In Mls-1b Mice, Fetal-Type β-Gene Rearrangements Are Frequent among Self-Anergic Vβ6 T Cells

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Summary

T lymphocytes generated in the fetal and neonatal period are characterized by T cell receptor (TCR) gene rearrangements that lack N region nucleotides (fetal-type TCR). Using fetal-type TCR, as a lineage marker, we show that such T cells are long-lived and persist in the periphery of adult mice. Moreover, in both neonatal and adult environments, upon encounter with self-antigens, they are less likely to be deleted. Inefficient clonal deletion could be due to the intrinsic properties of the T cells generated during this period, or to yet unknown properties of the perinatal thymus. Such anergic T cells constitute a subset that can further expand in vivo in an antigen-independent fashion, leaving open the possibility for self-aggression under the appropriate triggering conditions.

In newborn mice, most of the TCR rearrangements in thymocytes do not contain any addition of untemplated nucleotides in the N regions between the V, D, and J gene segments. This type of rearrangement is referred to as the fetal type (1, 2), in contrast to the adult-type rearrangements which are characterized by extensive addition of N nucleotides (3). Presumably, this is due to the absence of terminal transferase activity in the fetal and perinatal lymphoid precursors (4-6). If cells expressing fetal-type TCR are derived from precursors that lack at least one enzyme present in the lymphoid precursors of adult mice, they could be physiologically distinct from the other T cells. Little is known regarding the biological properties of α/β T cells which bear fetal-type TCR, other than that they appear to be diluted out by T cells which express adult-type TCR (1, 2). However, it has been reported that neonatal thymectomy in mice and rats greatly increases the incidence of autoimmune disease (7-11). One wonders whether, under such circumstances, T cells with fetal TCR rearrangements could be long-lived and contribute to autoimmunity.

In this study, we investigated the selection, antigen reactivity, and long-term fate of α/β T cells that are generated in the perinatal period and characterized by fetal TCR rearrangements. To assess their function, we exploited some unusual features of T cell reactivity to superantigens. Superantigens can induce T cell responses that are orders of magnitude larger than those generated by conventional antigens. The responder T cells consistently express a limited and specific set of Vβ TCR genes (for a review see reference 12). This is due to the fact that superantigens bind to TCR in a non-conventional fashion: they form a direct bridge between the MHC of the APC and the CDR4 of the TCR β subunit (13-15). The amino acid structure of the VDJ junction (CDR3) does not appear to affect this interaction. Mls-1b is an endogenous superantigen encoded by the retrovirus mammary tumor virus (Mtv7) (16). Mice that do not carry this endogenous virus (e.g., BALB/c, Mls-1b) express ~11% Vβ6 T cells in the peripheral T cell pool, whereas mice that do carry this viral sequence (e.g., DBA/2, Mls-1a) have <1% (17, 18). In mice that express a particular Mls antigen, the reactive T cells are deleted as they mature in the thymus (17, 19). Mature Mls-reactive T cells can also be deleted in the periphery after encounter with antigen (20). Clonal deletion is not the only way of establishing T cell tolerance to Mls antigens. Mls-1b stimulator cells have been shown to induce unresponsiveness in vivo (21). Based on more recent results obtained in euthymic mice, it was concluded that in vivo, subsequent to a primary response, some of the Mls-1b-reactive T cells became anergic (22). A significant proportion of self-Mls-1b anergic Vβ6 T cells was also detected in adult DBA/2 mice that were thymectomized during the first 4 d of life (23). In such mice, there were more Vβ6 T cells than in unmanipulated animals. In both cases, the anergic Vβ6 T cells could not be activated in vitro by Mls-1b stimulator cells, or by crosslinking with anti-Vβ6 antibody.

We reasoned that fetal-type Vβ6 T cells generated during the perinatal period could be retained in the adult T repertoire of DBA/2 mice, if they had been rendered anergic in...
the perinatal period and were long-lived. In particular, we expected to find them in adult DBA/2 mice that were thymectomized on the third day after birth (αTx). In these mice the few adult-type Vα6 T cells that might have been generated after thymectomy should be deleted in the periphery, as suggested by the experiments of Webb et al. (20). In contrast, the fetal Vα6 clonotypes, which were already anergic by day 3, should not be diluted out in the way that they were in normal adults. We show here that these assumptions are correct. In addition, we found that, even in normal DBA/2 mice, a significant fraction of fetal clonotypes was retained within the anergic Vα6 T cell subset. Our data also demonstrate that anergic T cells can survive and even expand in the periphery over long periods of time. Moreover, they indicate that either fetal clonotypes are predisposed to anergy, or that the neonatal environment favors anergy as an alternate path toward functional negative selection. Furthermore, the data reported below also reveal that “fetal” T cell clonotypes are anergy prone even in the adult environment, and suggest that a tendency toward acquiring nonresponsiveness is inherent in cells derived from this lineage. This view implies the accumulation of inactivated autoreactive cells, which can become self-aggressive under the appropriate triggering conditions, and could account for the observed tendency of neonatally thymectomized animals to develop autoimmunity.

Materials and Methods

Mice. All mice were purchased from the Jackson Laboratory (Bar Harbor, ME). In the case of 3-d-old mice, timed pregnant mice were purchased, and the day of birth was designated day 1.

