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Thymic Requirement for Clonal Deletion During T Cell Development

ALICIA M. FRY,* LORI A. JONES,* ADA M. KRUISBEEK, LOUIS A. MATIS†

During T cell differentiation, self tolerance is established in part by the deletion of self-reactive T cells within the thymus (negative selection). The presence of T cell receptor (TCR)–αβ$^+$ T cells in older athymic (nu/nu) mice indicates that some T cells can also mature without thymic influence. Therefore, to determine whether the thymus is required for negative selection, TCR V$_\beta$ expression was compared in athymic nu/nu mice and their congenic normal littermates. T cells expressing V$_\beta$3 proteins are specific for minor lymphocyte stimulatory (Mls^c) determinants and are deleted intrathymically due to self tolerance in Mls^{c+} mouse strains. Here it is shown that V$_\beta$3$^+$ T cells are deleted in Mls^{c+} BALB/c nu/+ mice, but not in their BALB/c nu/nu littermates. Thus, the thymus is required for clonal deletion during T cell development.

THE TCR REPERTOIRE IS DETERMINED through positive and negative selection by self antigens in association with major histocompatibility complex (MHC) molecules (*1*). Studies with TCR-αβ transgenic mice (*2*) indicate that both processes occur within the thymus. Similar evidence has emerged from experiments with monoclonal antibodies (MAb) specific for individual murine TCR V$_\beta$ proteins (*3–6*). For example, certain TCR V$_\beta$ proteins, independent of the other components of the receptor, are specific for self antigens (for example, Mls) in association with particular class II MHC molecules (*3, 4, 7*). It has been demonstrated that T cells expressing these V$_\beta$ proteins are deleted within the thymus in mouse strains that express the self antigens.

Whereas most T cell development takes place intrathymically, some T cell maturation can also occur extrathymically (*8–10*). Older athymic nu/nu mice develop TCR-αβ–bearing T cells that are capable of both MHC-specific cytotoxic function and lymphokine production (*10*). To compare the TCR repertoire of inbred athymic (nu/nu) mice with that of euthymic (nu/+) control mice, we examined lectin-stimulated T cells from aged nu/nu BALB/c and B10 mice, as well as normal BALB/c and B10 mice, for the surface expression of various TCR V$_\beta$ proteins (Fig. 1).

BALB/c mice express Mls^c determinants associated with V$_\beta$3 deletion (*4, 7*), and E$_\alpha^d$E$_\beta^d$ Ia molecules. V$_\beta$11$^+$ T cells are deleted in mouse strains expressing E$_\alpha$E$_\beta$ gene products in association with currently undefined non-MHC self antigens (*11*). BALB/c mice express no self antigens associated with V$_\beta$8 deletion. Accordingly, the lectin-stimulated BALB/c T cell populations contained numerous V$_\beta$8$^+$ T cells, but almost no V$_\beta$3$^+$ or V$_\beta$11$^+$ cells (Fig. 1). In contrast, V$_\beta$3$^+$ and V$_\beta$11$^+$ as well as V$_\beta$8$^+$ T cells were found in the concanavalin A (Con A)–stimulated population (Fig. 1, A and B) from BALB/c nu/nu mice. No V$_\beta$ deletions have been found in B10 mice, and as shown in Fig. 1, V$_\beta$3$^+$, V$_\beta$8$^+$, and V$_\beta$11$^+$ T cells were observed in both the B10 and B10 nu/nu lectin-activated T cells.

