In Mls-1^a Mice, Fetal-Type β-Gene Rearrangements Are Frequent among Self-Anergic V$_\beta$6 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 autoimmunity (7–11). One wonders whether, under such circumstances, T cells with fetal TCR rearrangements could be long-lived and contribute to autoaggression.

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 V$_\beta$ TCR genes (for a review see reference 12). This is due to the fact that superantigens bind to TCR in a nonconventional 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-1^a 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-1^b) express 11% V$_\beta$6 T cells in the peripheral T cell pool, whereas mice that do carry this viral sequence (e.g., DBA/2, Mls-1^a) 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-1^a 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-1^a-reactive T cells became anergic (22). A significant proportion of self-Mls-1^a anergic V$_\beta$6 T cells was also detected in adult DBA/2 mice that were thymectomized during the first 4 d of life (23). In such mice, there were more V$_\beta$6 T cells than in unmanipulated animals. In both cases, the anergic V$_\beta$6 T cells could not be activated in vitro by Mls-1^a stimulator cells, or by crosslinking with anti-V$_\beta$6 antibody.

We reasoned that fetal-type V$_\beta$6 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

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/11/1713/12 $2.00
Volume 178 November 1993 1713–1724

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 ($_{d3}$Tx)[1]. In these mice the few adult-type Vβ6 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 Vβ6 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 Vβ6 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 dissection microscope and by the reduction of the α/β 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 μM, Calbiochem Corp.), rIL-1 (10 U/ml, Amgen Biologicals, Thousand Oaks, CA) and rIL-2 (20 U/ml, Amgen). 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 Vβ TCR (25). For MLR, 2×10^6 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 IL-4 (30 U/ml, Amgen) for 24 h before irradiation. T cell blasts were harvested on day 5 from MLR cultures for both flow cytometry analysis 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-1^a 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-Vβ6 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 Vβ6 TCR were performed essentially according to our standard procedures (32). In this case, 5 pmol of an antisense Cβ-specific external primer (5′-CCAAGCACACGAGGGTAGC-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′-GATGGCTCAAACAAGGAGAC-3′) and 10 pmol of a sense Vβ6 specific external primer (5′-AACTGACCTTGAAATGTCAACAG-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 Vβ6 cDNA fragments were purified from low melting agarose gel and cloned into the SmaI site of PUC18 as described (32).

DNA Sequencing. Single colonies of independently derived Vβ6$^+$ 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 supernatant 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 Vβ6-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 μl of the PCR product was added to 40 μl 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 Vβ6 T Cells in Adult DBA/2 Mice Express Fetal-Type TCR Rearrangements. In DBA/2 mice, expression of the self superantigen Mls-1^a results in the deletion of most Vβ6 T cells. Nonetheless, ~1% of all α/β T cells in the periphery are Vβ6 positive. These T cells are anergic: they do not proliferate or produce lymphokines upon stimulation with anti-Vβ6 antibody or syngeneic Mls-1^a 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:* $_{d3}$Tx, mice thymectomized on the 3rd day after birth.

the presence of IL-1 and IL-2 (25). To compare the repertoire of the undeleted Vβ6 T cells in DBA/2 mice with a control population in which Vβ6 T cells were not subjected to negative selection, Vβ6 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 Vβ6 sequences lacked N region added nucleotides—the hallmark of fetal-type gene rearrangement. In contrast, in BALB/c mice, all Vβ6 sequences contained N region added nucleotides typical of the adult-type gene rearrangement (Fig. 1). We have also sequenced the Vβ11 cDNA clones obtained from the same BALB/c mRNA preparations from which we had ob-

	Vβ6	P	N	P	Dβ	P	N	P	Jβ	
BALB/C:										
1.10	AGCAGTATA		GC		ACAGGGG				ACAGAAGTC	Jβ 1.1
1.11	AGCAGTATA		GACG		AGG				CAAACACA	Jβ 1.1
1.17	AGCAGTATA		GGGG		CAGGGG				ACGAAAGA	Jβ 1.4
1.19	AGCAGTATA		CG		CT		C		CTCCTAT	Jβ 2.7
1.20	AGCA		CC	CC	GGGACTGGGG				TATGAACAG	Jβ 2.7
1.42	AGCAGTAT		CC		CAGGG		TTCGGC		CCAGCTCCG	Jβ 1.5
18.8	AGCAGTAT			C	GGGACA		A		AAAACACC	Jβ 2.4
21.1	AGCAGT		GA		GACAGGGGGC				TCCAACGAA	Jβ 1.4
21.6	AGCAGTAT		T		ACAGGG		CG		CACAGAA	Jβ 1.1
21.7	AGCAGTA		CT		AGGG		TCTCTCTTC		CAAACACA	Jβ 1.1
21.9	AGCAGTAT		TAAGGG		CTGGGG		AT	CT	AGTGCAGAA	Jβ 2.3
21.10	AGCAGTAT		C		GGA				AACTCCGAC	Jβ 1.2
21.12	AGCAG		CC		GACAG		AAT		AGTGCAGAACG	Jβ 2.3
21.13	AGCAGT		TCTTCGAC		GGGC		GATAGG		AACTATGCT	Jβ 2.1
21.14	AGCAGTA		TTT		CTGG				AAACACC	Jβ 2.2
21.15	AGCAGT		C		ACAGG				AACTCCGAC	Jβ 1.2
21.17	AGCAGTATA				GTC		AA		TGCAGAACG	Jβ 2.3
21.22	AGCAGTA		AC		GGGGG				TGGAAATACG	Jβ 1.3
21.23	AGCAGT				GGGA		G	T	AACCAAGAC	Jβ 2.5
21.24	AGCAGTATA		C		GGGG			T	AACACCGGG	Jβ 2.2
DBA/2:										
1.2	AGCAGTAT				GACAG				CAAACTCCGAC	Jβ 1.2
1.3	AGCAGT			CC	GGGACAGGGGGC	G			TATGAACAGGAG	Jβ 2.7
1.10	AGCAGTATA				GACA				CCCAGTACTTT	Jβ 2.5
1.11	AGCAGTAT				GGGACAGGG				TGGAAATACG	Jβ 1.3
8.1	AGCAG				ACTGGGGGGG				GCAGAACG	Jβ 2.3
8.2	AGCAGTAT				GGGGGGG				TATGAACAG	Jβ 2.7
8.12	AGCAGTAT				GGGGACTGG				CTCCTATGAA	Jβ 2.7
37.6	AGCAGTA				GGGAC				CAAGACACC	Jβ 2.5
37.8	AGCAGTATA				A			A	TTCTGGAAAT	Jβ 1.3
37.9	AGCAGTAT				GGGACA				AAACTCCGAC	Jβ 1.2
37.12	AGCAGTAT				GGGGG				CAGAAGTCTTC	Jβ 1.1
1.1	AGCAG		GA		GGACAG	C	A		AACTCCGACTAC	Jβ 1.2
1.6	AGCA		GA	CC	GGAC				AGTGCAGAACG	Jβ 2.3
1.7	AGCAGTATA				ACAGGGGG		A		AGTGCAGAATA	Jβ 2.3
1.12	AG		A		GGACAG		T	CT	AGTGCAGAA	Jβ 2.3
8.3	AGCAGT		CC		GGGGGGC		TTC		CAAGACACC	Jβ 2.5
8.6	AGCAGTA		AGA		ACA		T		TAACTAT	Jβ 2.1
8.7	GCCAG				GGGGC	G	A		CAAGACACC	Jβ 2.5
37.1	AGCAGT		CT	CC	GGGACAG		C		GACACCCAG	Jβ 2.5
37.4	GCCAG		AGG	C	GGG				AATTCGCCC	Jβ 1.6
37.5	AGCAGT		CTT		ACT		CTGA		CAAACACAGAA	Jβ 1.1
37.14	AGCAGTAT		T		ACAG				ATAATTCG	Jβ 1.6