Neonatal Thymectomy. Thymectomy was performed on anesthetized 3-d-old mice by sequential suction of both thymic lobes, as described by Miller (24). When the thymectomized mice were killed, the efficiency of thymectomy was ascertained for each mouse by the absence of remnant thymic tissue observed under the dissecting microscope and by the reduction of the T.cell percentage in LN cells (two- to threefold less than in euthymic mice).

Cells and Cultures. LN cells and splenocytes were isolated by standard procedures. For polyclonal T cell activation, cells were cultured in IMDM containing PMA (10 ng/ml, Calbiochem Corp., La Jolla, CA), ionomycin (0.25 #M, Calbiochem Corp.), rIL-2 (10 U/ml, Apen Biologicals, Thousand Oaks, CA) and rIL-2 (20 U/ml, Apen). After 2 d, cells were washed extensively and expanded in IMDM containing only rIL-1 and rIL-2 for an additional 3-5 d. Under these conditions of polyclonal activation and expansion, no alterations in the partial frequencies of T cell subsets were introduced, as monitored by either the expression of CD4/CD8, or various Vα TCR (25). For MLR, 2 x 10^8 LN cells from BALB/c mice were stimulated with irradiated (3,000 rad) DBA/2 B cell blasts. B cell blasts were prepared by culturing splenocytes with LPS (100 μg/ml; Difco, Inc., Detroit, MI) and IL4 (30 U/ml, Apen) for 24 h before irradiation. T cell blasts were harvested on day 5 from MLR cultures for both flow cytometry and RNA extraction. No attempt was made to analyze CD4+ or CD8+ T cell subsets separately in any of the experiments, in that both subsets display Mls-1a reactivity (17, 26, 27) and can be deleted or become anergic (23).

Flow Cytometry Analysis. Two-color immunofluorescence was performed on viable cells using biotin-conjugated anti-α/β antibody (H57-597, reference 28) or biotin-conjugated anti-Vα6 antibody (RR4-7, reference 29) followed by streptavidin-PE and FITC-conjugated anti-CD3 antibody (145-2C11, reference 30).

Nucleic Acids. Total cellular RNA was extracted from T cell blasts by the acid guanidium thiocyanate-phenol-chloroform method (31). cDNA synthesis and PCR amplification of Vα6 TCR were performed essentially according to our standard procedures (32). In this case, 5 pmol of an antisense Cβ-specific external primer (5'-CCAAGCAGAGGGTAC-3') was used to prime TCR-β-specific cDNA in a 20-μl reaction volume. 1 μl of the cDNA reaction mixture was directly added to 20 μl of the amplification reaction which contained 10 pmol of an antisense Cβ specific internal primer (5'-GATGGCTCAAAAACAAGGAG-3') and 10 pmol of a sense Vα6 specific external primer (5'-AACCTGACCTGAAA-TGTCACAG-3'). 30 cycles of PCR at 94°C, 30 s; 58°C, 30 s; and 72°C, 90 s were performed on a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) followed by an additional 9 min at 72°C. PCR-amplified Vα6 cDNA fragments were purified from low melting agarose gel and cloned into the Smal site of PUC18 as described (32).

DNA Sequencing. Single colonies of independently derived Vα6+ clones were each resuspended in 20 μl of sterile water, heated at 95°C for 5 min, centrifuged at 15,000 rpm at 4°C for 5 min, and 10 μl of each supernant was used as template in a 50 μl PCR reaction (25-30 cycles) where the TCR-β inserts were amplified. For this round of amplification, 20 pmol of a 5'-biotinylated Vα6-specific primer, synthesized on the oligonucleotide synthesizer (Applied Biosystems, Inc; Foster City, CA) using biotin phosphoramidite (Amersham Corp., Arlington Heights, IL), was used in conjunction with a normal antisense Cβ-specific internal primer under similar conditions as before. 40 ng of the PCR product was added to 40 ng of streptavidin-coupled Dynabeads M-280 suspension (Dynal Inc., Great Neck, NY) that had been equilibrated in 2 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.5, and gently agitated at room temperature for 15 min, and the beads were recovered on a magnetic particle concentrator (Dynal Inc.). The beads were washed once in the same buffer, then resuspended in 20 μl of 0.1 M NaOH at room temperature for 10 min. The nonbiotinylated negative strand was removed and the beads with the bound single-stranded PCR product served as templates in solid-phase sequencing by the dideoxynucleotide chain termination method (33) using Sequenase Version 2.0 (U.S. Biochemicals, Cleveland, OH). Sequence analysis was performed using the MacVector 3.5 software and GenBank database (IntelliGenetics, Inc., Mountain View, CA).