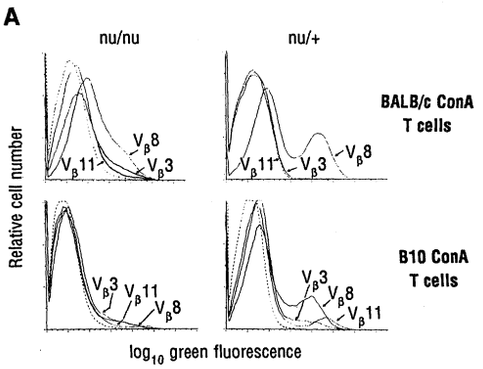
Thus, BALB/c nu/nu mice, unlike their thymus-bearing littermates, fail to delete V$_\beta$3$^+$ and V$_\beta$11$^+$ T cells (*12*). However, the implications of this result for autoimmunity rest largely on demonstrating whether these T cells can respond to antigen stimulation. To address this important question, we examined the ability of nu/nu-derived T cells to respond specifically to stimulation with superantigenic staphylococcal enterotoxins (SE). SE stimulation appears to be mediated through the TCR because SE preferentially activate T cells expressing particular V$_\beta$ proteins (*6, 13, 14*). T cells from normal and athymic mice were activated with staphylococcal enterotoxin B (SEB), A (SEA), or E (SEE). SEB selectively stimulates V$_\beta$3$^+$ and V$_\beta$8$^+$ T cells (*6*), SEA stimulates V$_\beta$3$^+$ and V$_\beta$11$^+$ T cells (*6*), and SEE stimulates V$_\beta$11$^+$ T cells. T cells from nu/nu mice mounted a V$_\beta$-specific proliferative response when stimulated with SE. SEB-stimulated B10 and B10 nu/nu, as well as BALB/c nu/nu T cell populations were enriched for V$_\beta$3$^+$ and V$_\beta$8$^+$ cells (Fig. 2). However, even after SEB activation, the normal BALB/c population, which was highly enriched for V$_\beta$8$^+$ T cells, failed to express V$_\beta$3.

Analogous results were obtained on activation with SEA (Fig. 3A). In the experiment shown, V$_\beta$3$^+$ T cells constituted 36% of the SEA-stimulated BALB/c nu/nu TCR-αβ$^+$ T cells (*15*). SEA-activated normal BALB/c T cells were >98% TCR-αβ$^+$ (Fig. 3A), but very few were V$_\beta$3$^+$ (<2%). The distinction in V$_\beta$11 expression between BALB/c nu/nu and nu/+ T cells seen after lectin stimulation (Fig. 1) was no longer apparent after SE activation, such that many SEE-responsive normal V$_\beta$11$^+$ BALB/c T cells were found (Fig. 3B). However, this result is consistent with the fact that V$_\beta$11 deletion is often incomplete in E$_\alpha$E$_b$ mouse strains (*11*), and SEE activation presumably produced a dramatic expansion of the residual V$_\beta$11$^+$ BALB/c T cells. Nonetheless, V$_\beta$11$^+$ T cells from BALB/c nu/nu mice, like V$_\beta$3$^+$ cells, also respond specifically to SE.

In normal mice, clonal deletion appears to occur among immature CD4$^+$CD8$^+$ (double positive) cells (*5, 6*). TCR-αβ$^+$ CD4$^+$ or CD8$^+$ nu/nu T cells expressing V$_\beta$ that are deleted in their nu/+ counterparts might therefore develop directly from CD4$^-$CD8$^-$ precursors. Alternatively, they could arise from CD4$^+$CD8$^+$ cells, but the

A. M. Fry and L. A. Matis, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.
L. A. Jones and A. M. Kruisbeek, Biological Response Modifiers Program, National Cancer Institute, NIH, Bethesda, MD 20892.

*Contributed equally to this work.
†To whom correspondence should be addressed.



B

Strain	V_β	Percentage of T cells nu/nu	nu/+
BALB/c	3	9.2	0.5
	8	19.1	32.2
	11	3.0	0.8
B10	3	4.3	6.7
	8	4.1	23.0
	11	1.7	8.1

