DELETION OF AUTOSPECIFIC T CELLS IN T CELL RECEPTOR (TCR) TRANSGENIC MICE SPARES CELLS WITH NORMAL TCR LEVELS AND LOW LEVELS OF CD8 MOLECULES

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The vast majority of mature T cells are either of the CD4⁺8⁻ or the CD4⁻8⁺ phenotype. The CD4 cell surface glycoprotein is expressed primarily on the surface of helper T cells, whose TCR recognize antigen on APCs in the context of class II MHC molecules. The CD8 molecule is primarily expressed on T cells, in particular CTL, whose TCR recognize antigen in the context of class I MHC molecules. The TCR on helper T cells and CTL has been identified as a disulfide-linked α/β heterodimer with a molecular weight of 80,000–90,000 (reviewed in reference 1). The binding of CD4 and CD8 molecules to nonpolymorphic regions of class II and class I MHC molecules, respectively, enhances the binding of the TCR to its ligand and may also contribute to signals leading to T cell activation (2, 3).

We have constructed TCR transgenic mice with the aim to study the selection of the T cell repertoire. To this end, α and β transgenes obtained from an HY-specific, H-2Kd-restricted cytolytic CD4⁺8⁺ T cell clone were injected into fertilized eggs. We have reported that in female α/β transgenic mice a large proportion of T cells is male specific and expresses both α and β transgenes. In male mice we found that cells with an abnormal CD4/CD8 phenotype accumulated in the periphery, with the vast majority of α/β T cells being CD4⁺8⁻ and a minority being CD4⁻8⁺. The latter expressed, however, low levels of CD8 accessory molecules. Due to the lack of an appropriate antibody we could not directly address the question as to whether or not these cells expressed the transgenic receptor. Here we describe the use of mAbs that detect cells expressing both α and β transgenes. Using these antibodies we find that in male H-2Db mice the vast majority of peripheral T cells express both α and β transgenes. Our studies indicate that despite the fact that precursor T cells, which express a low density of the transgenic TCR, are largely deleted in the thymus of male transgenic mice (4), the majority of T cells spared by the deletion express a high density of the transgenic TCR but a low density of CD8 molecules.
Materials and Methods

**Transgenic Mice.** H-2<sup>b</sup> mice expressing α and β TCR transgenes derived from the male-specific B6.2.16 clone were constructed as previously described (5).

**Normal Mice.** C57BL/6, CBA/J, and DBA/2 mice were purchased from IFFACREDO, St. Germain-sur-l'Arbresle, France. AKR/J and BALB.B mice were obtained from the animal colony at the Basel Institute for Immunology.

**T Cell Clones.** The transgenic α/β-expressing clone B6.2.16 and the transgenic β-expressing clone 93.2.20 were established as previously described (6). The α-expressing clone 264.2.5.3 was established by stimulating lymph node cells from an α-transgenic mouse with X-irradiated DBA/2 spleen cells in IL-2-containing medium. After 14 d the cells were washed and restimulated (10<sup>6</sup> responder cells and 10<sup>7</sup> X-irradiated stimulator cells) in IL-2-containing medium. Cloning was carried out by limiting dilution in 96-well microtiter plates containing 10<sup>6</sup> X-irradiated DBA/2 spleen cells per well and optimal concentrations of IL-2. After 1–2 wk, growing colonies were transferred together with 10<sup>7</sup> X-irradiated DBA/2 spleen cells per 1 ml of IL-2-containing medium.

**Surface Staining of Lymphocytes.** Single cell suspensions were prepared from thymus, lymph nodes (mesenteric, axillary, and inguinal), or spleen. The cells were washed once in RPMI 1640 and once in PBS with 5% FCS before staining. For staining the following mAbs were used: FITC-labeled and biotinylated CD8 antibodies (anti-mouse Lyt-2, Becton Dickinson & Co., Mountain View, CA), phycoerythrin (PE)-labeled CD4 antibody (anti-mouse L3T4, Becton Dickinson & Co.), unconjugated F23.1 (7), T3.70 (see below), and CD3 (clone 145.2, reference 8) mAbs. Optimal concentrations of staining reagents were predetermined and used in the experiments described in this report. For single staining of cells, the cells were incubated first with the unconjugated mAb for 20 min on ice, washed twice with PBS + 5% FCS, and incubated for a further 20 min on ice with FITC-conjugated sheep (Fab')<sub>2</sub> fragment anti-mouse Ig (Silenus Laboratories, Melbourne, Australia). The cells were washed three more times before being analyzed on the FACScan flow cytometer (Becton Dickinson & Co.).

