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Selection of γδ T cells with canonical T-cell antigen receptors in fetal thymus

(T-cell receptor γδ/mouse/positive selection/polymerase chain reaction)

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ABSTRACT **Two γδ T-cell subsets that are generated in the fetal thymus and selectively localize in epidermis and uterus–vagina–tongue epithelia exhibit remarkable homogeneity in their (T-cell) antigen receptors (TCR). In the present study, we show that cells expressing the canonical γδ TCR are also generated in fetal thymus organ cultures. Treatment of these cultures with anti-γδ TCR antibodies did not prevent γδ T-cell development but led to a striking increase in the frequency of noncanonical in-frame sequences. We conclude from this finding that cells expressing the canonical TCR accumulate selectively as a result of TCR-mediated positive selection in the fetal thymus.**

Previous studies have shown the sequential appearance of distinct γδ T-cell subsets in mouse ontogeny. The first two subsets to appear are unusual in that the vast majority of cells in each subset expresses one particular T-cell antigen receptor (TCR), which contains $V_{\delta 1}$–$J_{\delta 2}$-encoded and either $V_{\gamma 5}$–$J_{\gamma 1}$- or $V_{\gamma 6}$–$J_{\gamma 1}$-encoded variable and joining regions (1–3). While the in-frame junctions of the *V* gene segments encoding these regions exhibit almost complete homogeneity in the fetal thymus, the corresponding out-of-frame junctions were found to be more diversified (3). Therefore, we assumed that the predominance of the canonical sequences is not due to any form of instructional, sequence-specific gene rearrangements (4). If our assumption is correct we ought to find also diversified in-frame sequences in fetal thymocytes, if the accumulation of cells expressing the canonical TCR could be prevented. In the present study, we show that cells expressing the canonical γδ TCR are also generated in fetal thymus organ cultures. Treatment of these cultures with anti-γδ TCR antibodies did not prevent γδ T-cell development but led to a striking increase in the frequency of noncanonical in-frame sequences. We conclude from this finding that cells expressing the canonical TCR accumulate selectively as a result of TCR-mediated positive selection in the fetal thymus.

MATERIALS AND METHODS

Animals and Antibodies. C57BL/6J mice were purchased from The Jackson Laboratory. Anti-γδ TCR monoclonal antibody (mAb) 3A10 has been described (5). Anti-αβ TCR mAb H57-597 (6) and anti-CD3 mAb 2C11 (7) were gifts from Ralph Kubo and Jeff Bluestone, respectively. (CD3 is the invariant protein complex associated noncovalently in the TCR with the polymorphic glycosylated polypeptide chains α and β; it is also associated in T-cell subsets with a heterodimer of polymorphic γ and δ chains.)

Thymus Organ Culture. Thymuses were dissected under sterile conditions from C57BL/6J fetal mice at day 14.5 of gestation. The thymus lobes were cultured according to methods described by others (8) with minor modifications. Briefly, four to eight individual lobes were placed on a nitrocellulose filter (pore size, 3 μm; Nucleopore) supported by Collagen sponge (Helistat, United States Surgical) in 2 ml of RPMI 1640 medium (GIBCO) containing 10% (vol/vol) fetal calf serum, 25 mM Hepes, 4 mM glutamine, and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml) in a 6-cm dish. The antibodies were purified by using a protein A affinity column and were added at the beginning of the cultures at a concentration of 50–100 μg/ml. After being cultured for 3–7 days at 37°C in a 7% CO_2/93% air incubator, the lobes were rinsed with large volumes of the culture medium without antibodies, and single-cell suspensions were made by scrubbing the lobes with nylon meshes. The cells were washed and incubated at 37°C for 1 hr (5 hr in some experiments) in the culture medium containing recombinant interleukin 2 (100 units/ml) and recombinant interleukin 1 (2 units/ml) to allow the reexpression of the modulated TCR (9).

Flow Cytometric Analysis. Flow cytometric analyses were performed as described (5) by using FACScan with a software of FACSCAN (Becton Dickinson). The cells were stained with fluorescein isothiocyanate (FITC)-conjugated 2C11 (anti-CD3), and biotin-conjugated 3A10 (anti-γδ TCR) or biotin-conjugated H57-597 (anti-αβ TCR), followed by phycoerythrin (PE)-conjugated streptavidin (Becton Dickinson). As a control, the cells were stained with FITC-conjugated goat anti-hamster IgG (Caltag) and with PE-conjugated streptavidin.

