Clonal Populations of T Cells in Normal Elderly Humans: The T Cell Equivalent to "Benign Monoclonal Gammapathy"

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Summary

To determine whether T cells, like B cells, can become clonally expanded in normal individuals as a function of age, we compared the T cell Vβ repertoire of cord blood to that of peripheral blood from normal donors over 65 yr of age. T cells from elderly subjects contained expanded subsets (greater than the mean + three standard deviations) of T cell receptor (TCR) Vβ populations. These expanded subsets were observed primarily among CD8, but not CD4 cells, represented up to 37.5% of all CD8 cells, and were present in most elderly subjects. An expanded Vβ5.2/3 CD8 subset and a Vβ6.7a CD8 subset from separate donors were analyzed by reverse transcriptase–polymerase chain reaction, cloning and sequencing of the TCR β chain VDJ junction. In both cases the expanded subsets were mono- or oligoclonal while control CD4 populations were polyclonal. Using two-color flow cytometry it was possible to identify the expanded Vβ6.7a subset as $CD8^+$ $CD28^-CD11b^+$ cells. In three of five random old subjects similar expansions of Vβ subsets were found specifically in the $CD8^+$ $CD28^-$ subpopulation, an interesting subset of cytotoxic T lymphocytes, known to lack proliferative responses to TCR stimuli. It is common practice to use the demonstration of clonality as a diagnostic indicator for T cell lymphoma/leukemia. In view of the high frequency of expanded T clones of T cells in normal elderly subjects the diagnostic usefulness of this test should be reexamined.

Monoclonal expansions of nonmalignant B cells occur frequently in elderly individuals and can be readily detected due to the presence of monoclonal immunoglobulin peaks on serum electrophoresis, present in 10% of normal adults over 80 yr old (1). Clinically this condition is termed "Benign Monoclonal Gammapathy" and is thought to be a consequence of chronic stimulation of B cells by self-antigens or microbial antigens over a lifetime (2). We hypothesized that similar clonal expansions of T cells might occur in the elderly. Evidence is provided herein, that clonal TCR-α/β cell populations are frequent in normal subjects over age 65 and that these are usually clones of $CD8^+$ $CD28^-$ cells. These data should help in evaluating the significance of clonal expansions of T cells in inflammatory and autoimmune disorders (3–13). In addition, the clinical utility of demonstrating clonally expanded T cells in support of a diagnosis of T cell malignancy (14) should be reexamined.

Materials and Methods

Immunofluorescence. Indirect immunofluorescence was performed by incubating cells for 40 min at 25°C with the primary unlabeled antibody (usually an anti-TCR Vβ), followed by washing the cells three times with PBS, BSA 1%, followed by a 40-min incubation with goat anti-mouse Ig Fab'_2 FITC-labeled second antibody (Tago, Inc., Burlingame, CA), washing cells three times, a 40-min incubation with excess mouse IgG1, washing three times, and finally a 40-min incubation with PE-conjugated anti-CD4 mAb, anti-CD3, anti-CD8 (Upstate Biotechnology, Inc., Lake Placid, NY), or anti-CD28 (Becton Dickinson & Co., Mountain View, CA). The primary anti-TCR mAbs were S511-Vβ12, C37-Vβ5.2/3, OT145-Vβ6.7a, Ti3a-Vβ8, C1-Vβ17, LC4-Vβ5.1, F1-Vα2.3, 8F10-Vβ3 (15), and also included E22E7.2-Vβ2 (16), Vβ13.1 (17), Vβ13.2 (17), Vβ13.3 (18), IG125-Vβ21, and IMMU546-Vβ22 both kindly donated by Dr. A. Necker and Dr. F. Romagne (Immunotech, Marseille, France), and Vβ1 from Dr. O. Kanagawa (Washington University, St. Louis, MO). For the two-color immunofluorescence in Fig. 6 the goat anti-mouse Ig Fab'_2 antibody was PE conjugated and the final antibody was FITC-conjugated OT145 (anti-Vβ6.7a). The primary mAbs were a negative control (IgG1), CD3 (clone 454), CD4 (clone FFB2.3), CD8α (OKT8, CRL 8014; American Type Culture Collection [ATCC], Rockville, MD), HLA-DR (VG2.1, from Dr. S. M. Fu), CD11b (OKM1, CRL 8026; ATCC), anti-transferrin receptor (TRF, OKT9, CD71, CRL 8021; ATCC), CD45RA (4G10; from Dr. R. Steinman, Rockefeller University, New York), CD45RO (UCHL1), CD57 (HNK-1, TIB200; ATCC), Tac (CD25; from Dr. T. Waldman, National Institutes of Health, Bethesda, MD), CD28 (clone NE-51, Becton Dickinson & Co.). Cells were analyzed on an Elite flow cytometry system (Coulter Corp., Hialeah, FL).