Fig. 1. (**A**) Flow cytometry analysis of TCR V_β expression in lectin-stimulated peripheral T cells from BALB/c nu/nu, BALB/c, B10 nu/nu, and B10 mice. Inbred nu/nu mice greater than 6 months of age were obtained from the National Institutes of Health; control mice were purchased from the Jackson Laboratory. (Top) BALB/c nu/nu and normal T cells; (bottom) B10 nu/nu and normal T cells. T cells were purified from the lymph nodes and spleen of six mice in each group by passage over nylon wool columns and then cultured in complete medium for 4 days with Con A (2.5 μg/ml) and recombinant human interleukin-2 (50 U/ml; Cetus). Viable T cell blasts were isolated after centrifugation over Lympholyte-M (Cedarlane Labs.), stained with either fluorescein isothiocyanate (FITC)–labeled KJ16-133 [anti-V_β8.1 + V_β8.2 (*24*)], RR3-15 [anti-V_β11 (*11*)], or supernatant from the KJ25 hybridoma [anti-V_β3 (*4*)], and then with FITC-labeled goat antibodies to hamster immunoglobulin (Cappel). Flow cytometry analysis was performed on a B-D Dual Laser 440 interfaced to a PDP 11/24 computer. Data were collected on 50,000 viable cells (*25*). Dashed lines represent staining with irrelevant control MAb. (**B**) Percentage of T cells expressing individual TCR V_β proteins relative to the total number of $CD3^+$ cells examined. The percentage of V_β^+ cells in relation to total TCR-$\alpha\beta^+$ cells is somewhat underpresented for the nu/nu relative to the nu/+ cell populations because a higher percentage of $CD3^+$ nu/nu T cells are TCR-$\alpha\beta$ negative.

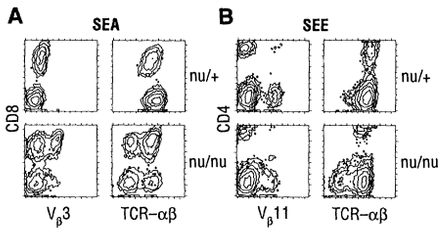


Fig. 3. Two-color flow cytometry analysis of SE-stimulated T cells from BALB/c nu/nu and nu/+ mice. (**A**) T cells from eight mice were prepared as in Fig. 2, except stimulation was performed with SEA (1 μg/ml) (Toxin Technologies). The cells were stained first with FITC-conjugated MAb to TCR-αβ, or MAb to V_β3 followed by FITC-conjugated goat antibodies to hamster immunoglobulin. After washing, cells were stained with biotinylated MAb to murine CD8 (Ab 2.43; ATCC), and then allophycocyanin-labeled avidin (Caltag). (**B**) Analysis of cells stimulated with SEE (1 μg/ml); (Toxin Technologies). The cells were stained with FITC-conjugated MAbs to TCR-αβ or V_β11, washed, and then stained with biotinylated MAb to murine CD4 (GK1.5, 27) and allophycocyanin-labeled avidin.

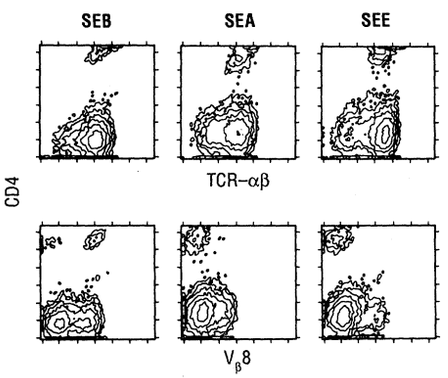


Fig. 4. TCR-αβ expression in $CD4^+$ BALB/c nu/nu T cells. Cells from seven mice were stimulated with either SEB, SEA, or SEE and then examined for TCR-αβ, V_β8, and CD4 expression. Cells were stained with FITC-labeled MAb to TCR-αβ or V_β8, washed, and then stained with biotinylated MAb to CD4 and allophycocyanin-labeled avidin.

negative selection of double positive T cells might require an appropriate thymic environment.