Polymerase Chain Reaction (PCR) and Nucleotide Sequence Analysis. Total DNA was extracted from the cell suspensions and recovered by ethanol precipitation with sonicated salmon sperm DNA as a carrier. The DNA was digested with *Eco*RI and subjected to PCR (10). The DNA from 5×10^4 thymocytes and 5 μg of salmon DNA was made up to 100 μl in a reaction mixture [10 mM Tris·HCl buffer, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/100 pmol of both primers/0.25 mM each dNTP/0.01% gelatin/2.5 units of *Thermus aquaticus* (*Taq*) polymerase]. Thirty PCR cycles were run with 1 min at 92°C, 2 min at 50°C for γ-chain genes or at 45°C for δ-chain genes, and 3 min at 72°C per cycle. The PCR products were separated by polyacrylamide gel electrophoresis, extracted, and cloned into the *Sma* I site of vector pUC13. The clones containing the rearranged TCR gene segment were identified by a colony hybridization technique with oligonucleotide probes that were end-labeled with ^{32}P. The cloned DNA was sequenced by the dideoxy chain-termination method using a kit of Sequenase version 2.0 (United States Biochemical). The PCR primers used have been described (3).

RESULTS AND DISCUSSION

We first studied the development of cells in cultures of fetal thymus lobes from 14.5-day-old C57BL/6 embryos. In the

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Abbreviations: TCR, T-cell antigen receptor; mAb, monoclonal antibody; PCR, polymerase chain reaction; V, variable; J, joining.