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Separation of Cell Subsets. CD4 and CD8 subsets were separated by negative selection from SRBC-rosetted T cells using the mAb followed by goat anti-mouse Ig-coated Dynabeads (Dynal Inc., Great Neck, NY). Populations were >90% purified. OT145 positive cells (Vβ6.7a) were isolated by positive selection using the same procedure and starting with the purified CD4 and CD8 subsets. This positive selection removed practically all OT145$^+$ cells (Fig. 3 *B*).

Molecular Analysis of Vβ Subsets. RNA was extracted from isolated cells by RNAzol (Biotecx Lab., Inc., Houston, TX). cDNA was synthesized with murine reverse transcriptase. PCR was performed with predetermined optimal amounts of cDNA, primers at 200 nM, $MgCl_2$ 1.5 mM, dNTPs at 0.5 mM in 0.05 ml of 10 mM Tris (pH 9 at 25°C), 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100 (36 cycles of 94°C × 1 min, 51°C × 1 min, 72°C × 1.5 min, with a final 72°C × 10 min). The primers used were Vβ5.2/3: 5′ GTCAGGGGCCCCAGTTTAT 3′ (sense), Vβ6: 5′ TCAGGTGTGATCCAATTTC 3′ (sense), which amplifies all Vβ6 family members, and a Cβ primer: 5′ CTTCTGATGGCTCAAACAC 3′ (antisense) (19). PCR products were ligated into the pCR2000 vector (Invitrogen Corp., San Diego, CA) and INV1αF′ *Escherichia coli* were transformed. Transformants containing appropriate sized inserts were sequenced by the dideoxy chain termination method.

Results

In a survey of the effect of age on the Vβ repertoire we analyzed the frequency of CD3$^+$ T cells expressing Vβ12, Vβ5.2/3, and Vβ6.7a in normal individuals grouped by age. There was no significant difference in Vβ expression between the groups of young, middle aged, or elderly subjects. However, the standard deviations of the mean were consistently higher in the group of elderly donors (Fig. 1). Thus variation in the percentage of cells expressing a given Vβ gene increases with age within a population, which can be interpreted as the effect of environmental influences on the Vβ repertoire.

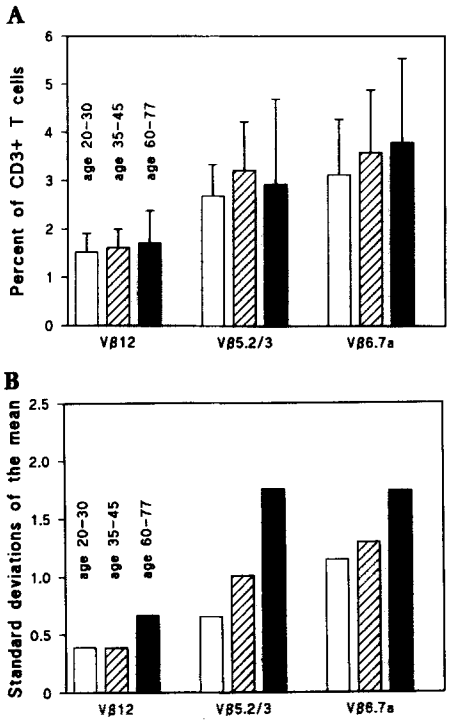


Figure 1. Percentages of Vβ subsets are more variable in the elderly. Normal donors were divided into three subsets by age (n = 15 for each subset). (*A*) The mean percentage of cells expressing the indicated Vβ does not differ significantly from one age group to another. (*B*) The standard deviation of the mean is consistently higher in the older age groups.

The frequency of T cells expressing 6 Vβ and 1 Vα gene was determined in CD4 and CD8 subsets from cord blood lymphocytes (n = 12) and from lymphocytes obtained from donors over age 65 (n = 11) (Table 1). To select outlying values an arbitrary cutoff was used: the mean + 3 SD of the Vβ percentage in either CD4 or CD8 cells, derived from cumulative data obtained on normal subjects of all ages, who had served as control populations in prior studies, n = 48 (15, 20). The only outlying values (above the mean + 3 SD) occurred among the CD8 cells from 4 of 11 old donors (Table 1).