Figure 1. Vβ6-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. Vβ6 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 Vβ6 clones in the earlier analysis. In this Vβ11 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 α/β 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 Vβ6 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^a-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 Vβ6 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 Vβ6 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 Vβ6 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 Vβ6 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 Vβ6 T cells on day 3 than later in life. Nonetheless, at day 3, the peripheral pool of Vβ6 T cells in DBA/2 represents <40% of the Vβ6 T cell fraction in BALB/c mice, indicating that many Vβ6 T cells are already deleted in DBA/2 mice at this age. The Vβ6 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 Vβ6 T cells is higher in $_{d3}$Tx DBA/2 than in euthymic mice for a long time (4% compared to 1% at 16 wk).

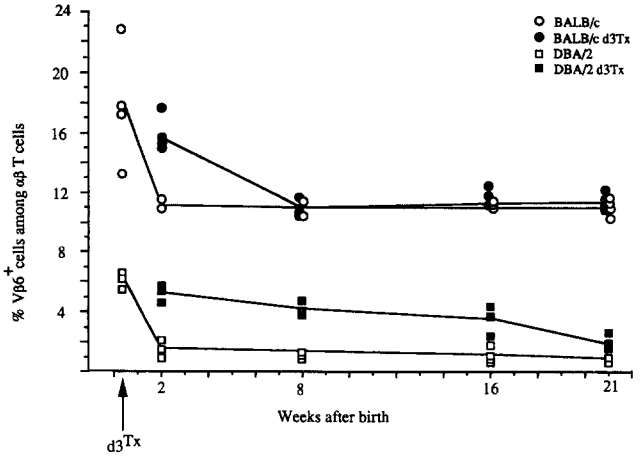
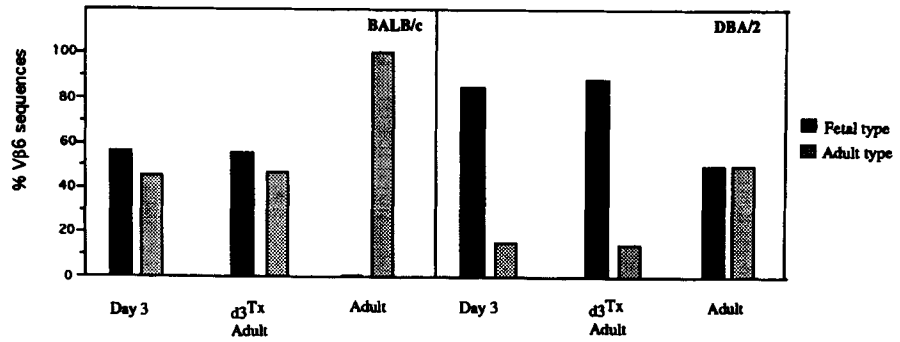


Figure 2. Relative frequency of peripheral Vβ6 T cells in euthymic and $_{d3}$Tx mice at various ages. Splenocytes from 3-d-old mice and LN cells from euthymic and $_{d3}$Tx BALB/c and DBA/2 mice of various ages were analyzed. The percentages of Vβ6 T cells were determined by two-color flow cytometry in cell suspensions prepared ex vivo and, in younger mice, also after culturing with PMA, ionomycin, IL-1, and IL-2 for 5–7 d. This allowed for reliable measurements in situations where the α/β T cell frequency in the initial suspensions was low. For day 3 mice, each measurement was obtained from lymphocytes pooled from five to seven mice, whereas for older mice, measurements were made on lymphocytes from individual animals.

The ratios of fetal to adult-type Vβ6 TCR rearrangements in the neonatal and adult mice were estimated by DNA sequence analysis. For both DBA/2 and BALB/c, the productive Vβ6 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, Vβ6 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 Vβ6 sequences with adult type rearrangements in day 3 DBA/2 mice (not shown), confirming that adult-type Vβ6 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 Vβ6 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 Vβ6 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 Vβ6-D-Jβ cDNA sequences were derived from T cell blasts. (*A*) Percentages of fetal and adult type Vβ6 rearrangements in 3-d-old, adult, and $_{d3}$Tx DBA/2 and BALB/c mice. (*B*) Vβ6-D-Jβ 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.