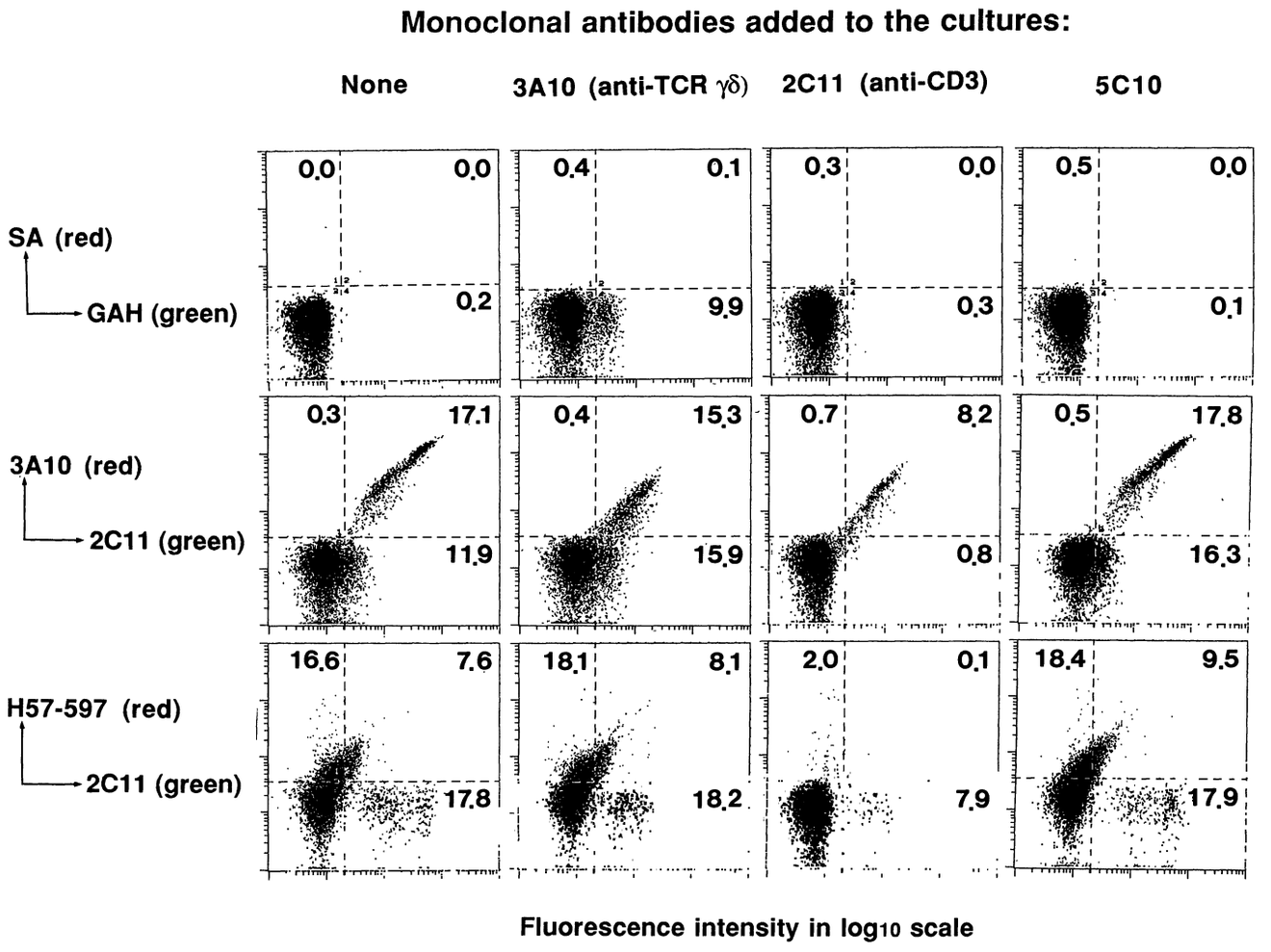

FIG. 1. Two-color flow cytometric analysis of T cells from mouse fetal thymuses that have been cultured in the absence or presence of various antibodies. The percentage of cells is shown in three of the four quadrants. SA, phycoerythrin-conjugated streptavidin; GAH, fluorescein isothiocyanate-conjugated goat anti-hamster IgG.

first 4–5 days, the total cell number continuously increased up to 2.5×10^5 cells per lobe and then remained constant for at least 7 more days. Cells expressing $\alpha\beta$ TCR or $\gamma\delta$ TCR developed as in age-matched thymuses *in vivo* (data not shown) except that the proportion of $\alpha\beta$ TCR-expressing cells remained slightly lower *in vitro*. We then examined the development of T cells in cultures to which we added mAb directed against all $\gamma\delta$ TCR (mAb 3A10) (5), all $\alpha\beta$ TCR (mAb

Table 1. The effects of mAbs on the development of thymocytes *in vitro*

Exp.	Incubation time, days	mAb treatment	Cells per lobe,* no. $\times 10^{-4}$ (% of control)		
			Total	$\gamma\delta$ TCR$^+$	$\alpha\beta$ TCR$^+$
1	3	None	9.3 (100%)	1.6 (100%)	2.2 (100%)
		3A10	9.0 (97%)	1.5 (94%)	2.4 (109%)
		2C11	7.5 (80%)	0.6 (38%)	0.1 (5%)
		5C10	9.8 (105%)	1.7 (106%)	2.6 (118%)
2	5	None	23.3 (100%)	1.4 (100%)	10.8 (100%)
		3A10	23.8 (102%)	1.3 (93%)	10.4 (96%)
		2C11	15.0 (64%)	0.6 (43%)	0.3 (3%)
		5C10	22.2 (94%)	1.6 (114%)	8.3 (77%)
3	5	None	20.0 (100%)	1.0 (100%)	7.6 (100%)
		3A10	23.8 (109%)	2.2 (220%)	7.4 (97%)
		2C11	14.3 (72%)	1.0 (100%)	0.3 (4%)
4	7	None	27.8 (100%)	2.1 (100%)	8.9 (100%)
		3A10	25.8 (93%)	1.5 (71%)	10.6 (119%)
		2C11	14.5 (52%)	1.0 (48%)	0.3 (3%)

*The number of cells with $\gamma\delta$ TCR or $\alpha\beta$ TCR were calculated from the total number of viable cells recovered, and the data were obtained by flow cytometry.

Table 2. DNA clones isolated from the cultured fetal thymuses

TCR genes	Exp.		DNA clones with various types of junctional sequences, no. In-frame: Canonical	In-frame: Noncanonical	In-frame: Total	With *N* nucleotides*	Total†
			No mAb in culture				
$V_{\delta 1}$–$J_{\delta 2}$	1		13	0	13	1	16
	2		21	1	22	1	29
		Subtotal	**34 (97%)**	**1 (3%)**	**35 (100%)**	**2**	**45**
$V_{\gamma 6}$–$J_{\gamma 1}$	1		14	1	15	1	35
	2		7	0	7	2	34
		Subtotal	**21 (95%)**	**1 (5%)**	**22 (100%)**	**3**	**69**
$V_{\gamma 5}$–$J_{\gamma 1}$	1		12	0	12	1	31
	2		13	0	13	1	34
		Subtotal	**25 (100%)**	**0 (0%)**	**25 (100%)**	**2**	**65**
		Total	**80 (98%)**	**2 (2%)**	**82 (100%)**	**7**	**179**
			mAb 3A10 in culture				
$V_{\delta 1}$–$J_{\delta 2}$	1		7	7	14	6	28
	2		14	5	19	3	26
		Subtotal	**21 (64%)**	**12 (36%)**	**33 (100%)**	**9**	**54**
$V_{\gamma 6}$–$J_{\gamma 1}$	1		4	6	10	8	32
	2		9	2	11	4	40
		Subtotal	**13 (62%)**	**8 (38%)**	**21 (100%)**	**12**	**72**
$V_{\gamma 5}$–$J_{\gamma 1}$	1		10	3	13	3	42
	2		10	3	13	1	32
		Subtotal	**20 (77%)**	**6 (23%)**	**26 (100%)**	**4**	**74**
		Total	**54 (68%)**	**26 (32%)**	**80 (100%)**	**28**	**200**