This type of analysis was extended using a larger panel of TCR mAb in seven further donors above the age of 65 (Table 2). Expansions of Vβ subsets beyond the cutoff value were observed in five of seven donors, again primarily in the CD8 subset. Thus, the use of a larger panel of Vβ antibodies, covering ~35% of the total Vβ repertoire (15), demonstrates the high frequency of the observed expansions, highlighted in Tables 1 and 2, and suggests that these expansions occur in virtually all individuals as a function of age.

Fluorescence histograms are shown for donor 15 (Fig. 2) and donor 18 (Fig. 3). Donor 15 had three expanded Vβ subsets (13.0% Vβ5.2/3, 12.0% Vβ8, 11.5% Vβ22) among the CD8 cells and one borderline expanded subset (10.4% Vβ6.7a) among the CD4 cells, each highlighted in Fig. 2. Donor 18 had a particularly large subset of Vβ6.7a cells, 37.5% of CD8 cells, and an additional expansion of Vβ5.1 cells representing 20.9% of CD8 cells (Fig. 3 *A*). Repeated analysis of both donors over a 6-mo period showed that these subsets remained constantly expanded. These two donors were chosen for further studies of the clonality of the Vβ5.2/3 subset (donor 15) and the Vβ6.7a subset (donor 18) by RT-PCR[1] and sequencing of the VDJ junction of the relevant Vβ chains.

The CD8 cells of donor 15 contain a Vβ5.2 clone represented by 12 of 20 sequenced clones (Fig. 4). By contrast the Vβ5.2/3 clones obtained from the CD4 cells of the same donor, a nonexpanded subset (Fig. 2), were polyclonal (Fig. 4). Even the CD4 cells from donor 15 that were cultured in vitro with IL-2 for 6 d remained polyclonal. 12 of 23 clones used the Vβ5.3 gene segment and the rest used Vβ5.2 in the CD4 subset, while only 1 clone derived from the CD8 subset had a Vβ5.3 sequence. This indicates skewed representation of these two Vβ genes in the CD8 cells of this individual.

The expanded Vβ6.7a CD8 subset from donor 18 was posi-

[1] *Abbreviations used in this paper:* IEL, intestinal intraepithelial lymphocytes; RT-PCR, reverse transcriptase-polymerase chain reaction.

Table 1. *Vβ Analysis in Cord Blood and in Elderly Subjects*

CD4	Vβ12	Vβ5.2/3	Vβ6.7a	Vβ8	Vβ5.1	Vβ17	Vα2.3
m + 3 SD	5.7	5.5	10.6	7.3	13.1	14.1	6.5
Cord blood							
1	2.1	2.1	4.2	4.2	2.1	3.2	2.1
2	2.0	4.0	4.0	5.3	7.3	1.3	4.0
3	1.2	0.0	1.2	1.2	2.3	3.5	2.3
4	0.5	3.1	2.7	1.7	3.5	1.4	1.2
5	2.2	2.8	4.8	4.2	1.3	4.0	3.6
6	3.6	1.8	9.5	3.6	3.6	4.8	4.8
7	2.8	3.2	4.9	5.7	7.8	3.6	2.3
8	1.9	1.9	1.4	2.8	7.0	4.2	2.8
9	2.4	5.0	5.7	7.0			
10	1.4	2.1	1.4	3.6	9.3	3.6	
11	2.4	2.7	5.1	5.5	7.1	4.7	3.5
12	1.2	1.7	6.6	5.5	11.4	6.0	6.0
Elderly subjects							
1	0.8	1.8	3.9	2.2	2.5	8.0	3.5
2	0.0	2.6	2.8	3.5	7.4	14.0	2.9
3	1.7	2.3	2.8	2.8	3.7	7.7	3.8
4	1.4	3.3	4.0	4.4	5.6	6.4	3.2
5	0.7	2.5	5.9	4.6	5.9	4.9	3.4
6		4.2	3.8	2.8	4.5		
7	2.9	3.8	7.7	5.7	5.3	5.7	
8	0.5	0.6	1.7	4.2	9.0	4.5	2.4
9	0.3	0.0	3.6	2.0	2.3	1.0	2.7
10	4.3	3.4	3.9	4.8	3.0	9.7	2.4
11	1.9	1.9	0.0	2.7	3.4	3.9	1.5
CD8	Vβ12	Vβ5.2	Vβ6.7a	Vβ8	Vβ5.1	Vβ17	Vα2.3
m + 3 SD	4.7	7.3	7.7	9.2	9.7	13.2	8.9
Cord blood							
1	1.0	4.1	1.0	3.1	1.0	4.1	3.1
2	3.5	2.1	2.1	7.1	5.9	4.7	8.2
3	4.5	6.0	4.5	1.5	3.0	4.5	7.5
4	0.8	0.5	0.9	2.0	0.9	1.2	1.3
5	0.6	2.5	1.7	2.4	2.8	3.2	4.8
6	4.7	4.7	4.7	2.3	2.3	7.0	2.3
7	0.3	2.5	2.0	6.4	3.1	3.1	4.2
8	1.5	3.8	1.5	3.8	3.8	7.4	6.1
9	3.6	3.6	1.3	7.2	3.6	12.4	
10	2.8	4.3	3.6	2.1	2.1	1.4	
11	1.2	4.2	3.0	6.0	4.5	5.4	0.6
12	1.8	3.0	3.0	5.5	6.1	4.9	6.7