**Dead cell exclusion.** The results are presented as density plots. Where applicable, percentages were calculated using FACScan research software programs. Markers were set against negative controls which involved cells that were incubated with PBS + 5% FCS and second stage-labeled reagents, i.e., FITC-labeled anti-mouse Ig or streptavidin PE, and analyzed in the same manner as the double stained cells.

**Production of an mAb, T3.70 that Detects the Transgenic α chain.** Five BALB.B (H-2<sup>b</sup>) mice were immunized with four intraperitoneal injections of 2 × 10<sup>7</sup> B6.2.16 cells given a week apart. A week after the fourth injection the mice were tail bled and the sera were used to stain B6.2.16 (α/β-expressing) and 93.2.20 (β-expressing) cells. One of the five immunized BALB.B mice yielded an antiserum that gave a 5% differential in staining of B6.2.16 and 93.2.20 cells. This mouse was rested for 4 wk and given an intravenous injection of 2 × 10<sup>7</sup> B6.2.16 cells 3 d before fusion with the HAT-sensitive, non-Ig-producing myeloma cell line, AG8.653. A total of 140 wells from this fusion contained growing hybridoma cells and supernatants from these wells were used to stain B6.2.16 and 93.2.20 cells. 20 supernatants gave good staining of B6.2.16. 10 of these supernatants also stained 93.2.20 and were omitted from further analysis. The remaining 10 supernatants were then used to stain thymocytes from B6 and female α/β transgenic H-2<sup>b</sup> mice. Of these 10 supernatants only one stained the transgenic thymocytes in
the manner shown in Fig. 3 and did not stain B6 thymocytes. The other nine supernatants did not stain transgenic or normal thymocytes. The hybridoma cells producing this antibody were subcloned at 0.3 cells/well. All subclones (20 of 20 tested) gave the same staining pattern of female transgenic thymocytes as indicated in Fig. 3. This mAb is referred to as T3.70 and is of the IgG1 subclass.

**Immunoprecipitation of TCR.** B6.2.16 and 93.2.20 cells were labeled with 125I using the lactoperoxidase/glucose oxidase method. Cells (2–5 × 10⁷) were washed three times with PBS and suspended in 1 ml of PBS. 50 μl of lactoperoxidase (30 U/ml)/glucose oxidase (10 U/ml) mixture was added followed by 1 mCi of Na 125I and 50 μl of n-glucose (200 mM). The cells were mixed and incubated for 30 min at room temperature. The cells were washed five times in PBS/0.1% NaN₃/5% FCS before lysing in lysis buffer containing 20 mM NaH₂PO₄ (pH 7.6), 150 mM NaCl, 2 mM EDTA, 8 mM iodoacetamide, 2% Triton X-100, 1 mM MgCl₂, 1 mM PMSF, 40 KIU/ml aprotinin, and 5 μg/ml leupeptin.

The solid-phase immuneisolation technique (9) was used to immunoprecipitate TCRs. The V-bottomed wells on an ELISA plate were coated with 100 μl of 0.1 mg/ml affinity-purified rabbit anti-mouse Ig at 4°C overnight. The wells were washed three times with PBS. 100 μl of 20 μg/ml of protein A-purified F23.1 or T3.70 mAbs were added to each well and incubated for 4 h at room temperature. The wells were washed three times with PBS and incubated with 200 μl of PBS + 5% FCS for further 4 h at room temperature. The wells were washed three times with PBS and 100 μl of 125I-labeled B6.2.16 or 93.2.20 cell lysates (equivalent to 2 × 10⁷ cells/ml) were added to each well. The wells were incubated at 4°C overnight. The wells were then washed six times with PBS/Tween 20.