*DNA clones containing *N* nucleotides among total (in-frame and out-of-frame) clones.
†Total in-frame and out-of-frame DNA clones analyzed.

H57-597) (6), CD3 (mAb 2C11) (7), or an idiotypic determinant of γδ TCR from the hybridoma KN6 (mAb 5C10) (5). Single-cell suspensions were prepared from the cultured thymus lobes at various times after initiation of the cultures. The cells were washed and cultured for 1 to 5 hr in antibody-free medium to allow the regeneration of surface TCR (9). Fig. 1 shows the staining of cells from 3-day cultures with anti-CD3/TCR mAb. We confirmed the previous finding (11) that treatment with anti-αβ TCR mAb significantly reduced the number of both mature and immature αβ TCR-expressing cells (data not shown). In contrast, cultures treated with anti-γδ TCR mAb still contained as many γδ TCR- and αβ TCR-expressing cells as untreated cultures. Cultures treated with anti-CD3 mAb still contained a normal number of γδ TCR-expressing cells but no or very few αβ TCR-expressing cells. The anti-idiotypic mAb that served as a control did not have any significant effect. Similar results were obtained with cells from 5- and 7-day-old cultures (Table 1).

To examine the γδ TCR repertoire that developed in cultures with and without anti-TCR mAb, we analyzed the junctional sequences of rearranged $V_{\delta 1}$–$J_{\delta 2}$, $V_{\gamma 6}$–$J_{\gamma 1}$, and $V_{\gamma 5}$–$J_{\gamma 1}$ gene segments. The PCR method (10) was used to amplify these rearranged gene segments for sequencing. Data obtained in two separate experiments are shown in Table 2 and Fig. 2. In untreated cultures, almost all (80 of 82) in-frame sequences were identical with the canonical sequences that we have previously observed *in vivo* (3). However, this was not the case in anti-γδ TCR-treated cultures, where a significant proportion of the in-frame sequences were noncanonical—namely, 36% of $V_{\delta 1}$–$J_{\delta 1}$ joints, 38% of $V_{\gamma 6}$–$J_{\gamma 1}$ joints and 23% of $V_{\gamma 5}$–$J_{\gamma 1}$ joints. Addition of the antibody had no discernable effect on the occurrence of out-of-frame sequences (Table 2).

The appearance of noncanonical in-frame sequences in antibody-treated cultures could result from a positive selection of cells expressing rare noncanonical TCR and/or negative selection of cells expressing the canonical TCR by the antibody. However, such differential effect of the antibody is highly unlikely because it is specific for a determinant in the δ chain constant region gene (5), and the TCR variability is restricted to the V_γ–J_γ and V_δ–D–J_δ junctional regions (D = diversity region). It is much more likely that the noncanonical in-frame sequences in antibody-treated culture resulted from a partial blocking by the antibody of the selection of cells expressing the canonical sequence. Thus, our results are against the hypothesis that the predominance of the canonical TCR in fetal γδ T cells is due to a predominance of "canonical" γ and δ gene rearrangements and strongly support our view that fetal γδ T cells that express the canonical TCR are positively selected from a pool of TCR with junctional diversity through the interaction of the canonical TCR with self determinants in the thymus (4). The putative selection of cells expressing canonical TCR is already well underway by day 14.5 of gestation (Table 2, Fig. 2, and ref. 3) at which time the fetal thymuses were placed in culture. This explains why the canonical sequences were still more frequent than the noncanonical in-frame sequences in the antibody-treated cultures. The somewhat more effective blocking of the accumulation of the canonical $V_{\gamma 6}$ TCR (38%) than of the canonical $V_{\gamma 5}$ TCR (23%) supports this explanation (Table 2), since the appearance of the $V_{\gamma 6}$ subset is known to be delayed as compared with the $V_{\gamma 5}$ subset during ontogeny (3).

