Self-tolerance to transgenic γδ T cells by intrathymic inactivation

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DURING their intrathymic differentiation, T lymphocytes expressing αβ T-cell receptors (TCR) are negatively and positively selected. This selection contributes to the establishment of self-tolerance and ensures that mature CD4+ and CD8+ cell populations are restricted by the self major histocompatibility complex. Little is known, however, about γδ T-cell development. To investigate whether selection operates in the establishment of the γδ T-cell class, we have generated transgenic mice using γ and δ-transgenes encoding a TCR that is specific for a product of a gene in the Tλ-region of the Tλ haplotype. Similar numbers of thymocytes expressing the transgenic TCR were generated in mice of Tλb and Tλa haplotypes. But γδ thymocytes from Tλb and Tλa transgenic mice differed in cell size, TCR density and in their capacity to respond to Tλb stimulator cells or interleukin-2 (IL-2). In contrast to γδ T cells from Tλa transgenic mice, γδ T cells from Tλb transgenic mice did not produce IL-2 and did not proliferate in response to Tλb stimulator cells, but they did proliferate in the presence of exogenous IL-2. These results indicate that functional inactivation of self-antigen-specific T cells could contribute to the establishment of self-tolerance to thymic determinants.

Several γδ TCR-positive T hybridomas were obtained by fusing CD4-CD8- thymocytes from adult C57BL/6 (H-2b, Tλb) mice with the thymoma BW5147 (ref. 9). Using a hybridoma growth inhibition assay, we identified one hybridoma (KN6) that was specific for syngeneic cells. It recognized a product of the Tλ haplotype, but not a product of the Tλa haplotype, that was expressed not only on peritoneal and spleen cells, but also in the thymus. We were therefore interested in whether the KN6 hybridoma originated from an immature γδ T cell destined to be eliminated in the thymus, but was rescued by the fusion. To investigate this possibility, we generated transgenic mice by introducing into the germ line the rearranged Vγ1Jγ1 and Vδ1Jδ1 chain genes encoding the KN6 TCRγδ. We backcrossed CBA/J × C57BL (Tλa) transgenic mice several times to C57BL/6 (Tλb) or BALB/c (Tλb) mice, and analysed homozygous Tλb and Tλa transgenic mice, as well as heterozygous Tλb/d transgenic mice. We studied γδ TCR expression by immunofluorescence using monoclonal antibodies that react

**FIG. 1.** Transgene-encoded γδ TCR are expressed on the surface of γδ T cells from KN6 transgenic mice of Tλb and Tλa haplotypes. αγ Thymus and spleen cell suspensions were stained with fluorescein isothiocyanate (FITC)-conjugated 3A10 monoclonal antibody (anti-pan γδ) and biotinylated 8D6 monoclonal antibody (anti-Vγ4Vδ3-encoded TCRγδ), followed by streptavidin coupled to phycoerythrin. The dot-plot histograms shown (3A10, horizontal axis; 8D6, vertical axis) were obtained after flow cytometric analysis of thymocytes and splenocytes from KN6 transgenic mice of Tλb (TGb/d) and Tλa (TGd/d) haplotypes and from nontransgenic litterate is (LM) of Tλa haplotype mice. Fluorescence intensity is expressed in log₂ units. The percentage of cells in right quadrants are indicated. bγ, TCR density and size of γδ T cells from KN6 transgenic mice. Thymus and spleen cell suspensions were stained with 3A10 and 8D6 monoclonal antibodies according to a. The forward scatter (FSC) and the 8D6 fluorescence histograms (Tλb/g transgenic, dotted line; Tλa/g transgenic, solid line) were obtained from gated γδ T cells. c. Total number of KN6 γδ TCR-positive cells in thymus and spleen from Tλb, Tλb/d and Tλb/d KN6 transgenic mice. Single cell suspensions of thymic and splenic cells from KN6 transgenic mice (4–10 week-old) were stained with biotinylated monoclonal antibody 5C10 (KN6 clonotypic) followed by streptavidin coupled to phycoerythrin, and the number of KN6 γδ TCR-positive cells was calculated from the percentage of 5C10 cells and the total cell number. The obtained mean values (horizontal bars) and s.d.s. were the following (in 10⁶ cells): thymus (Tλb), 6.0 ± 5.1; Tλb/d, 5.9 ± 2.4; Tλa/d, 7.5 ± 4.4; spleen (Tλb), 2.2 ± 1.9; Tλb/d, 1.9 ± 1.0; Tλa/d, 22.4 ± 9.4. MHC03S. KN6 γδ transgenic mice were generated after injection into fertilized eggs of purified Vγ1Jγ1 and Vδ1Jδ1 cosmid DNA from the KN6 hybridoma as previously described. Flow cytometric analysis was performed on a FACScan (Becton Dickinson) using Consort C30 and LYSYS programs. The percentages of 8D6- and 5C10- cells were virtually identical (data not shown).
with all γδ TCR (3A10)13, with most or all Vγ4Vδ5-encoded TCR (8D6)13, or with the K6N γδ TCR only (5C10)13. Virtually all thymic and splenic γδ T cells from TLδ/δ or TLδ/δ transgenic mice expressed the transgene-encoded TCR (Fig. 1a), due to blockade of endogenous γ- and δ-gene rearrangements10-12. Anti-CD4 and anti-CD8 monoclonal antibodies did not stain any K6N TCR-positive cells (data not shown). The total number of peripheral and thymic αβ T cells varied in different litters, and will not be further considered here. By contrast, the numbers of K6N TCR-positive thymocytes was rather constant and not significantly different between transgenic mice of the TLδ/δ and TLδ/δ haplotypes (Fig. 1c). But the numbers of splenic K6N TCR-positive cells was on average 10 times lower in TLδ/δ or TLδ/δ transgenic mice than in TLδ/δ transgenic mice. Also γδ T cells in TLδ/δ or TLδ/δ transgenic mice were larger than those in TLδ/δ transgenic mice, and expressed surface Kn6 TCR at a lower density. This difference was more pronounced in thymocytes than in spleen cells (Fig. 1b).