A



B

	Vβ6	P	N	P	Dβ	P	N	P	Jβ	
BALB/c Day 3 :										
36.2	GCC AGC A				GG GAC TGG GG				CTAT GAA CAG	Jβ 2.7
36.3	AGC A				GG GAC AG				CTAT AAT TGG CCC	Jβ 1.6
36.8	AGC AGT				GAC TGG GGG GGC				TAT GCT GAG	Jβ 2.1
36.12	AGC AGT				GGG ACT GGG GGG				CAA AAC ACC	Jβ 2.4
36.13	AGC AGT ATA				GGG GGG G				AA GAC ACC CAG	Jβ 2.5
36.14	AGC AGT A				ACT GG GGG				GCA GAA ACG CTG	Jβ 2.3
36.15	AGC AG				A CTG GGG				GAA ACG CTG TAT	Jβ 2.3
36.16	AGC AGT ATA				GGG GGC				TCC TAT GAA CAG	Jβ 2.7
36.17	AGC AGT				GGA CAG				GCT GAG CAG TTC	Jβ 2.1
36.25	AGC AGT ATA				GGG ACA GGG GG				T GAA CAG TAC TTC	Jβ 2.7
36.4	AGC		C		GGA		TA AAA A	TT	AAC CAA GAC ACC	Jβ 2.5
36.5	AGC AGT A		CC		GGG GGG				GAA CAG TAC TTC	Jβ 2.7
36.7	AGC AGT				GGG ACA GGG G		AG		GCT GAG CAG	Jβ 2.1
36.9	AGC A		A		A CA		AG		GT CAA AAC ACC	Jβ 2.4
36.10	AGC AGT ATA				GGG ACA GGG GC		A		TAT GCT	Jβ 2.1
36.18	AGC AGT		TTA G		CT GGG GGG				TAT GAA CAG	Jβ 2.7
36.19	AGC AGT ATA		GC		G GAC A				GC TCC TAT GAA	Jβ 2.7
36.20	AGC AGT A		CC C		CA				AAC TCC GAC TAC	Jβ 1.2
36.21	AGC AGT A		CT		CTG GGG GGG C	G	G		GAC ACC CAG TAC	Jβ 2.5
36.24	AGC AGT		C		AC AGG G				AC ACC GGG CAG	Jβ 2.2
BALB/c d3Tx :										
5.1	AGC AGT			CC	G GGA CTG GGG				TAT GAA CAG	Jβ 2.7
5.2	AGC AGT ATA				GGG GGG G				GT CAA AAC	Jβ 2.4
5.4	AGC AG				A CAG G				GT CAA AAC	Jβ 2.4
5.5	AGC AGT ATA				CA				A AAC ACA	Jβ 1.1
5.6	AGC AGT ATA				TGG				GAA CAG TAC	Jβ 2.7
5.11	AGC AG				A CAG GGGG				CA GAA GTC	Jβ 1.1
6.1	AGC AGT A				GG GGG C				CC AAC GAA	Jβ 1.4
6.3	AGC AGT ATA				GGC				AAC TAT GCT	Jβ 2.1
6.5	AGC AGT AT				G GGA CTG GG				CTAT GCT	Jβ 2.1
6.6	AGC AG				G ACA GGG GGC				TAT AAT TGG	Jβ 1.6
6.7	AGC AGT ATA				CAG G				CA AAC ACC	Jβ 2.2
6.9	AGC AGT ATA				GGA CAG GG				T AAT TGG	Jβ 1.6
7.3	AGC AGT				CAG GGG GC	G			CAA AAC ACC	Jβ 2.4
7.7	AGC AGT A				GG GAC A				AC ACA GAA	Jβ 1.1
5.3	AGC AGT		CCT		CTG GGG G		C	T	AGT GCA GAA	Jβ 2.3
5.9	AGC A		CC T		CA GGG G		TT		GTC TTC TTT	Jβ 1.1
5.10	AGC A		CA CAG G		AG GGG G				AT TGG CCC	Jβ 1.6
6.4	AGC AG		C		GGA				TAT GAA CAG	Jβ 2.7
6.8	AGC AGT AT		T		GGG G		T		TGG AAT	Jβ 1.3
7.1	AGC AGT ATA		GC		A CAG GGG				AAC ACA GAA	Jβ 1.1
7.2	AGC AGT ATA		CCC		GGA				AAC CAA GAC	Jβ 2.5
7.4	AGC AGT		CCC GTC C		GG GAC A		C	TG	CA AAC	Jβ 1.1
7.5	AGT A		CT TT		G GGA CTG GGG G				AC CAA GAC	Jβ 2.5
7.6	AGC AG		AA		GG GC				A AAC TCC	Jβ 1.2
7.9	AGC AGT ATA	TA			TG		CC		CAG TAC TTC	Jβ 2.7
7.10	AGC AGT ATA		GT		G GGA CA		A	G	CA AAC TCC	Jβ 1.2