Results

A Large Fraction of Vα6 T Cells in Adult DBA/2 Mice Express Fetal-Type TCR Rearrangements. In DBA/2 mice, expression of the self superantigenic Mls-1a results in the deletion of most Vα6 T cells. Nonetheless, ~1% of all α/β T cells in the periphery are Vα6 positive. These T cells are anergic: they do not proliferate or produce lymphokines upon stimulation with anti-Vα6 antibody or syngeneic Mls-1a stimulator cells (23; Rajasekar, unpublished observation). However, all T cells can proliferate when polyclonally activated by the appropriate doses of PMA and ionomycin in

1 Abbreviation used in this paper: αTx, mice thymectomized on the 3rd day after birth.
the presence of IL-1 and IL-2 (25). To compare the repertoire of the undeleted V$\gamma$6 T cells in DBA/2 mice with a control population in which V$\gamma$6 T cells were not subjected to negative selection, V$\gamma$6 cDNA sequences from polyclonally activated LN cells of 11-wk-old DBA/2 and BALB/c mice were analyzed. As shown in Fig. 1, in DBA/2, half of the V$\gamma$6 sequences lacked N region added nucleotides—the hallmark of fetal-type gene rearrangement. In contrast, in BALB/c mice, all V$\gamma$6 sequences contained N region added nucleotides typical of the adult-type gene rearrangement (Fig. 1). We have also sequenced the V$\gamma$11 cDNA clones obtained from the same BALB/c mRNA preparations from which we had ob-

| BALB/C: | | | | | | J$\beta$
|---|---|---|---|---|---|---|
| 1.10 | AOC AGT ATA | GC | A C A G G O G | A C A G A A G T G | J$\beta$ 1.1 |
| 1.11 | AOC AGT ATA | G A C G | AG G | C A A C A C A | J$\beta$ 1.1 |
| 1.17 | AOC AGT ATA | G O G G | C A G G G | A C G A G A | J$\beta$ 1.4 |
| 1.19 | AOC AGT ATA | C G | CT | C T | C T C T C T A T | J$\beta$ 2.7 |
| 1.20 | AOC A | C C | C C | G O G A C T G O O G | T A T G A A C A G | J$\beta$ 2.7 |
| 1.42 | AOC AGT AT | C C | C A G G | T T C G O G | O C A G T C C T | J$\beta$ 1.5 |
| 1.88 | AOC AGT AT | G O G G | A C A | A A A C A C C | J$\beta$ 2.4 |
| 21.1 | AOC AGT | G A C A G G O G G | A C C A A G A | T C C A A C A G A | J$\beta$ 1.4 |
| 21.6 | AOC AGT AT | A C A G G | C G | C A C A G A | J$\beta$ 1.1 |
| 21.7 | AOC AGT AT | A C G G | A A G G | T C T C T C T C T | C A C A C A | J$\beta$ 1.1 |
| 21.9 | AOC AGT AT | T A A G G G | C T T G O G | A T | C T | A C G A C A G A | J$\beta$ 2.3 |
| 21.10 | AOC AG | C | G A A | A A C T C C G A C | J$\beta$ 1.2 |
| 21.12 | AOC AG | C C | G A C A G | A A T | A C T C A A G A | J$\beta$ 2.3 |
| 21.13 | AOC AGT AT | T C T C T G G | G O G G | G A T A G G | A A C T C C C T | J$\beta$ 2.1 |
| 21.14 | AOC AGT AT | T T T | C T G G | A C G | A A C | J$\beta$ 2.2 |
| 21.15 | AOC AG | C | C A C O G | A A C T C C G A C | J$\beta$ 1.2 |
| 21.17 | AOC AGT AT | G T C | G A A | T C A C A A G A | J$\beta$ 2.3 |
| 21.22 | AOC AGT AT | A C | O O G G | T C O A A T C A O | J$\beta$ 1.3 |
| 21.23 | AOC AGT | G O G G A | G | T | A C C A C A G A | J$\beta$ 2.5 |
| 21.24 | AOC AGT AT | C | O O G G | T | A C C A C C G G | J$\beta$ 2.2 |

| DBA/2: | | | | | | J$\beta$
|---|---|---|---|---|---|---|
| 1.2 | AOC AGT AT | G A C A G | G A C A | C A A C T C C | G A C | J$\beta$ 1.2 |
| 1.3 | AOC AG | G C C | G O G A C A G O O G | G | T A T G A A C A G A O G | J$\beta$ 2.7 |
| 1.10 | AOC AGT AT | G A C A | G A C | G C A C A T C T | J$\beta$ 2.5 |
| 1.11 | AOC AGT AT | G O G A C A G | G G G | T C O A A T C O | J$\beta$ 1.3 |
| 37.6 | AOC AGT AT | G O G G | C A G A C | C A A G A C C | J$\beta$ 2.3 |
| 37.8 | AOC AGT AT | A | A | T C O A A T C O A | J$\beta$ 1.5 |
| 37.9 | AOC AGT AT | G O G A C A | A A A C T C C | J$\beta$ 1.2 |
| 37.12 | AOC AGT AT | G O G G | C A A G A G A | C A A C T C C | J$\beta$ 1.1 |
| 1.1 | AOC AG | G A | G A C A G | C A | A A C T C C G A C T | J$\beta$ 1.2 |
| 1.6 | AOC AG | G A | G C C | G A C | A C G | J$\beta$ 2.3 |
| 1.7 | AOC AGT ATA | A C A G G O G G | A A C T C C G A C | J$\beta$ 2.3 |
| 1.12 | A O | A | G A C A G | T | C T A A | J$\beta$ 2.3 |
| 3.3 | AOC AGT AT | G G G G | G G G | C A A G A C C | J$\beta$ 2.5 |
| 8.6 | AOC AGT AT | A G A | C A C | T A A C T A T | J$\beta$ 2.1 |
| 8.7 | G O G G | G O G G | G A | C A A C A C C | J$\beta$ 2.5 |
| 37.1 | AOC AGT AT | C T G G | G G G A C A G | C | G A C A C C G | J$\beta$ 2.5 |
| 37.4 | AOC AG | G O G G | C C | C A C A C C | J$\beta$ 1.6 |
| 37.5 | AOC AGT AT | C T T | G A C A G | C T G A | C A A C A C A | J$\beta$ 1.1 |
| 37.14 | AOC AGT AT | T | A C A | A T A C T C T G | J$\beta$ 1.6 |