To assess functional properties of γδ T cells in the various TCR transgenic mice, we purified these cells from thymus and spleen by treatment with anti-CD4 and anti-CD8 monoclonal antibodies and complement. The resulting cell population (<1% αβ T cells, >90% γδ T cells) was cultured either in medium alone or in medium with TLδ stimulator cells or IL-2, or both. No spontaneous proliferation was observed in medium alone (Table 1 and Fig. 2a). IL-2 receptor α-chain expression (Fig. 3) and proliferation (Fig. 2) were induced in thymocytes from TLδ/δ or TLδ/δ transgenic mice by IL-2 but not by TLδ stimulator cells. TLδ stimulator cells also failed to induce IL-2 or IL-4 production, or both, by TLδ/δ thymocytes (Table 1). Opposite results were obtained with thymocytes from TLδ/δ transgenic mice: IL-2 or IL-4 production, or both, proliferation and IL-2

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<th>Table 1: Functional properties of γδ T cells from TLδ/δ KN6 transgenic mice</th>
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<td><strong>Transgenic responder cells</strong></td>
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When exposed to specific antigen-bearing cells, γδ T cells from TLδ/δ KN6 transgenic mice are unable to proliferate or secrete lymphokines, CD4⁺CD8⁻ thymocytes and splenocytes from TLδ/δ and TLδ/δ KN6 transgenic mice were cultured with or without irradiated peritoneal cells of C57BL/6J origin (TLδ). Proliferative activity of responder cells, assessed by 3H-labelled thymidine uptake, was assayed at day 2 after the initiation of the culture. Culture supernatants were collected at day 1 and tested for their ability to trigger proliferation of IL-2/4-dependent HT-2 cells. The experiment was performed three times, obtaining similar results. A representative result from one of the three experiments is shown. All measurements were made in triplicate and the data are expressed as mean ± s.d. Spleen and thymus cell suspensions from 6-week-old TLδ/δ and TLδ/δ KN6 transgenic mice were incubated for 1 h at 37°C with anti-CD4 (RIL2T4.2) and anti-CD8 (3.155.D14) monoclonal antibodies and complement, and live cells were recovered by centrifugation over Ficoll-hypaque. Double-negative responder cells (5 x 10³) were cultured in 96-well plates in 200 μl complete medium culture (RPMI 1640, 10% FCS, 2 mM L-glutamine) with or without 2.5 x 10⁴ irradiated (1,500 rad) peritoneal cells from C57BL/6J mice. Culture supernatant (100 μl) was collected at day 1. At day 2, cultures were pulsed for 6 h with [3H-methyl] thymidine (1 μCi per well), collected and counted in a scintillation counter. To determine IL-2/4 production, dilutions of day-1 culture supernatants were added to culture of IL-2/4-dependent HT-2 cells. [3H-methyl] thymidine incorporation by these cells was determined on day 2. The results shown are the mean ± s.d. of triplicates that were obtained with 1/4 final dilutions.