The putative interaction between the canonical γδ TCR and their thymic ligands may be a prerequisite for γδ T-cell survival and/or maturation or may result in the clonal expansion of already mature cells. The ligands appear to be expressed in the thymus only during fetal and early postnatal life (14). However, the same ligands are probably expressed or inducible throughout life in peripheral epithelia such as the epidermis in case of the ligand for the $V_{\gamma 5}$ subset (15) and the mucosal surfaces of vagina, uterus, and tongue in case of the ligand for the $V_{\gamma 6}$ subset (16).

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Germline	Vδ1 GGG TCA GAT AT Dδ1 cactgtg GTGGCATATCA cacaggt Dδ2 caccgtg ATCGGAGGGATACGAG cacagtg Jδ2 taacgtg C TCC TGG GAC		Frequency
No treatment	GGG TCA GAT ATCGGAGGGA GC TCC TGG GAC		33/34 *
	GGG TCA GAT CGGAGGGATACG TCC TGG GAC		1/34
3A10	GGG TCA GAT ATCGGAGGGA GC TCC TGG GAC		21/33 *
	GGG TCA GAT A GC TCC TGG GAC		1/33
	GGG TCA G GT ATCGGAGGGA GCC CC TGG GAC		1/33
	GGG TCA GAT ATCGGAGGGATACGA AGC TCC TGG GAT		1/33
	GGG TCA GAT CGGAGGGATAC C AGC TCC TGG GAC		1/33
a	GGG TCA GAT CGGAGGGATACGAG C TCC TGG GAC		3/33
	GGG TCA GAT ATCGGAGGGATACG C TCC TGG GAC		1/33
	GGG TCA GAT AT ATATCGGAGGGATACGAG C TCC TGG GAC		1/33
	GGG TCA GAT AT ATCGGAGGGATAC TCC TGG GAC		1/33
	GGG TCA G ATATC G ATCGGAGGGATACGAG C TCC TGG GAC		1/33
	GGG TCA GAT ATCGGAGGGATACG C TCC TGG GAC		1/33

Germline	Vγ6 TGT GCA TGC TGG GAT A cactcta Jγ1 cactgtg AT AGC TCA GGT TTT		Frequency
No treatment	TGT GCA TGC TGG GAT AGC TCA GGT TTT		21/22 *
	TGT GCA TGC TGG GAT GA C TCA GGT TTT		1/22
3A10	TGT GCA TGC TGG GAT AGC TCA GGT TTT		13/21 *
	TGT GCA TGC TGG GAT GGG AGC TCA GGT TTT		3/21
	TGT GCA TGC TGG GAT CGGG AT AGC TCA GGT TTT		1/21
b	TGT GCA T AT AGC TCA GGT TTT		1/21
	TGT GCA TGC TGG G GC TCA GGT TTT		1/21
	TGT GCA TGC TGG GA C TTT		1/21
	TGT GCA TGC TGG AGC TCA GGT TTT		1/21

Germ line	Vγ5 TGT GCC TGC TGG GAT CT cacagtg Jγ1 cactgtg AT AGC TCA GGT TTT		Frequency
No treatment	TGT GCC TGC TGG GAT AGC TCA GGT TTT		25/25 *
3A10	TGT GCC TGC TGG GAT AGC TCA GGT TTT		20/26 *
	TGT GCC TGC TG CCT ATAT AGC TCA GGT TTT		1/26
	TGT GCC TGC T AT AGC TCA GGT TTT		1/26
c	TGT GCC TGC TGG GAT CTA AGC TCA GGT TTT		1/26
	TGT GGC TGC TGG G C ATAT AGC TCA GGT TTT		1/26
	TGT GGC T AT AGC TCA GGT TTT		1/26
	TGT GGC TGC TGG GAT CTAG AT AGC TCA GGT TTT		1/26

FIG. 2. $V_{\delta 1}$–$J_{\delta 2}$ (*a*), $V_{\gamma 6}$–$J_{\gamma 1}$ (*b*), and $V_{\gamma 5}$–$J_{\gamma 1}$ (*c*) in-frame DNA sequences present in thymocytes from organ cultures. The junctional sequences are aligned with germ-line sequences of TCR δ-chain (12) and γ-chain gene segments (3, 13). The recombination signal sequences are shown in small letters. The sequences derived from germ-line and non-germ-line sequences are shown in boldface and regular type, respectively. The possible P (palindromic) nucleotide(s) (3) are underlined. The frequency with which a particular sequence was found among DNA from randomly chosen clones is listed in the last column. The canonical sequences that have been observed previously *in vivo* (3) are marked with asterisks.

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