Figure 1. V$\gamma$6-D-J junctional sequences from DBA/2 and BALB/c LN T cells. LN cells of 11-wk-old DBA/2 and BALB/c mice were polyclonally activated in vitro with PMA, ionomycin, rIL-1, and rIL-2 as described in Materials and Methods. Polyclonal activation was essential in order to ensure that all T cells present were transcribing their TCR genes at similar levels. V$\gamma$6 cDNA sequences were derived from T cell blast mRNA. Cells from five mice were pooled for each group. Within each set, fetal type rearrangements are presented before the adult type. The criteria used for defining a type of rearrangement are: (a) in fetal rearrangements there is no template independent addition of nucleotides (N region) at the junctions of rearranging V, D, and J segments, and (b) both fetal and adult rearrangements can display P nucleotides. One or two P nucleotides added to an intact, adjacent V, D, or J segment are derived from a tetranucleotide palindrome which appears as an intermediate during gene rearrangement (36).
tained the adult V\(_{\beta}6\) clones in the earlier analysis. In this V\(_{\gamma}11\) subset, which is tolerized in BALB/c mice because of the expression of the endogenous Mtv-9 products in conjunction with the I-E\(^d\) class II antigen, over 30% of the sequences showed typical fetal rearrangements (data not shown). This indicates that the appearance of fetal clonotypes in the pool of an anergic T cell subset in either DBA/2 or BALB/c mice is due to the specific retention of such clonotypes under conditions of self tolerance.

Considering that \(\alpha/\beta\) fetal clonotypes are generated in large numbers during fetal and perinatal life and are progressively outnumbered afterwards by adult clonotypes, the high proportion of fetal type V\(_{\beta}6\) sequences detected in the anergic T cell population of adult DBA/2 is unexpected. There are two possible explanations for this observation. Cells bearing fetal-type TCR may be produced at a low frequency in the thymus throughout life, but are more likely to be rendered nonresponsive to Mls-1\(^a\) than the other T cells. Therefore, the fetal-type T cells accumulate in the periphery, whereas the others are preferentially deleted. Alternatively, if cells bearing fetal-type rearrangements only originate in the perinatal period, then it follows that at that time, such T cells recognizing Mls-1\(^a\) superantigen are easily rendered anergic, whereas Mls-1\(^b\)-reactive T cells bearing adult rearrangements and appearing later in life are deleted. Both explanations imply that at least part of the fetal T cell pool must be long-lived. To test these assumptions and to estimate the life span of fetal clonotypes, we studied the V\(_{\beta}6\) repertoire of Mls-1\(^a\) and Mls-1\(^b\) neonatal mice, and followed the fate of these T cells in normal adult and in adult mice that had been thymectomized on the 3rd day after birth (\(d3\)Tx).

Most of the Anergic V\(_{\beta}6\) T Cells in DBA/2 Mice Thymectomized on Day 3 Express Fetal-Type TCR Rearrangements. Based on previous measurements on thymocytes (1, 2) we anticipated that the day 3 peripheral T cell repertoire of BALB/c and DBA/2 mice would consist of both fetal and adult V\(_{\beta}6\) clonotypes. Furthermore, the fate of both subsets could be followed under various experimental circumstances after day 3 thymectomy. First, we established the percentages of peripheral V\(_{\beta}6\) T cells in BALB/c and DBA/2 mice at day 3. These measurements were essential since no information was available on the composition of the peripheral T cell repertoire at this age. Fig. 2 shows that in both BALB/c and DBA/2 mice, there is a larger proportion of V\(_{\beta}6\) T cells on day 3 than later in life. Nonetheless, at day 3, the peripheral pool of V\(_{\beta}6\) T cells in DBA/2 mice represents <40% of the V\(_{\beta}6\) T cell fraction in BALB/c mice, indicating that many V\(_{\beta}6\) T cells are already deleted in DBA/2 mice at this age. The V\(_{\beta}6\) T cells in the periphery of day 3 DBA/2 mice are probably already anergic and could be retained in mice thymectomized at this time. Indeed, as shown in Fig. 2, the fraction of V\(_{\beta}6\) T cells is higher in \(d3\)Tx DBA/2 than in euthymic mice for a long time (4% compared to 1% at 16 wk).

The ratios of fetal to adult-type V\(_{\beta}6\) TCR rearrangements in the neonatal and adult mice were estimated by DNA sequence analysis. For both DBA/2 and BALB/c, the productive V\(_{\beta}6\) sequences obtained from peripheral T cells of 3-d-old mice and 8-wk-old \(d3\)Tx mice are presented in Fig. 3 B. The relative proportions of fetal- and adult-type TCR from various sources are illustrated in Fig. 3 A. In day 3 DBA/2 mice, V\(_{\beta}6\) fetal clonotypes were predominant in the periphery. In BALB/c mice of the same age, however, equal proportions of fetal- and adult-type rearrangements were present, indicating that adult-type TCR were already being produced in large numbers but that in DBA/2 mice they were preferentially deleted. Moreover, we found several out-of-frame V\(_{\beta}6\) sequences with adult type rearrangements in day 3 DBA/2 mice (not shown), confirming that adult-type V\(_{\beta}6\) sequences were produced at that time. From Fig. 3 A, it appears that the proportions of fetal to adult-type TCR did not change in either mouse strain for at least 8 wk after \(d3\)Tx. Among the undeleted V\(_{\beta}6\) T cells present in 8-wk-old \(d3\)Tx DBA/2 mice, over 80% were of fetal type (Fig. 3, A.