FIG. 2. Addition of recombinant IL-2 (rIL-2) partly restores the proliferation of γδ T cells from TLδ/δ KN6 transgenic mice to TLδ stimulator cells. CD4⁺CD8⁻ thymocytes (TLδ/δ transgenic, ●; TLδ/δ transgenic, ▲) were cultured without (a, c) or with (b, d) irradiated TLδ peritoneal cells (Ag), in the absence (a and b) or the presence (c and d) of rIL-2. Their proliferative activity, assessed by [3H-labelled thymidine] production, was assayed at day 2, 3 and 5 after the initiation of the culture. METHODS. CD4⁺CD8⁺ double-negative responder cells and irradiated TLδ peritoneal cells were prepared and cultured as described in Table 1. IL-2 was added to a final concentration of 100 U/ml. All cultures were pulsed with 1 μCi of [3H-methyl] thymidine 6 h before collection.
the thymus of TL<sup>b</sup> mice. But it is clear that most of these cells are not eliminated even though they interact with products of the TL<sup>b</sup> haplotype in the thymus. As a result of this interaction, KN6 TCR-positive cells increase in size, down-modulate their TCR and probably express IL-2-receptor β-chains, which would confer on them the ability to respond to IL-2 alone<sup>14</sup>. This effect of the TL<sup>b</sup> product does not reflect an essential positive selection step, because KN6 TCR-positive cells do survive in, and are exported from, the thymus of TL<sup>ε</sup> transgenic mice. Whereas KN6 TCR-positive cells from TL<sup>ε</sup> thymi respond to TL<sup>b</sup> stimulator cells with IL-2 production and proliferation, KN6 TCR-positive cells from TL<sup>ε</sup> thymi lose the capacity to produce IL-2 and, as a result, respond to TL<sup>b</sup> stimulator cells only in the presence of exogenous IL-2. Therefore the response of KN6 TCR-positive cells from TL<sup>b</sup> mice to TL<sup>b</sup> stimulator cells is dependent on helper cells that supply IL-2. The lack of such helper cells in TL<sup>ε</sup> mice could explain the lack of destructive autoimmune responses in TL<sup>b</sup> transgenic mice and the lower number of transgenic TCR-positive cells in the periphery of TL<sup>b</sup> mice versus TL<sup>ε</sup> mice. The unresponsive state of the TG-positive cells that is generated in the thymus of the transgenic mice used in the study described here is reminiscent of the state of clonal energy that can be induced in peripheral αβ T cells<sup>15–18</sup>, as well as in the thymocytes of P→F<sub>1</sub> chimerae<sup>19</sup>.

Our study shows that certain T cells may become dependent on helper cells if they encounter self-antigen in the thymus as well as in the periphery. Like self antigen-specific B cells, self antigen-specific T cells are not necessarily harmful to the host. In fact they could have beneficial functions provided that helper cells are absent, which would facilitate their proliferation and differentiation to potentially harmful effector cells in response to host components.

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A second B cell-specific enhancer 3′ of the immunoglobulin heavy-chain locus

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The expression of immunoglobulin heavy-chain (IgH) genes is generally thought to be regulated by the combination of the VH promoter with the enhancer element which is located in the J<sub>H</sub>–CH<sub>1</sub> intron<sup>1–4</sup>. This is probably an oversimplification: there are cell lines that transcribe IgH genes despite the deletion of the intron-enhancers<sup>5–8</sup>. These findings could imply that other enhancer element(s) exist in the IgH locus<sup>9,10</sup>. Here we show that a strong B-cell-specific enhancer is indeed located at the 3′-end of the rat IgH locus, 25 kilobases downstream of Ca. This enhancer should be retained downstream of all rearranged IgH genes, regardless of the VH or CH segment used. Taken together with analogous findings for the mouse κ locus<sup>9</sup>, the results prompt a re-evaluation.

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