The maturation of self-reactive V_β-expressing T cells has also been observed after the administration of cyclosporine A (CSA) to developing T cells in vivo (*16*), or after neonatal thymectomy (NTX) (*17*). Thus, by several independent approaches it appears that the disruption of normal thymic function interferes with the induction of self tolerance by clonal deletion. Because both CSA-treated and NTX mice frequently develop autoimmune disease (*16, 17*), it has been inferred that the nondeleted V_β-expressing T cells have a causal role. However, the functional reactivity of the potentially autoreactive V_β^+ T cells has not yet been assessed directly (*16, 17*).

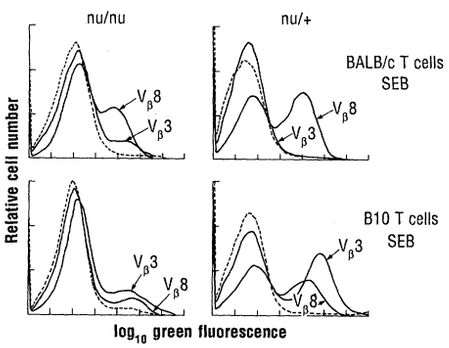


Fig. 2. Expression of V_β3 and V_β8 by SEB-stimulated T cells from nu/nu and nu/+ mice. In this and subsequent experiments, the nu/+ littermates of the nu/nu mice were used as the control euthymic animals. (**Top**) BALB/c nu/nu and nu/+ T cells; (**bottom**) B10 nu/nu and nu/+ T cells. T cells were stained with MAbs to V_β3 or V_β8. Dashed lines indicate staining with irrelevant MAb. Approximately 2×10^7 nylon wool purified splenic and lymph node T cells from six mice in each group were cultured for 4 days with SEB (10 μg/ml; Sigma) plus 3×10^7 irradiated (2000 rad) T cell–depleted syngeneic spleen cells in a total volume of 50 ml. Cells were then further expanded in fresh medium with interleukin-2 (100 U/ml) for an additional 3 days. Viable T cell blasts were isolated and stained as in Fig. 1.

Hodes and colleagues (*18*) similarly report that T cells from nu/nu mice express potentially self-reactive TCR V_β. However, in contrast to our observation that a preponderance of these cells are $CD4^-CD8^+$ (for example, >80% of the $V_\beta3^+$ SEA-stimulated BALB/c nu/nu T cells are $CD4^-CD8^+$ (Fig. 3A) (*19*), they find an equal distribution of the undeleted $V_\beta3^+$ and $V_\beta11^+$ T cells among the $CD4^+CD8^-$ and $CD4^-CD8^+$ cell subsets. It may be relevant that Hodes *et al.* examined freshly harvested lymphocytes, whereas our analyses were performed on lectin or SE-stimulated T cell populations. Therefore, the differences in the data could reflect the preferential activation of $CD4^-CD8^+$ T cells by lectin and SE, or alternatively the fact that $CD4^+CD8^-$ nu/nu T cells are relatively insensitive to mitogenic signaling (*20*). To address this latter question, we examined more closely the response of $CD4^+CD8^-$ nu/nu T cells to SE (Fig. 4). After SE stimulation of nu/nu T cell populations, we consistently find that 5 to 20% of the responding cells that stain brightly for TCR-αβ are $CD4^+CD8^-$ (Figs. 3 and 4) (*21*). Moreover, although roughly equal numbers of $CD4^+$ TCR-$\alpha\beta^+$ T cells were generated after activation of BALB/c nu/nu cells with SEB, SEA, or SEE, only the SEB-activated $CD4^+$ cells were enriched for V_β8 (Fig. 4). We have also found that SEA-activated $CD4^+$ B10 nu/nu T cells are enriched for V_β11 (*21*). Thus, at least relative to $CD4^-CD8^+$ nu/nu T cells, $CD4^+CD8^-$ nu/nu T cells can respond specifically to SE and appear to be functional.