	Vβ6	P	N	P	Dβ	P	N	P	Jβ	
DBA/2 Day 3 :										
9.1	AGC AGT A				GG GAC				AAC ACA GAA	Jβ 1.1
9.11	AGC AGT ATA				GGA CAG GG				T AAT TGG	Jβ 1.6
9.14	AGC AGT A				GA CAG GGG G				CA GAA GTC	Jβ 1.1
12.6	AGC AGT AT				G GGA CAG				CAA GAC ACC	Jβ 2.5
32.2	AGC AGT AT				G GGC				AAC TAT GCT GAG	Jβ 2.1
32.6	AGC AGT AT				GGA				TTCT GGA AAT ACG	Jβ 1.3
32.7	AGC AGT				GAC AGG GG				A GAA GTC TTC	Jβ 1.1
32.11	AGC AGT ATA				GGA CAG GGG GC				C GAA AGA TTA	Jβ 1.4
32.12	AGC AGT ATA				GGA CAG				TAT GCT GAG CAG	Jβ 2.1
32.16	AGC AGT AT				T GGG GG				T GAA CAG TAC TTC	Jβ 2.7
32.17	AGC AGT				TGG GGG GGC	G			CC AAC GAA AGA	Jβ 1.4
32.21	AGC AGT				GAC AGG				TCC GAC TAC ACC	Jβ 1.2
32.26	AGC AGT ATA				GGG GGC				TCC GAC TAC ACC	Jβ 1.2
32.28	AGC AGT ATA				GGA CA				CTAT GCT GAG CAG	Jβ 2.1
32.31	AGC AGT ATA				CAG				TAT GAA CAG TAC	Jβ 2.7
32.32	AGC AGT ATA				CTG				TAT GAA CAG TAC	Jβ 2.7
32.33	AGC AGT AT				G GAC AG				CTCC TAT GAA CAG	Jβ 2.7
32.8	AGC AG			C CC	CTGG GGG GG				CTAT GCT GAG	Jβ 2.1
32.30	AGC AG			CT	CC GGG ACA GGG GGC				TAT GAA CAG TAC	Jβ 2.7
32.34	AGC A				CC GGG ACT GGG GG			T	TAT GAA CAG	Jβ 2.7
DBA/2 d3Tx:										
2.2	AGC AGT				GGG ACT GGG G				CC TAT GAA	Jβ 2.7
2.3	AGC AGT				T AC				CAA GAC	Jβ 2.5
2.4	AGC AGT AT				G GGG GG				TGAA CAG	Jβ 2.7
2.7	AGC AGT ATA				CTG GG				TGCT GAG	Jβ 2.1
2.8	AGC AGT AT				G GGA CAG GG				TGGA AAT	Jβ 1.3
2.9	AGC AGT ATA				GGG ACT				AGT CAA AAC	Jβ 2.4
2.13	AGC AGT A				AC				TAT GCT GAG	Jβ 2.1
2.14	AGC AG				A CTG GGG GGG C	GC			GAA CAG TAC	Jβ 2.7
2.16	AGC AGT ATA				GAC AG				A AAC ACA	Jβ 1.1
2.18	AGC AGT AT				G GG				TGAA CAG	Jβ 2.7
2.19	AGC AGT ATA				GGG GGC				GAA CAG TAC	Jβ 2.7
2.20	GCC AG				A GGG GGC				TCC GAC TAC	Jβ 1.2
2.21	AGC AGT				ACA				AAC ACA GAA	Jβ 1.1
2.22	AGC AGT AT				G GAC TGG GGG				TAT GAA CAG	Jβ 2.7
3.2	AGC AGT ATA				GGA C				ACT AT GCT	Jβ 2.1
3.3	AGC AGT				CAG GGG GGC				AAC TCC GAC	Jβ 1.2
3.4	AGC AGT A				GG GAC TGG GGG				GAC ACC CAG	Jβ 2.5
3.5	AGC AG				G GGG GGC				GAA CAG TAC	Jβ 2.7
3.6	AGC AGT ATA				CAG GG			T	AAC CAA GAC	Jβ 2.5
3.7	AGC A				GG GAC AGG GG				A AAC TCC GAC	Jβ 1.2
4.1	AGC AGT AT				G GAC A				CA AAC ACA	Jβ 1.1
4.3	AGC AGT A				AG G				AG TAC TTC	Jβ 2.7
4.6	AGC AGT AT				G GGG G				CC TAT AAT	Jβ 1.6
4.7	AGC AGT AT				G GGG				TAT GAA CAG	Jβ 2.7
4.8	AGC AGT AT				G GGA CAG GG				CTCC GAC	Jβ 1.2
2.1	AGC AGT			TTA	ACA				CAA CCA GCT	Jβ 1.5
2.5	AGC AGT ATA			AC	GGC				A AAC TCC	Jβ 1.2
2.11	AGC AGT ATA			G	C G GGA CA				A AAC ACA	Jβ 1.1

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 Vβ6 rearrangements were deleted, whereas the fetal type Vβ6 T cells were preferentially retained.

Fetal Vβ6 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 $_{d3}$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 Vβ6 T cell pool present in the periphery at the time of thymectomy had expanded ~20–25-fold in $_{d3}$Tx BALB/c. In DBA/2, although the percentage of Vβ6 T cells decreased from 6% at day 3 to 4% in 8-wk-old $_{d3}$Tx mice, the absolute number of Vβ6 T cells increased ~10–15-fold during that time. The proportion of fetal- and adult-type Vβ6 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 Vβ6 cDNA clones derived from 16-wk-old BALB/c nude (nu/nu) mice. In such mice, there are essentially no detectable α/β T cells at birth. Thus, the α/β 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 Vβ6 rearrangements were detected (data not shown). This shows that fetal Vβ6 T cells detected in the adult $_{d3}$Tx mice are indeed long-lived, and represent expanded descendants of T clonotypes that rearranged their TCR elements during the perinatal period. Vβ6 T cells of $_{d3}$Tx DBA/2 mice cannot be induced to proliferate by either Mls-1^a stimulator cells or by crosslinking with anti-Vβ6 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 Jβ 2.7 usage in the Vβ6 TCR pool. As shown in Fig. 4, in both DBA/2 and BALB/c, 30% of Vβ6 T cells present before thymectomy (day 3) used the Jβ 2.7 gene segment. The fraction represented by this subset remained unchanged in adult $_{d3}$Tx DBA/2. However, in adult $_{d3}$Tx BALB/c mice, the fraction of Jβ 2.7$^+$ Vβ6 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 Vβ6 T Cells Are Mls-1^a Reactive but Can Be Preferentially Rendered Anergic by In Vivo Challenge with DBA/2 Splenocytes. To ascertain that fetal-type Vβ6 T cells exhibit a TCR which accommodates interactions with Mls-1^a, we set up MLR, in which the responder cells were from $_{d3}$Tx BALB/c (H-2^d, Mls-1^b) which contained equal proportions of fetal- and adult-type Vβ6 T cells. Activated DBA/2 B cells (H-2^d, Mls-1^a) were used as stimulators. The proliferative responses of Vβ6 T cells were estimated by their enrichment in culture (Fig. 5). It appeared that Vβ6 T cells of $_{d3}$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 Vβ6 T cells from $_{d3}$Tx BALB/c mice were Mls-1^a reactive. Moreover, among the Vβ6 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 Vβ6 T cells were capable of reacting to the Mls-1^a superan-