The bound proteins from each well were eluted by incubating with 100 μl of SDS sample buffer for 30 min at room temperature. The eluted proteins were analyzed on a 10% polyacrylamide gel under nonreducing or reducing (with 2-ME) conditions. Autoradiography was accomplished with Kodak X-Omat AR film.

**Results**

**Production of a mAb that Detects the Transgenic α chain.** The β transgene uses the Vβ8.2 gene segment, and its expression on T cells can be monitored by the F23.1 mAb (7), which detects all three members of the Vβ8 gene family (10). It has been shown that the β transgene prevents both functional and nonfunctional Vβ rearrangements in the endogenous β chain loci but not Dβββ rearrangements. Thus, T cells from β as well as α/β transgenic mice expressed almost exclusively the transgenic β chain on their cell surface (4, 6). It has also been shown that in H-2b α/β transgenic mice, rearrangement of endogenous α chain loci is also suppressed, although to a lesser extent than that observed for β chain loci (5). To address more directly the question of expression of the transgenic α or endogenous α genes on various T cell subsets, we prepared a mAb that permits the detection of the transgenic α chain on T cells. For this purpose we immunized BALB.B mice with the B6.2.16 clone from which the α and β transgenes were isolated. An mAb, referred to as T3.70, was obtained (see Materials and Methods). The staining patterns of T3.70 for the parental B6.2.16 T cell clone, the 93.2.20 T cell clone derived from a β transgenic mouse, and the 264.2.5.3 T cell clone derived from an α transgenic mouse are shown in Fig. 1. It is clear that the B6.2.16 (α/β-expressing) clone is stained equally well by the F23.1 and T3.70 mAbs. In contrast, the 93.2.20 (β-expressing) clone is stained only by F23.1 and not at all by T3.70. T3.70 also gave weak staining of the 264.2.5.3 (α-expressing) clone. These experiments are compatible with the notion that T3.70 binds either to an idiotypic determinant that is dependent on expression of both transgenic α and β chains or to a determinant on the α chain only. This would mean that the 264.2.5.3 clone expressses endogenous α chains in addition to the trans-
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Figure 1. Preferential binding of the T3.70 mAb to T cells expressing transgenic α chains. The B6.2.16 (α/β-expressing), 93.2.20 (β-expressing) and 264.2.5.3 (α-expressing) T cell clones were stained with F23.1, T3.70, or CD3 Abs and analyzed as described in Materials and Methods. The x-axis indicates log fluorescence and the y-axis relative cell number in linear scale.

genic α chain. Recent experiments in fact support the latter notion: After transfection of the α and β genes into cell lines that already express a different α/β heterodimer we observed cells that stained ~10 times better with the T3.70 than the F23.1 antibody. This suggests that the idiotype seen by the T3.70 antibody does not depend on the transgenic β chain.

mAbs to α/β TCR are expected to immunoprecipitate from detergent lysates of appropriate T cells a disulfide-linked heterodimer of 80,000–90,000 molecular weight. By using the solid-phase immunoprecipitation technique (9) we were able to immunoprecipitate the TCR α/β heterodimer from B6.2.16 with both F23.1 and T3.70 mAbs (Fig. 2). On the other hand, the TCR from the 93.2.20 (β-expressing) clone was precipitated only by F23.1 but not by T3.70 (Fig. 2). These data confirmed that T3.70 binds to the α/β heterodimer on B6.2.16 cells.

