chen, R. Kubo, and K.S.K. Tung. 1989. Neona-
tal thymectomy results in a repertoire enriched in T cells deleted in adult thymus. Science (Wash. DC). 245:749.
15. Yunis, E.J., R. Hong, M.A. Gerwe, C. Martinez, E. Cornelius, and R.A. Good. 1967. Postthymectomy wasting associated with autoimmune phenomena. Antiglubulin-positive anaemia in A and C57BL/6 Ks mice. J. Exp. Med. 125:947.
16. Kojima, A., and R.T. Prehn. 1981. Genetic susceptibility to post-thymectomy autoimmune disease in mice. Immunogenetics. 14:15.
17. Sjödin, K., A.P. Dalamasso, J.M. Smith, and C. Martinez.
1963. Thymectomy in newborn and adult mice. Transplantation (Baltimore). 1:521.
18. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J. Exp. Med. 153:1198.
19. Ozato, K., N. Mayer, and D.H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. J. Immunol. 124:533.
20. Janeway, C.A., P.J. Conrad, E.A. Lerner, J. Babich, P. Wettstein, and D.B. Murphy. 1984. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cellbound Ia antigens as targets of immunoregulatory T cells. J. Immunol. 132:2496.
21. Kubo, R.T., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine alpha-beta T cell receptors. J. Immunol. 142:2665.
22. Pullen, A.M., P. Marrack, and J.W. Kappler. 1988. The T cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. Nature (Lond.). 335:796.
23. MacDonald, H.R., R.K. Lees, and Y Chvatchko. 1990. Characterization of a mononuclear cell marker which correlates with the use of specific members of the Vβ8 gene segment subfamily. J. Exp. Med. 165:257.
24. Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. Kappler, and P. Marrack. 1984. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. J. Exp. Med. 160:452.
25. Behlke, M.A., T.J. Henkel, S.A. Anderson, N.C. Lan, L. Hood, V.L. Braciale, T.J. Braciale, and D.Y. Loh. 1987. Expression of a murine polyclonal T cell receptor marker correlates with the use of specific members of the Vβ8 gene segment subfamily. J. Exp. Med. 165:257.
26. Staerz, U., H.G. Rammensee, J. Benedetto, and M. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. J. Immunol. 134:3994.
27. Liao, N.S., J. Maltzman, and D.H. Raulet. 1989. Positive selection determines T cell receptor Vβ14 gene usage by CD8+ T cells. J. Exp. Med. 170:135.
28. Dialynas, D.P., Z.S. Quan, K.A. Wall, A. Pierres, J. Quintans, M.R. Loken, M. Pierres, and E.W. Fitch. 1983. Characterization of the murine T cell surface molecule designated L3T4a, identified by the monoclonal antibody GK1.5: similarity of L3T4 to the human Leu/T4 molecule. J. Immunol. 131:2445.
29. Webb, S.R., and J. Sprent. 1990. Response of mature unprimed CD8+ T cells to Mlsα determinants. J. Exp. Med. 171:953.
30. MacDonald, H.R., R.K. Lees, and Y. Chvatchko. 1990. CD8+ T cells respond clonally to Mls1-a-encoded determinants. J. Exp. Med. 71:1381.
31. Gao, E.K., O. Kanagawa, and J. Sprent. 1989. Capacity of unprimed CD4+ and CD8+ T cells expressing Vβ11 receptors to respond to I-E alloantigens in vivo. J. Exp. Med. 170:1947.
32. Crispe, I.N., M.J. Bevan, and U.D. Staerz. 1985. Selective activation of Lyt 2+ precursor T cells by ligation of the antigen receptor. Nature (Lond.). 317:627.
33. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J. Exp. Med. 165:302.
34. Schneider, R., R.K. Lees, T. Pedrazzini, R.M. Zinkernagel, H. Hengartner, and H.R. MacDonald. 1989. Postnatal disappearance of self-reactive (Vβ6+) cells from the thymus of Mlsα mice. Implications for T cell development and autoimmunity. J. Exp. Med. 169:2149.
35. Pircher, H., K. Bürke, R. Lang, H. Hengartner, and R.M. Zinkernagel. 1989. Tolerance induction in double specific T cell receptor transgenic mice varies with antigen. Nature (Lond.). 342:559.
36. Fowlkes, B.J., R.H. Schwartz, and D.M. Pardoll. 1988. Deletion of self-reactive thymocytes occurs at a CD4+CD8+ precursor stage. Nature (Lond.). 334:620.
37. MacDonald, H.R., H. Hengartner, and T. Pedrazzini. 1988. Intrathymic deletion of self-reactive T cells prevented by anti-CD4 antibody treatment. Nature (Lond.). 335:174.
38. Zöllig-Pilücker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1989. Both TCR-MHC and accessory molecule-MHC interactions are required for positive and negative selection of mature T cell in the thymus. Cold Spring Harbor Symp. Quant. Biol. LIV:153.
39. Berg, L.J., B. Fazekas de St. Groth, A.M. Pullen, and M.M. Davis. 1989. Phenotypic differences between αβ versus β T cell receptor transgenic mice undergoing negative selection. Nature (Lond.). 340:559.
40. Matzinger, P., and S. Guerder. 1989. Does T cell tolerance require a dedicated antigen-presenting cell? Nature (Lond.). 338:74.
41. MacDonald, H.R., R.K. Lees, and J.A. Louis. 1989. Clonal deletion of autoreactive T lymphocytes: insights from neonatal ontogeny. Progress in Immunology. VII:561.
42. Taguchi, O., and Y. Nishizuka. 1987. Self tolerance and localized autoimmunity. J. Exp. Med. 165:146.
43. Sakaguchi, S., T. Takahashi, and Y. Nishizuka. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. J. Exp. Med. 156:1565.
44. Sakaguchi, S., T. Takahashi, and Y. Nishizuka. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. J. Exp. Med. 156:1577.