Table 1. *Vβ6 T Cells from DBA/2 $_{d3}$Tx Mice Cannot Be Stimulated by Either Mls-1^a B Cell Blasts or by Crosslinking with Anti-Vβ6 Antibody*

	Percentage Vβ6$^+$ T cells among α/β T cells		
	Medium alone	Anti-DBA/2 MLR	Anti-Vβ6 antibody
BALB/c $_{d3}$Tx	13	60	67
DBA/2 $_{d3}$Tx	3	1	6

BALB/c $_{d3}$Tx and DBA/2 $_{d3}$Tx LN cells (3 × 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-Vβ6 mAbs (RR 4-7). 5 d later, the percentage of Vβ6$^+$ T cells was estimated by two-color flow cytometry.

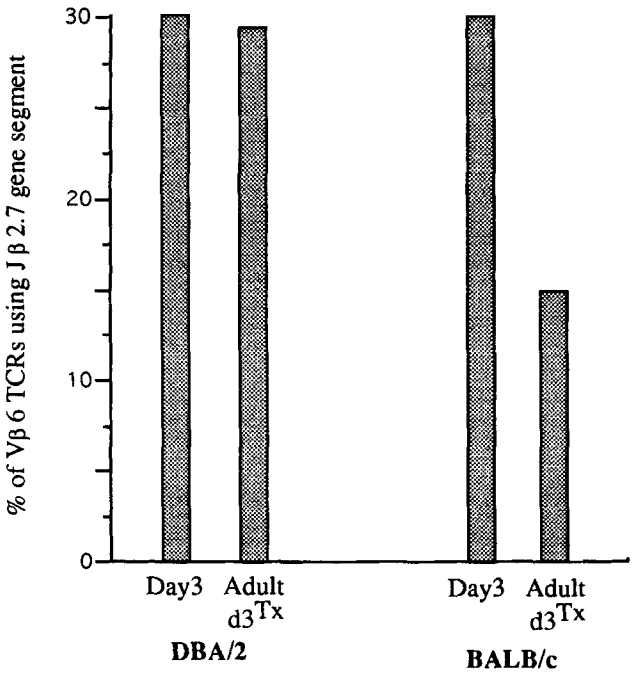


Figure 4. Utilization of Jβ 2.7 gene segment in Vβ6 T cells. The percentages of Jβ 2.7 containing sequences were calculated from the data presented in Fig. 3 *B*. 20–28 sequences were analyzed for each group.

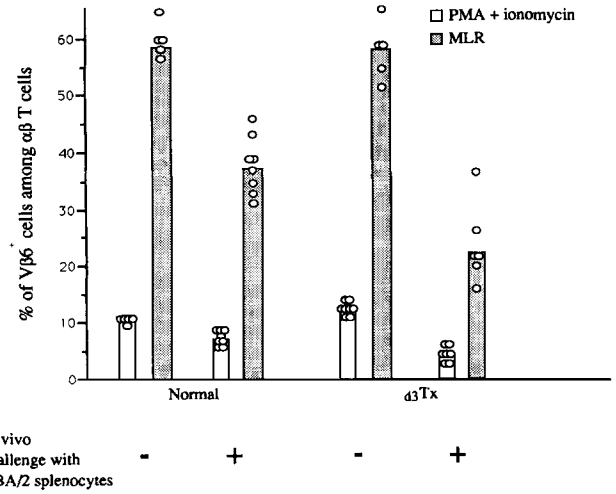


Figure 5. Enrichment of BALB/c $V_\beta 6$ T cells in anti–DBA/2 MLR. 8-wk-old, euthymic and $_{d3}$Tx BALB/c mice were challenged with an intravenous injection of 1.5×10^7 live splenocytes from DBA/2 mice. 3 wk later, LN cells were prepared from challenged and unchallenged mice. Cells were either polyclonally activated with PMA and ionomycin, and expanded in the presence of IL-1 and IL-2 for 7 d or stimulated in MLR with irradiated DBA/2 cells for 5 d. Percentages of $V_\beta 6$ T cells were determined by two-color flow cytometry. (○) Measurements for individual mice; columns represent mean values for each group, as indicated.

tigen. Therefore, the persistence of numerous fetal $V_\beta 6$ T clonotypes in $_{d3}$Tx or euthymic DBA/2 mice was not due to the structural inability of their TCR to recognize Mls-1^a.