Expression of the α and β Transgenes in Transgenic Thymocytes. The staining of thymo-

Figure 2. Immunoprecipitation of the TCR by F23.1 and T3.70 mAbs. B6.2.16 and 93.2.20 were surface labeled with 125I. Detergent lysates of labeled cells were used for the isolation of the TCR by the solid-phase immunoprecipitation technique (see Materials and Methods). Proteins eluted from wells to which no mAb (A), the F23.1 (B), or the T3.70 (C) mAb was attached were analyzed under reduced or nonreduced conditions on a 10% polyacrylamide SDS gel.
cytes from normal and α/β transgenic H-2b mice by F23.1 and T3.70 are shown in Fig. 3. As previously reported (4) and shown here for comparison, female transgenic thymocytes gave a high and a low level of staining by F23.1. A similarly high and low level of staining of female transgenic thymocytes by T3.70 is also observed. Furthermore, it is clear that some female transgenic thymocytes were not stained by T3.70 (11). In contrast to F23.1, which stained ~10% of C57BL/6 thymocytes, T3.70 did not stain any thymocytes from C57BL/6 mice nor did it stain thymocytes from CBA/J or AKR/J mice (data not shown). These observations suggest that the T3.70 idiotype is relatively uncommon.

The thymocytes in female transgenic H-2b mice that express low density of F23.1 and T3.70 idiotypes correspond to immature CD4+8+ thymocytes that are progenitors of mature CD4−8− and CD4−8+ T cells; in male α/β transgenic mice, this population is largely deleted (4). As indicated in Fig. 3, most of the male thymocytes that escaped deletion only expressed a high density of the F23.1 and T3.70 idiotypes. To further analyze the CD4/CD8 phenotype of thymocytes that expressed this high level of transgenic α and β chains, male transgenic thymocytes were double stained with combinations of CD4, CD8, F23.1, and T3.70 mAbs. We know from previous studies that female transgenic thymocytes, like normal C57BL/6 thymocytes, have well-defined CD4−8−, CD4+8+, CD4−8+ and CD4+8− populations; the

**THYMOCYTES**

![Thymocytes from different genotypes](image)

**Figure 3.** Staining of female and male transgenic thymocytes by F23.1 and T3.70. Thymocytes from a female or a male H-2b α/β transgenic mice and a normal C57BL/6 mouse were stained with F23.1 and T3.70 mAb and analyzed as described in Materials and Methods.
principle difference from normal C57BL/6 thymocytes is in the increase in number of CD4−8− T cells (5). In contrast, male transgenic thymocytes do not segregate into well-defined subsets on the basis of labeling with CD4 and CD8 antibodies. The most distinctive features of the male transgenic thymus is the large CD4−8− population and the lack of a well-defined CD4+8+ population (4).

Double staining of male thymocytes with CD4 and F23.1 or T3.70 on the one hand, and with CD8 and F23.1 or T3.70 on the other, is shown in Fig. 4. It is evident from such double staining that the most prominent population in the male transgenic thymus is the CD4−8− population (80% of male thymocytes) that expresses high levels of the transgenic TCR. Double staining with CD4 and CD8 indicated that in the male thymus the CD4+8+ population makes up ~8% of male thymocytes and expresses low levels of CD4 and CD8 (data not shown). Fig. 4 shows that most thymocytes expressing low CD4 and CD8 levels also express high levels of the transgenic TCR. Very few thymocytes however express high levels of CD4 and high levels of the transgenic TCR. In fact, a significant number of CD4+8− thymocytes (4% of total) express no detectable levels of the transgenic α and β chains. They presumably express endogenous α and β genes, which has been shown to be possible in cells that have deleted both transgenes (5). On the other hand, most of the CD8+
FIGURE 5. Staining of nylon wool nonadherent male transgenic spleen cells by F23.1, T3.70, CD4, and CD8 mAbs. Nylon wool nonadherent spleen cells were prepared as previously described (12). The nonadherent cells were double stained with F23.1 and CD4 or CD8 and with T3.70 and CD4 or CD8 and analyzed as described in Materials and Methods.

FIGURE 6. Comparison of lymph node cells from female and male α/β transgenic mice by staining with Thy-1, F23.1, CD4, and CD8 antibodies.
thymocytes that express high levels of the receptor express lower levels of CD8 molecules (~10-20% of that observed in female cells (see below).