Figure 3. Expression of fetal- and adult-type V\(_{\beta}6\) rearrangements in 3-d-old, adult, and \(d3\)Tx DBA/2 and BALB/c mice. Lymphocytes were prepared from 20-30 3-d-old BALB/c and DBA/2 spleens as well as from LN of five \(d3\)Tx, 8-wk-old BALB/c or DBA/2 mice. T cells were polyclonally activated with PMA and ionomycin, and expanded in culture for 7 d in the presence IL-1 and IL-2. The V\(_{\beta}6\)-D-J\(\beta\) cDNA sequences were derived from T cell blasts. (A) Percentages of fetal and adult type V\(_{\beta}6\) rearrangements in 3-d-old, adult, and \(d3\)Tx DBA/2 and BALB/c mice. (B) V\(_{\beta}6\)-D-J\(\beta\) junctional sequences from the T cell populations in A; sequences derived from LNT cells of adult nonthymectomized BALB/c and DBA/2 mice were presented in Fig. 1.
and B), indicating that anergic fetal clonotypes survived for a long time. We concluded that in DBA/2 mice, most of the T cells expressing adult-type $\delta$6 rearrangements were deleted, whereas the fetal type $\delta$6 T cells were preferentially retained.

**Fetal $\delta$6 T Clonotypes Are Long-lived and Can Be Expanded in the Periphery.** By counting the T cells in 3-d-old and 8-wk-old $\kappa$Tx mice, it became apparent that the total number of T cells increased severalfold in spite of the removal of the thymus. We estimated that the $\delta$6 T cell pool present in the periphery at the time of thymectomy had expanded $\sim$20-25-fold in $\kappa$Tx BALB/c. In DBA/2, although the percentage of $\delta$6 T cells decreased from 6% at day 3 to 4% in 8-wk-old $\kappa$Tx mice, the absolute number of $\delta$6 T cells increased $\sim$10-15-fold during that time. The proportion of fetal- and adult-type $\delta$6 sequences did not change during T cell expansion in either mouse strain (Fig. 3). One could argue that this observation actually reflects the accumulation of T cells generated extrathymically in these thymectomized mice, and that some of these extrathymically generated T cells carry fetal rearrangements. To investigate this, we established the sequences of $\delta$6 cDNA clones derived from 16-wk-old BALB/c nude (nu/nu) mice. In such mice, there are essentially no detectable $\alpha\beta$ T cells at birth. Thus, the $\alpha\beta$ T cells that appear later represent extrathymically generated T cells that have accumulated over an extended period of time. Out of 19 in-frame rearrangements derived from such cells, no fetal-type $\delta$6 rearrangements were detected (data not shown). This shows that fetal $\delta$6 T cells detected in the adult $\kappa$Tx mice are indeed long-lived, and represent expanded descendants of T clonotypes that rearranged their TCR elements during the perinatal period. $\delta$6 T cells of $\kappa$Tx DBA/2 mice cannot be induced to proliferate by either Mls-1stimulator cells or by crosslinking with anti-$\delta$6 antibody (Table 1), and therefore are bona fide anergic cells. It follows that the post-thymic expansion of these anergic T cells must be antigen independent.

Another indication that the expansion of anergic T cells is antigen independent comes from the analysis of $\beta$2.7 usage in the $\delta$6 TCR pool. As shown in Fig. 4, in both DBA/2 and BALB/c, 30% of $\delta$6 T cells present before thymectomy (day 3) used the $\beta$2.7 gene segment. The fraction represented by this subset remained unchanged in adult $\kappa$Tx DBA/2. However, in adult $\kappa$Tx BALB/c mice, the fraction of $\delta$6 $^{\beta}2.7$ $\delta$6 T cells decreased to 15%. This observation could be a reflection of the difference between a strictly antigen-independent expansion in DBA/2, and a selective expansion in BALB/c, where T cells could have been subjected to antigen-driven selection.

**BALB/c Fetal-type $\delta$6 T Cells Are Mls-1* Reactive but Can Be Preferentially Rendered Anergic by In Vivo Challenge with DBA/2 Splenocytes.** To ascertain that fetal-type $\delta$6 T cells exhibit a TCR which accommodates interactions with Mls-1*, we set up MLR, in which the responder cells were from $\kappa$Tx BALB/c (H-2d, Mls-1k) which contained equal proportions of fetal- and adult-type $\delta$6 T cells. Activated DBA/2 B cells (H-2d, Mls-1k) were used as stimulators. The proliferative responses of $\delta$6 T cells were estimated by their enrichment in culture (Fig. 5). It appeared that $\delta$6 T cells of $\kappa$Tx BALB/c mice responded well, because their relative enrichment was comparable to that of T cells from euthymic BALB/c mice. This indicated that most $\delta$6 T cells from $\kappa$Tx BALB/c mice were Mls-1 reactive. Moreover, among the $\delta$6 sequences derived from T cell blasts harvested from such cultures, we identified 46% fetal rearrangements (Fig. 6), a frequency close to the 54% fetal rearrangements detected before MLR (Fig. 3). We concluded that fetal-type $\delta$6 T cells were capable of reacting to the Mls-1* superan-