Another possibility is that because $CD4^+$ $V_\beta3^+$ cells would be predominantly responsible for Mls^c-specific self-reactivity in BALB/c nu/nu mice (*22*), and these mice display no obvious signs of autoreactivity, it is possible that the $CD4^+$ $V_\beta3^+$ cells have been selectively inactivated. Functional inac-

tivation of T cells without clonal deletion has been demonstrated recently as a mechanism for establishing peripheral T cell tolerance in vivo (*23*). Thus, during T cell development, cells with high-affinity self-reactive TCR that escape clonal deletion in the thymus might nevertheless be tolerized peripherally by the induction of clonal energy. Experiments with V_β-specific activating antibodies would directly address this possibility.

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DNA Topoisomerase I–Targeted Chemotherapy of Human Colon Cancer in Xenografts

BEPPINO C. GIOVANELLA, JOHN S. STEHLIN, MONROE E. WALL, MANSUKH C. WANI, ALLAN W. NICHOLAS, LEROY F. LIU, ROBERT SILBER, MILAN POTMESIL*

Drug development is needed to improve chemotherapy of patients with locally advanced or metastatic colon carcinoma, who otherwise have an unfavorable prognosis. DNA topoisomerase I, a nuclear enzyme important for solving topological problems arising during DNA replication and for other cellular functions, has been identified as a principal target of a plant alkaloid 20(*S*)-camptothecin. Significantly increased concentrations of this enzyme, compared to that in normal colonic mucosa, were found in advanced stages of human colon adenocarcinoma and in xenografts of colon cancer carried by immunodeficient mice. Several synthetic analogs of camptothecin, selected by tests with the purified enzyme and tissue-culture screens, were evaluated in the xenograft model. Unlike other anticancer drugs tested, 20(*RS*)-9-amino-camptothecin (9-AC) induced disease-free remissions. The overall drug toxicity was low and allowed for repeated courses of treatment.

THE ANTITUMOR ACTIVITY OF 20(*S*)-camptothecin, a plant alkaloid isolated from *Camptotheca acuminata* (*1*), was studied in the early 1970s (*2*). Its water-soluble sodium salt, substantially less effective than the lactone form (*3*), was briefly tested in phase I clinical trials. Leukopenia was the dose-limiting toxic effect, and hemorrhagic cystitis was the most prominent nonhematological complication. Since the purpose of phase I trials is to establish drug toxicity, therapeutic responses were evaluated only in some patients. Partial remissions were noted in patients with advanced gastrointestinal cancer, which had been refractory to other treatments. Further development of camptothecins was hampered by the unavailability of compounds with better antitumor efficacy and by the lack of understanding of its mechanism of action. The recent demonstration that DNA topoisomerase I is the main, if not exclusive, target of camptothecin (*4*) has revived interest in research on camptothecin analogs as anticancer drugs. Human topoisomerase I, a monomeric protein of 100 kD (*5*), acts by relaxing supercoiled DNA. Its activity is likely to be important for semiconservative replication of double-helical DNA and for other DNA functions such as transcription, recombination, and chromosomal decondensation (*6*). The 20(*S*)-camptothecin interferes with the DNA breakage-reunion reaction catalyzed by topoisomerases I, by trapping a key covalent enzyme-DNA intermediate termed the "cleavable complex" (*4, 7*). Topoisomerase I levels are lower in normal cells than in cells of chronic lymphocytic leukemia as well as in several types of lymphoma (*8*).

Human colon cancer was selected as a model for solid tumors of epithelial origin, because colonic cancer is a major problem in clinical oncology. One of 25 Americans will develop this disease during their lifetime (*9*).

B. C. Giovanella and J. S. Stehlin, The Stehlin Foundation for Cancer Research, St. Joseph Hospital, Houston, TX 77002.
M. E. Wall, M. C. Wani, A. W. Nicholas, Research Triangle Institute, Research Triangle Park, NC 27709.
L. F. Liu, Johns Hopkins University School of Medicine, Department of Biological Chemistry, Baltimore, MD 21206.
R. Silber, and M. Potmesil, New York University School of Medicine, Departments of Medicine and Radiology, and Kaplan Cancer Center, New York, NY 10016.

*To whom correspondence should be addressed.