Expression of the α and β Transgenes on Male Transgenic T Cells. Splenic T cells stained by the same method used to stain thymocytes are shown in Fig. 5. The splenocytes were enriched for T cells by passing over a nylon wool column. The nonadherent cells were 66% Thy-1+ and 8% Ig+, with 26% of the spleen cells expressing neither Thy-1 nor Ig markers. Double staining with CD4 and CD8 indicates that CD4-8+ T cells constitute the major T cell population in the spleen (58% of Thy-1+ cells); 20% of Thy-1+ were CD4+8- and <2% of the T cells were CD4+8+. Double staining of splenic T cells with CD4 and F23.1 or T3.70 or with CD8 and F23.1 or T3.70 showed that the most prominent T cell population was the CD8+ population that expresses high levels of both the α and β transgenic chains but low

\[ \text{CD4}^{-8^{-}} \text{LYMPH NODE CELLS} \]
\[ \alpha \beta \text{TRANSGENIC MALE} \]

\[ \text{CD4 PE} \]
\[ \text{CD8 FITC} \]

\[ \text{F23.1 FITC} \]
\[ \text{T3.70 FITC} \]

Figure 7. Staining of CD4-8- male transgenic lymph node cells with F23.1 or T3.70 mAbs. Lymph node cells from a male transgenic mouse were stained with PE-labeled CD4, FITC-labeled CD8, and FITC-labeled anti-mouse Ig antibodies. The labeled cells were then separated from the unlabeled cells using a FACS IV (Becton Dickinson & Co.). The sorted CD4-8- cells were then stained with either the F23.1 or the T3.70 mAb and analyzed as described in Materials and Methods.
levels of CD8. Fig. 6 shows that among lymph node cells the most prominent population contains CD4⁻⁸⁺ T cells and that the CD4⁻⁸⁺ T cells in male mice express lower CD8 levels when compared with T cells from female mice (Figs. 5 and 6). Very few T cells were CD4⁺⁻⁸⁻ and transgenic TCR high. The majority of the CD4⁺⁻⁸⁻ cells (15% of Thy-1⁺ cells) expressed undetectable levels of both of the α and β transgenes. We also deduced from this analysis that ~18% of the Thy-1⁺ spleen cells were CD4⁻⁸⁻ and expressed high levels of transgenic α and β chains.

Lymph node cells from male transgenic mice contained ~80% Thy-1⁺ cells, of which 24% were CD4⁺⁻⁸⁺, 5% were CD4⁺⁻⁸⁻, and 71% were CD4⁺⁻⁸⁻. To directly determine whether CD4⁺⁻⁸⁻ T cells express the α and β transgenic chains rather than γ/δ TCRs, these cells were enriched by staining with CD4, CD8, and anti-Ig antibodies and sorting for unstained cells using the FACS. As indicated in Fig. 7, the sorted cells were phenotypically CD4⁺⁻⁸⁻. All the CD4⁺⁻⁸⁻ cells were Thy-1⁺ (data not shown) and expressed high levels of both α and β transgenic chains (Fig. 7).

CD4⁻⁸⁺ T Cells from Male Mice Are Tolerant to HY Antigen. The proliferative responses of CD8⁺ T cells from female and male α/β transgenic mice induced by Con A or H-2b male stimulator cells were compared. As shown in Fig. 8 both types of T cells responded well to Con A but only the T cells from female α/β transgenic
mice responded to male antigen (Fig. 8). Neither of the T cells showed any significant response when cultured with female stimulator cells (not shown).

Discussion

The analysis of T cell subset composition in male transgenic H-2^b mice allows the direct evaluation of the impact of the nominal antigen on the development of T cells in these mice (4, and this report). The observations can be summarized as follows: (a) In male transgenic mice CD4^+8^+ precursors expressing low levels of the transgenic TCR have been largely deleted in the thymus; (b) the major T cell population in the thymus is of the CD4^-8^- phenotype and expresses high levels of the transgenic TCR; (c) in the periphery, the two major T cell subsets are CD4^-8^-low and CD4^-8^- cells and both subsets express high levels of the transgenic TCR; (d) the number of CD4^-8^- cells in the periphery is greatly reduced when compared with normal or α/β transgenic female mice. Very few of these CD4^-8^- cells express the transgenic TCR.