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**Table 1.** $\delta$6 T Cells from DBA/2 $\kappa$Tx Mice Cannot Be Stimulated by Either Mls-1* B Cell Blasts or by Crosslinking with Anti-$\delta$6 Antibody

|                      | Percentage $\delta$6 + T cells among $\alpha\beta$ T cells |
|----------------------|----------------------------------------------------------|
|                      | Medium alone Anti-DBA/2 MLR Anti-$\delta$6 antibody       |
| BALB/c $\kappa$Tx    | 13 60 67                                                  |
| DBA/2 $\kappa$Tx     | 3 1 6                                                    |

BALB/c $\kappa$Tx and DBA/2 $\kappa$Tx LN cells (3 x 10^6/ml) were cultured in the presence of rIL-1 (10 U/ml) and rIL-2 (20 U/ml) either with irradiated DBA/2 B cell blasts as stimulators, or in wells containing immobilized anti-$\delta$6 mAbs (RR 4-7). 5 d later, the percentage of $\delta$6 + T cells was estimated by two-color flow cytometry.

**Figure 4.** Utilization of $\beta$2.7 gene segment in $\delta$6 T cells. The percentages of $\beta$2.7 containing sequences were calculated from the data presented in Fig. 3 B. 20-28 sequences were analyzed for each group.
wk later, LN cells were prepared from challenged and unchallenged mice.

Figure 5. Enrichment of BALB/c V~6 T cells in anti-DBA/2 MLR.

Table 1. Comparison of V~6 T cell frequencies in various experimental groups.

| Group                  | % of V~6 T cells |
|------------------------|-----------------|
| Normal                 | 20              |
| a~Tx                  | 40              |
| a~Tx + PMA + ionomycin | 60              |
| MLR                   | 80              |

Discussion

The generation of T cells that bear fetal-type TCR rearrangements was first observed in the /~6 T cell pool (32, 34–36). Many of the fetal /~6 T cells display invariant TCR sequences and are partitioned in various epithelial tissues. Moreover, for the dendritic epidermal cells of the skin, the canonical Vγ5/Jβ1 fetal rearrangement occurs only in fetal T cell precursors which differentiate in a fetal thymus (39, 40). Recently, it was shown that for /~6 T cells, fetal-type TCR rearrangements also predominate in the fetal and neonatal thymus, but that their frequency decreases rapidly after birth (1, 2). By day 4, over 70% of the thymocytes already have adult TCR rearrangements. However, there is little information regarding the biological properties of this first set of /~6 T cells, or their fate in the normal animal. As a matter of fact, it is not clear whether /~6 T cells with fetal-type rearrangements do exit the thymus and become functional lymphocytes. Data presented here indicate that fetal-type /~6 T cells exist as functional immunological entities in the periphery, that they are similar to fetal-type /~6 T cells in the ability to survive for long periods of time, but that physiologically, they can differ from cells with adult-type TCR.

Among the anergic remnants of the V~6 T cell pool subjected to incomplete deletion by the self superantigen
BALB/C d3Tx MLR:

19.2 AC T9 GOGA CAC AC TATGCT J2 3.1
19.4 AC T9 GAC AOD GC GC TCA CAC J2 3.1
19.5 AC T9 GOGA GOGA CTGAA J2 3.1
19.13 AC T9 GAC AOD CAC TACT J2 3.1
19.19 AC T9 GOGA TAC CAC J2 3.1
20.3 AC T9 GAC AOD AGCG ACG CAC J2 3.1
20.7 AC T9 GAC AOD AGCG AGCG CAC J2 3.1
20.9 AC T9 GOGA GOGA CAC J2 3.1
20.10 AC T9 GOGA AOD AOD CAC J2 3.1
20.11 AC T9 GOGA TATGCT J2 3.1
19.3 AC T9 GOGA GOGA GOGA J2 3.1
19.13 AC T9 GAC AOD AOD CAC J2 3.1
19.14 AC T9 GAC AOD GOGA J2 3.1
19.16 AC T9 GOGA AOD GOGA J2 3.1
19.18 AC T9 GOGA GOGA J2 3.1
20.20 AC T9 GOGA GOGA GOGA J2 3.1
21.2 AC T9 TAC CAC J2 3.1
20.1 AC T9 TAC CAC J2 3.1
20.4 AC T9 TAC CAC J2 3.1
20.5 AC T9 TAC CAC J2 3.1
20.6 AC T9 TAC CAC J2 3.1
20.13 AC T9 TAC CAC J2 3.1
20.14 AC T9 TAC CAC J2 3.1
20.15 AC T9 TAC CAC J2 3.1

BALB/C d3Tx Challenged Polyclonal:

11.4 AC T9 GOGA GOGA J2 3.1
11.7 AC T9 GAC AOD GOGA AOD AOD J2 3.1
11.8 AC T9 GOGA GOGA J2 3.1
11.9 AC T9 GOGA GOGA J2 3.1
11.10 AC T9 GOGA GOGA J2 3.1
11.11 AC T9 GOGA GOGA J2 3.1
11.12 AC T9 GOGA GOGA J2 3.1
11.13 AC T9 GOGA GOGA J2 3.1
11.14 AC T9 GOGA GOGA J2 3.1
11.15 AC T9 GOGA GOGA J2 3.1
11.16 AC T9 GOGA GOGA J2 3.1
11.19 AC T9 GOGA GOGA J2 3.1
11.20 AC T9 GOGA GOGA J2 3.1
11.24 AC T9 GOGA GOGA J2 3.1
11.27 AC T9 GOGA GOGA J2 3.1
11.28 AC T9 GOGA GOGA J2 3.1
11.29 AC T9 GOGA GOGA J2 3.1
11.30 AC T9 GOGA GOGA J2 3.1
34.2 AC T9 GOGA GOGA J2 3.1
34.4 AC T9 GOGA GOGA J2 3.1
34.20 AC T9 GOGA GOGA J2 3.1

BALB/C d3Tx Challenged MLR:

10.19 AC T9 GOGA GOGA J2 3.1
10.1 AC T9 GOGA GOGA J2 3.1
10.6 AC T9 GOGA GOGA J2 3.1
10.7 AC T9 GOGA GOGA J2 3.1
10.8 AC T9 GOGA GOGA J2 3.1
10.9 AC T9 GOGA GOGA J2 3.1
10.10 AC T9 GOGA GOGA J2 3.1
10.11 AC T9 GOGA GOGA J2 3.1
10.12 AC T9 GOGA GOGA J2 3.1
10.13 AC T9 GOGA GOGA J2 3.1
10.14 AC T9 GOGA GOGA J2 3.1
10.15 AC T9 GOGA GOGA J2 3.1
10.16 AC T9 GOGA GOGA J2 3.1
10.17 AC T9 GOGA GOGA J2 3.1
10.18 AC T9 GOGA GOGA J2 3.1
10.21 AC T9 GOGA GOGA J2 3.1
10.22 AC T9 GOGA GOGA J2 3.1
10.23 AC T9 GOGA GOGA J2 3.1
10.24 AC T9 GOGA GOGA J2 3.1
10.25 AC T9 GOGA GOGA J2 3.1
10.26 AC T9 GOGA GOGA J2 3.1

Figure 6. Fetal-type V\p6 T cells of d3Tx BALB/c are Mls-1 reactive but can be preferentially anergized upon in vivo challenge with DBA/2 splenocytes. cDNA sequences were derived from mRNA of T cell blasts produced in the experimental groups described in Fig. 5. Each set of sequences represents data pooled from three to five mice.
Jones et al. (23), who found that, in DBA/2 mice that were thymectomized no later than day 4 of life, the proportion of V\textsubscript{a}6 T cells in adults is higher than in nonthymectomized animals. An analogous observation was made by Smith et al. (41) on the persistence of V\textsubscript{a}11 T cells in the adult repertoire of B6AF1 mice thymectomized on day 3. Schneider et al. (42) also reported that in Mls-1\textsuperscript{a} mice only a fraction of the V\textsubscript{a}6 thymocytes are deleted during the first days of life, but did not follow the fate of these cells in the periphery. Our data indicate that deletion of self-reactive clonotypes appears in the periphery soon after birth. In day 3 DBA/2 mice, V\textsubscript{a}6 T cells represent only 6% of the total \(\alpha/\beta\) splenic T cell pool, while in BALB/c mice they represented 17% (Fig. 2). Thus, at day 3, 60% of the V\textsubscript{a}6 T cell subset is already deleted in DBA/2 mice. We have investigated whether during the first days of life, deletion equally affects fetal and adult T cell clonotypes. A comparison between DBA/2 and BALB/c shows that the skewing of the DBA/2 V\textsubscript{a}6 peripheral repertoire towards fetal type is already pronounced at day 3 (90% vs. 55%). We interpret this as an indication that in DBA/2 mice deletion operates soon after birth, preponderantly on adult V\textsubscript{a}6 T clonotypes, whereas fetal clonotypes are frequently rendered anergic. This conclusion is enforced by the presence of out-of-frame adult V\textsubscript{a}6 rearrangements in DBA/2 day 3 peripheral T cells, indicating that at this time, adult-type V\textsubscript{a}6 rearrangements are being produced but that a substantial fraction of the T cells expressing in-frame adult V\textsubscript{a}6 rearrangements are deleted.

In adult life, it appears that deletion is the main tolerogenic mechanism. Otherwise, the fetal V\textsubscript{a}6 clonotypes would be diluted out by anergized adult V\textsubscript{a}6 cells that would accumulate in the peripheral T cell pool of DBA/2 mice. In contrast, Fig. 1 shows that V\textsubscript{a}6 adult-type rearrangements have not become predominant in 11-wk-old DBA/2 mice. Moreover, the frequency of V\textsubscript{a}6 fetal sequences in adult \(\alpha/\beta\) BALB/c or DBA/2 mice is almost identical to that present in the day 3 peripheral T cell pool of the corresponding mouse strain (Fig. 3 A). Surprisingly, in \(\alpha/\beta\) DBA/2 mice, the anergic V\textsubscript{a}6 T cell pool expands between day 3 and adulthood. The peripheral expansion of anergic T cells should be antigen independent. Thus, in the expanding V\textsubscript{a}6 T cell pool of \(\alpha/\beta\) DBA/2 mice the usage of the J\textsubscript{a}2.7 gene segment remains unchanged, whereas in \(\alpha/\beta\) BALB/C mice where V\textsubscript{a}6 T cells are not anergic, the usage of this J\textsubscript{a} segment is adjusted in time (Fig. 4). This observation is consistent with the data of Candeias et al. (43) who noticed distinct patterns of J\textsubscript{a} usage between V\textsubscript{a17} T cell clonotypes selected in the presence or in the absence of self-surface class II-IE dimers.