Because the sequence analysis of $V_\beta 6$ rearrangements in DBA/2 mice (Fig. 3) suggested that fetal clonotypes could have been anergy prone, we asked whether this tendency could be conserved among fetal clonotypes in adult $_{d3}$Tx BALB/c mice. To investigate this, we took advantage of the fact that a partial tolerance to Mls-1^a could be achieved in BALB/c mice injected with DBA splenocytes (22). The in vivo exposure to Mls-1^a antigen induced a series of changes in the host antigen-reactive $V_\beta 6$ T cell pool, beginning with an expansion between days 2 and 4 after injection, followed by a progressive deletion. 2–3 wk after injection, the fraction of $V_\beta 6$ T cells was substantially reduced (20). In our experiments, live DBA/2 splenocytes were injected in both euthymic and $_{d3}$Tx BALB/c mice. 3 wk later we measured: (*a*) the fraction of $V_\beta 6$ T cells among α/β T cells, (*b*) their Mls-1^a–specific reactivity, (*c*) the ratio of fetal- to adult-type TCR. As shown in Fig. 5, this treatment resulted in a decrease of host $V_\beta 6$ T cell frequency, in both euthymic and $_{d3}$Tx BALB/c mice. The decrease was marked and consistent in thymectomized hosts, in agreement with the observation of Webb et al. (20). In $_{d3}$Tx BALB/c mice, both fetal and adult clonotypes were present among the remaining $V_\beta 6$ T cells which were not deleted after in vivo challenge (Fig. 6). However, the fraction of fetal-type $V_\beta 6$ T cells (24%) was less than in unchallenged mice (54%). There are two possible explanations for this difference. There could be a preferential deletion of the fetal clonotypes in the adult environment. This is unlikely because the data from adult DBA/2 mice indicate that there is no preferential deletion of fetal clonotypes in either newborn or adult animals (Fig. 3). Alternatively, if during the in vivo response, the progeny of Mls-1^a-reactive fetal clonotypes were preferentially inactivated and failed to proliferate further, they would finally represent a smaller fraction of the remaining $V_\beta 6$ T cells. When we tested the reactivity of the remaining $V_\beta 6$ T cells to Mls-1^a, it appeared that indeed, a vast majority of the fetal clonotypes were anergic. Although $V_\beta 6$ T cells were enriched four- to fivefold after 5 d in MLR (Fig. 5), only one fetal-type rearrangement was present among the 22 sequences derived from day 5 MLR blasts (Fig. 6). Thus, these results suggest that anergy to Mls-1^a antigen could be preferentially induced in fetal T cell clonotypes in the adult environment.

CDR3 Regions of Fetal-Type $V_\beta 6$ T Cells. Diversity within a given set of V_β TCR is achieved by (*a*) combinatorial use of different $J\beta$ and $D\beta$ gene segments; (*b*) nucleotide deletions at the $V\beta$-$D\beta$ and $D\beta$-$J\beta$ junctions; (*c*) addition of "P" nucleotides, and (*d*) addition of non–germline-encoded N nucleotides at the above junctions (for a review see reference 3). Although the fetal-type sequences lack N nucleotides, there is no substantial difference in the degree of combinatorial usage of germline gene elements, or the extent of deletions observed between the fetal- and adult-type sequences analyzed in this study. In addition, >90% of the sequences from either set contain between 8 to 12 amino acid residues in the CDR3 region (Fig. 7). There is no significant difference in the length of the CDR3 region between the fetal- and adult-type $V_\beta 6$ rearrangements. Thus, we concluded that despite the lack of untemplated N nucleotides, T cells that exited the thymus carrying fetal-type $V_\beta 6$ TCR rearrangements expressed TCR β chains which did not differ significantly from their adult counterparts.

Discussion

The generation of T cells that bear fetal-type TCR rearrangements was first observed in the γ/δ T cell pool (32, 34–38). Many of the fetal γ/δ T cells display invariant TCR sequences and are partitioned in various epithelial tissues. Moreover, for the dendritic epidermal cells of the skin, the canonical $V\gamma5/J\gamma1$ fetal rearrangement occurs only in fetal T cell precursors which differentiate in a fetal thymus (39, 40). Recently, it was shown that for α/β 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 α/β T cells, or their fate in the normal animal. As a matter of fact, it is not clear whether α/β T cells with fetal-type rearrangements do exit the thymus and become functional lymphocytes. Data presented here indicate that fetal-type α/β T cells exist as functional immunological entities in the periphery, that they are similar to fetal-type γ/δ 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 $V_\beta 6$ T cell pool subjected to incomplete depletion by the self superantigen