On the basis of these observations we conclude that the presence of the nominal antigen in H-2^b transgenic mice leads to deletion of precursor cells expressing the self-reactive TCR. The deletion affects mostly CD4^+8^+ cells or their CD4^-8^- precursors (13) and this is reflected in the reduced number of thymocytes and the high proportion of CD4^-8^- thymocytes expressing the transgenic receptor. A reduction in the number of all thymocytes in TCR transgenic mice expressing a particular TCR and the relevant class I MHC antigen simultaneously was not observed by Sha et al. (14). This is not in conflict with our results because in the mice described by Sha et al. only a fraction of the thymocytes express the transgenic receptor. For the same reason the authors do not observe a reduction in the number of CD4^-8^- T cells since they can be selected from CD4^+8^- thymocytes expressing endogenous receptor chains. In fact, the authors (14) cite their own unpublished data on transgenic mice with higher levels of TCR expression confirming essentially the results reported here and in a previous publication (4). One may wonder why CD4^-8^- precursor cells expressing low levels of the transgenic receptor are deleted but CD4^-8^-low cells expressing high levels of the receptor can escape the deletion process. One could argue that the precursors of the CD4^-8^-low cells are initially CD4^+8^-low TCR^-low^. The low CD8 expression allows them to escape deletion but this phenotype can still be positively selected (11) to differentiate into CD4^-8^-low TCR^-high^ T cells. These cells, because of their low CD8 levels, cannot be activated by male cells, i.e. they are harmless for the animal. The alternative possibility is that tolerance can not only be achieved by deletion of immature CD4^+8^- T cells but can also be achieved by downregulation of CD accessory molecules. At present we cannot determine whether the CD4^-8^-low, TCR high cells survive because of an inherent CD8^-low^ phenotype or whether their CD8 molecules are actively downregulated when they get into contact with male cells. The latter mechanism would not necessarily result in drastically reduced thymocyte numbers whereas a deletion, which spares a minor population, would be reflected in smaller numbers of thymocytes. Our future experiments are aimed at testing the stability of the CD4^-8^-low TCR^-high^ phenotype by transferring these cells into female and male recipients.

The results shown here demonstrate that tolerance does not necessarily require the elimination of all T cells expressing an autospecific TCR. Obviously the few
cells that escape the deletion mechanism, because of reduced levels of accessory molecules or even lack of accessory molecules, can migrate from the thymus and expand enormously in the periphery such that the thymus contains only few cells of this phenotype but the periphery contains almost normal numbers of Thy-1+ cells. This view of peripheral expansion of thymus migrants is supported by the finding that one often observes rare phenotypes in the periphery of male but not female α/β transgenic mice. We have previously noted peripheral T cells that had deleted both transgenes and express endogenous α/β genes (5) and here we find that a significant proportion of CD4+8- cells seem to lack both transgenes but express an endogenous receptor. In summary, tolerance to T cells expressing an autospecific TCR in the male transgenic mouse is achieved through deletion of CD4+8+ precursor cells expressing low levels of this transgenic TCR and the lack of development of mature T cells that express high levels of this autospecific TCR and normal levels of CD4/CD8 molecules.

Our experiments are compatible with recent experiments concerned with tolerance of CD4+8- cells (13, 15). These authors used CD4 antibodies to interfere with tolerance induction and concluded that also in their system the deletion occurred at the level of CD4+8+ thymocytes. In addition, the authors provided evidence that the accessory molecules played a crucial role in tolerance induction: CD4-8- cells expressing the autospecific α/β receptor could be found in these animals without any signs of autoimmunity (13, 15).

Summary

Transgenic mice that carry on a large fraction of their T cells an α/β T cell receptor that recognizes the male antigen in the context of H-2Db molecules were constructed. An mAb specific for the transgenic receptor was developed and used to analyze T cell subsets in male transgenic H-2b mice. The vast majority of immature CD4+8- T cells that express the transgenic TCR were deleted in the male transgenic mouse. Nevertheless, the majority of T cells spared by this deletion process expressed a high level of the transgenic TCR. These T cells, however, had an abnormal CD4/CD8 phenotype in that they expressed either no CD8 molecules or only low levels.

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