The essential conclusion that emerges from the data commented above is that in DBA/2 mice, V\textsubscript{a}6 fetal T clonotypes are preferentially retained in the anergic pool. One could explain this observation in two ways: First, deletion operates randomly on both groups of clonotypes before day 3 of life,
at a time when fetal VDJ rearrangements are predominant in the V\beta 6 T cell pool. Thus, the high proportion of fetal V\beta 6 clonotypes in day 3 DBA/2 splenocytes would be due to their accumulation in the periphery over a period of several days. However, as an intrinsic part of this hypothesis, one also has to assume that, during the perinatal period, Mls-1\alpha-APC are unable to induce deletion but are capable of inducing anergy in V\beta 6 T cells. Second, the alternate explanation of these observations would be that during the perinatal period, fetal V\beta 6 T cells are anergy prone. In contrast, in the same time period, the V\beta 6 adult clonotypes would be preferentially deleted upon encounter with the self superantigen Mls-1\alpha. Although there is no direct experimental evidence confirming this second hypothesis, we favor it because long-lived V\beta 6 fetal T clonotypes manifest a tendency toward anergy in atTx BALB/c mice, after such mice reach adulthood. This is reflected in the reactivity of V\beta 6 T cells obtained from atTx BALB/C mice, challenged in vivo with Mls-1\alpha (see Results and Figs. 5 and 6). Among such cells only a few (<5%) display fetal V\beta 6 TCR after in vitro selection with Mls-1\alpha stimulator cells. In contrast, polyclonal activation of T cells from the same atTx BALB/C mice shows that 24% of V\beta 6 sequences contain fetal-type rearrangements (Figs. 5 and 6).

While investigating the behavior of fetal (day 19) thymocytes after TCR engagement, Takahama et al. (44) recently determined that early fetal CD4-CD8\alpha thymocytes are affected in their differentiation, but are not subjected to "programmed death" via apoptosis. Similar effects of TCR engagement were obtained with either a defined antigen (D\beta 8 + H-Y) on transgenic T cells or with a standard anti-TCR antibody on polyclonal thymocytes in vitro. Finkel et al. (45) also showed that in the fetal thymus immature thymocytes were protected from depletion early in ontogeny. In these experiments, the triggering of fetal thymocytes was also achieved by crosslinking with anti-TCR and anti-CD3 antibodies, which excluded antigen presentation as a factor determining the differential behavior of early and late thymocytes. In the perspective of our data, we propose that the pattern of behavior detected upon triggering in fetal early thymocytes is typical for fetal cells and not for a stage of differentiation within every T cell lineage. Such behavior could be responsible for the accumulation of the anergic fetal T cells that we detected. On the other hand, as demonstrated by Teh et al. (46), the expression of functional, immunogenic H-Y antigen in the male thymus precedes any deletion of H-Y reactive thymocytes in this organ by at least 2 d. These data point at the fetal thymocyte itself as being responsible for its anergy-prone behavior. They contrast with the lack of any indication that antigen presentation in the fetus could be of a different kind.

If many T cells expressing fetal V\beta 6 rearrangements are Mls-1\alpha anergic, this is probably not due to a distinct structure of their TCR. Fetal and adult \beta polypeptide chains are similar in terms of the length of the CDR3 (Fig. 7). Moreover, no sequence motif specific for either adult or fetal rearrangements has been detected among Mls-1\alpha-stimulated T cells. It is also generally accepted that superantigens bind to a portion of the V\beta which does not include the diversified VDJ junction (13–15). Thus, one should consider an alternate possibility: that the tendency of fetal-type V\beta 6 T cells towards anergy in the perinatal life and, at a lesser extent, even late in adult life is due to their lineage-specific signal transduction properties. Such properties should allow them to respond to TCR signalling by becoming anergic rather than undergoing programmed cell death. This is not an unlikely hypothesis, since we already know that rearranging fetal and adult clonotypes must differ in at least one enzymatic function, namely terminal transferase activity (4–6), reflected in the presence or absence of the N region. As an extension of our view, one could consider that fetal T cell clonotypes retained in the adult peripheral T cell pool could escape from the state of self-tolerance and become self-aggressive. Indeed, this could be the case in some strains of neonatally thymectomized mice which develop autoimmune conditions late in adult life (7, 10). Interestingly, T cells which react to myelin basic protein and participate in the onset of experimental allergic encephalomyelitis in rats, display a well-conserved V\beta DBJ\beta region (CDR3) which could be generated by typical "fetal rearrangements" (47). Such cells could be the progeny of tolerized fetal clonotypes which escaped from anergy after hyperimmunization. One might conclude that compared to deletion, the quality of tolerance achieved via anergy is only second best.

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