	Vβ6	P	N	P	Dβ	P	N	P	Jβ	
BALB/C $_{d3}$Tx MLR:										
19.2	AGC AG				G GGA CAG				AAC TAT GCT	Jβ 2.1
19.4	AGC AGT				GAC AGG GG				TGAA CAG	Jβ 2.7
19.5	AGC AGT AT				GGGGG				CT GGA AAT	Jβ 1.3
19.15	AGC AGT ATA				GAC AGG				AAC TCC GAC	Jβ 1.2
19.19	AGC AGT ATA				GGG				TAT GAA CAG	Jβ 2.7
20.3	AGC AGT AT				G GGC GGG				AAC CAA GAC	Jβ 2.5
20.7	AGC AGT ATA				CTG GGG GGG C	GC			CAA AAC ACC	Jβ 2.4
20.9	AGC AGT			CC	GGGA				AAC TCC GAC	Jβ 1.2
20.10	AGC AGT A				AC AGG GC				A AAC TCC	Jβ 1.2
20.11	AGC AGT AT				GGGG				TAT GCT GAG	Jβ 2.1
19.3	AGC AGT A		GT A		GG GGC	G			GT CAA AAC	Jβ 2.4
19.13	AGC AGT A				AG GGG		AAC GTG GG		A AAC ACC	Jβ 2.4
19.14	AGC AGT ATA		C		GG GG				A AAC ACA	Jβ 1.1
19.16	AGC AGT AT		G AAA		AGG GGG C		A		TGGA AAT	Jβ 1.3
19.18	AGC AGT A		G	C	GGG		T	CT	AGT GCA GAA	Jβ 2.3
19.20	AGT		CCC G		GG GAC TGG GGG G				GT CAA AAC	Jβ 2.4
19.21	AGC AG		A		GGA CTG GGG G			TT	AAC CAA GAC	Jβ 2.5
20.2	AGC		TCC T		AC TGG GG		CT		CA AAC ACC	Jβ 2.2
20.4	AGC AT			CC	GG GAC AGG GGG		AAG GAC		CAA CCA GCT	Jβ 1.5
20.5	AGC AGT		C	CC	GGG ACA GGG		AT		TGGA AAT ACG	Jβ 1.3
20.8	AGC AGT ATA		CGG T		GG GA		G ACG		GCA GAA ACG	Jβ 2.3
20.13	AGC AGT		CCC C		GG ACA GGG				CAA AAC ACC	Jβ 2.4
20.14	AGC AGT AT		G A		AC AGG GGG C			A	TGGA AAT	Jβ 1.3
20.15	AGC AGT AT		C TC		G GAC		GT		C ACA GAA	Jβ 1.1
BALB/C $_{d3}$Tx Challenged Polyclonal:										
11.4	AGC AG				G GGC	G			AA AAC ACC	Jβ 2.4
11.7	AGC AGT A				AC AGG GGC				GAA AGA TTA	Jβ 1.4
11.18	AGT				GGGG			TT	AAC CAA GAC	Jβ 2.5
11.25	AGT AG				AG				GT CAA AAC	Jβ 2.4
11.27	AGC AGT			C	GG GAC A			TA	CAA CCA GCT	Jβ 1.5
34.8	AGC AGT ATA				CTG GGG GGG C				AT GAA CAG TAC	Jβ 2.7
34.12	AGC AGT ATA				GGA				TAT AAT TCG	Jβ 1.6
11.1	AGC AGT AT		C		AC		C	G	CA AAC ACA	Jβ 1.1
11.3	AGC AGT AT		CC		GG GG				CTAT GAA	Jβ 2.7
11.5	AGC AGT		C		GG ACA GGG GGG				CAA GAC ACC	Jβ 2.5
11.6	AGT				GGG GC		T		TAT GAA CAG	Jβ 2.7
11.8	AGC AGT		CC		C AGG GGG COC	G	G		A GAC ACC	Jβ 2.5
11.10	AGC AGT		CCC		ACA		ATA		AAC GAA AGA	Jβ 1.4
11.11	AGC AGT AT		TC		GG ACA G			CT	AGT GCA GAA	Jβ 2.3
11.12	AGC AGT A		GT		GGA CAG		CA		C AAC GAA	Jβ 1.4
11.13	AGC AGT		CCA	CC	G GGA CAG		AAGG		AT TCG CCC	Jβ 1.6
11.14	AGC A		T	C	GGG AC		TTT		T AAC TAT	Jβ 2.1
11.15	AGC AGT A		GC		GGGG				CA AAC ACA	Jβ 1.1
11.16	GCC A		TC TC		G GGG		A	A	T AAC TAT	Jβ 2.1
11.19	AGC A		CC TT	CC	GG GAC A		T		C TCC TAT	Jβ 2.7
11.20	AGC AGT AT		T		GGA CTG G		AG		TAT GCT GAG	Jβ 2.1
11.26	AGC AGT A		C		G GAC AGG G		AG AAG GCT AGG		AGT GCA GAA	Jβ 2.3
11.28	AGC AG				A CAG GGG		A		AA GAC ACC	Jβ 2.5
11.29	AGC AGT A		A	CC	GG GAC				AAC CAA GAC	Jβ 2.5
11.30	AGC AGT AT		T		GGGG			A	T AAC TAT	Jβ 2.1
34.2	AGC AGT ATA		AA		A CTG GGG GGG C		TAC		AT GCT GAG	Jβ 2.1
34.10	AGC AGT ATA		GG		A GGG GGC				TAT GCT GAG	Jβ 2.1
34.14	AGC AGT ATA				CA		AA		AC ACC CAG TAC	Jβ 2.5
34.20	AGC AGT A				GC		A	A	T AAC TAT GCT	Jβ 2.1
BALB/C $_{d3}$Tx Challenged MLR:										
10.19	AGC AGT A				AC A				CC TAT GAA	Jβ 2.7
10.1	AGC AGT		CT	CC	GG GAC AGG G		TT		TAT GAA CAG	Jβ 2.7
10.5	AGC AGT AT			C	GGG ACA GGG G		AGC		CA GAA GTC	Jβ 1.1
10.6	AGC AGT		CCC		GGG			A	TT TCC AAC	Jβ1.4
10.7	AGC A		AG GGA GC		GGGG G		AG		GAC ACC CAG	Jβ 2.5
10.8	AGC AGT ATA		GGT		GGG C		GT		TAT GAA CAG	Jβ 2.7
10.9	GCC AG		G TCC ACC		GGC		GG	T	AAC CAA GAC	Jβ 2.5
10.10	AGC A		TG GC		GGGG		AAG GG		CTAT AAT	Jβ 1.6
10.11	AGC AGT		CC		G AC		C TTT		AGT CAA AAC	Jβ 2.4
10.12	TGT GCC A		T		C TGG GGG		AA		T AAC TAT	Jβ 2.1
10.13	AGC AGT		G		CA G				TT TCC AAC	Jβ 1.4
10.14	AGC AGT AT		C		GAC AGG G				AT AAT TCG	Jβ 1.6
10.15	AGC AGT		CCT	CC	G GGA CAG GG		T		TAT GAA CAG	Jβ 2.7
10.16	AGC		CGT ACG ACT CAT		GGGG				CT GGA AAT	Jβ 1.3
10.17	AGC A		CC A		GA CAG GGG GC				A AAC TCC	Jβ 1.2
10.18	AGC AG		C C		GG GC		C TTT		CAA GAC ACC	Jβ 2.5
10.21	AGC AG		C TCC		CTG GGG G		T		C CAA GAC	Jβ 2.5
10.22	TGT GCC A		TC TC		GGGG		A	A	T AAC TAT	Jβ 2.1
10.23	AGC AG		C C		GG ACA GGG G				AC TCC GAC	Jβ 1.2
10.24	AGC AGT AT		T		ACT GGG GG		A		TAT GCT GAG	Jβ 2.1
10.25	AGC AG		ATGG		GGG ACA G		TA AA		T TCT GGA	Jβ 1.3
10.26	AGC AGT		CCT	CC	G GGA CAG GG		T		TAT GAA CAG	Jβ 2.7

Figure 6. Fetal-type Vβ6 T cells of $_{d3}$Tx BALB/c are Mls-1^a 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.

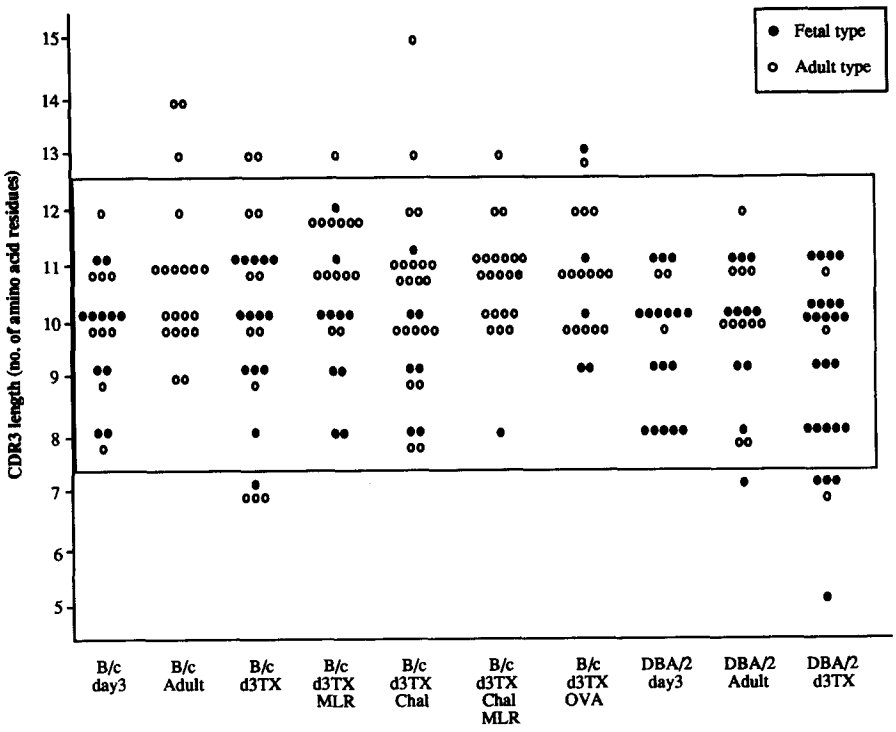


Figure 7. CDR3 lengths of fetal- and adult-type $V_\beta 6$ rearrangements. The V_β-D_β-J_β junctional region was considered as the CDR3 region of the TCR β chain, based on the homologies with the V_H region of Ig (see reference 48). The border limits of this region accommodate all the junctional variabilities observed in this study: between amino acid positions 94 in the $V_\beta 6$ segment and 106 in the J segment. At position 93 there is a conserved alanine in all of the sequences, and the amino acid residues after position 106 are part of the framework region beginning with the FGxG motif present in 11 of the 12 J_β gene segments.

Mls-1^a, T cells of fetal origin are retained in the adult peripheral T cell pool. Thus, in 11-wk-old DBA/2 mice, 50% of the peripheral $V_\beta 6$ clones show typical fetal rearrangements, whereas in BALB/c mice of the same age, the peripheral $V_\beta 6$ T cell pool consists essentially of adult type rearrangements (Fig. 1). Two conclusions can be drawn from this observation: (*a*) that fetal T cell clonotypes can be long-lived cells, and (*b*) that in the neonatal period the transition to an anergic state is a frequent event leading to self-tolerance. The second conclusion is in line with the observations of Jones et al. (23), who found that, in DBA/2 mice that were thymectomized no later than day 4 of life, the proportion of $V_\beta 6$ T cells in adults is higher than in nonthymectomized animals. An analogous observation was made by Smith et al. (41) on the persistence of $V_\beta 11$ T cells in the adult repertoire of B6AF1 mice thymectomized on day 3. Schneider et al. (42) also reported that in Mls-1^a mice only a fraction of the $V_\beta 6$ 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, $V_\beta 6$ T cells represent only 6% of the total α/β splenic T cell pool, while in BALB/c mice they represented 17% (Fig. 2). Thus, at day 3, 60% of the $V_\beta 6$ 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 $V_\beta 6$ 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 $V_\beta 6$ T clonotypes, whereas fetal clonotypes are frequently rendered anergic. This conclusion is enforced by the presence of out-of-frame adult $V_\beta 6$ rearrangements in DBA/2 day 3 peripheral T cells, indicating that at this time, adult-type $V_\beta 6$ rearrangements are being produced but that a substantial fraction of the T cells expressing in-frame adult $V_\beta 6$ rearrangements are deleted.

In adult life, it appears that deletion is the main tolerogenic mechanism. Otherwise, the fetal $V_\beta 6$ clonotypes would be diluted out by anergized adult $V_\beta 6$ cells that would accumulate in the peripheral T cell pool of DBA/2 mice. In contrast, Fig. 1 shows that $V_\beta 6$ adult-type rearrangements have not become predominant in 11-wk-old DBA/2 mice. Moreover, the frequency of $V_\beta 6$ fetal sequences in adult $_{d3}$Tx 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 $_{d3}$Tx DBA/2 mice, the anergic $V_\beta 6$ T cell pool expands between day 3 and adulthood. The peripheral expansion of anergic T cells should be antigen independent. Thus, in the expanding $V_\beta 6$ T cell pool of $_{d3}$Tx DBA/2 mice the usage of the Jβ2.7 gene segment remains unchanged, whereas in $_{d3}$Tx BALB/C mice where $V_\beta 6$ T cells are not anergic, the usage of this J_β 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_β usage between $V_\beta 17^+$ 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, $V_\beta 6$ 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^a-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^a. 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 $_{d3}$Tx BALB/c mice, after such mice reach adulthood. This is reflected in the reactivity of $V_\beta 6$ T cells obtained from $_{d3}$Tx BALB/C mice, challenged in vivo with Mls-1^a (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^{a+} stimulator cells. In contrast, polyclonal activation of T cells from the same $_{d3}$Tx 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 $CD4^-CD8^{lo}$ 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^b + 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 deletion 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^a anergic, this is probably not due to a distinct structure of their TCR. Fetal and adult β 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^a–stimulated T cells. It is also generally accepted that superantigens bind to a portion of the V_β 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 $V\beta D\beta J\beta$ 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.

We are grateful to K. Karjalainen and R. Palacios for their critical reading of the manuscript.

This work was supported by National Institutes of Health grant to A. Augustin (RO1 AI 19775). The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd., Basel.

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Received for publication 16 April 1993 and in revised form 21 July 1993.

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