continued

Table 1. *(continued)*

CD4	Vβ12	Vβ5.2/3	Vβ6.7a	Vβ8	Vβ5.1	Vβ17	Vα2.3
m + 3 SD	5.7	5.5	10.6	7.3	13.1	14.1	6.5
Elderly subjects							
1	0.3	1.5	2.1	0.0	0.3	5.6	2.1
2	0.6	1.1	2.1	2.9	**16.1**	3.8	2.6
3	0.2	2.7	0.0	2.0	2.0	1.8	0.0
4	0.7	0.5	2.9	2.3	1.5	2.3	1.4
5	0.0	1.1	1.1	2.6	2.6	0.4	1.9
6	**27.4**	4.6	5.1	9.0	0.3	**17.0**	
7	0.6	5.5	7.3	3.7	0.6	7.9	2.0
8	0.6	1.2	0.3	3.1	3.2	**14.7**	1.3
9	1.4	0.4	1.6	3.4	3.8	1.0	1.6
10	0.0	**10.1**	1.7	3.4		5.1	6.8
11	0.0	1.8	0.0	1.8		2.6	

The numbers indicate percentages of CD4+ (or CD8+), CD3+ T cells that express the indicated Vβ. Values above the mean + 3 SD are boldfaced.

tively selected with mAb OT145 and goat anti-mouse-coated magnetic beads. As seen in Fig. 3 *B*, the remaining CD8 cells from donor 18 were depleted of the large Vβ6.7a subset but not of other Vβ subsets, indicating high efficiency and specificity of the positive selection. Cellular RNA was then isolated from the selected cells, reverse transcribed and used for a PCR with a pan-Vβ6 sense primer and a Cβ anti-sense primer. Here also a large fraction of the sequenced clones

Table 2. *Extended Vβ Analysis in Elderly Subjects*

CD4	Vβ12	Vβ5.2/3	Vβ6.7a	Vβ8	Vβ5.1	Vβ17	Vβ2	Vβ13.1	Vβ13.2	Vβ13.3	Vβ22	Vβ21	Vβ3
m + 3 SD	5.7	5.5	10.6	7.3	13.1	14.1	13.2	9.0	6.7	12.6	9.9	2.9	9.4
12	2.2	2.2	6.0	4.1	5.3	5.9	8.3	4.0	4.4		4.9	2.0	
13	1.0	**7.6**	1.2	**8.8**	4.8	2.8	6.8	2.8	0.4	4.6	2.2	2.0	5.0
14	1.2	3.6	4.6	5.2	5.6	5.6	8.2	4.4	3.0	7.6	4.0	2.4	5.4
15	1.2	2.6	10.4	4.8	5.4	6.4	6.8	7.2	2.6	9.8	3.2	2.4	3.6
16	1.6	0.1	4.3	5.2	4.4	5.5	11.1	4.1	2.2	5.9	3.2	2.5	6.2
17	2.1	0.1	1.0	4.6	7.5	5.8	9.4	4.5	0.5	5.8	7.6	2.3	4.1
18	2.4	1.4	7.6		5.7	5.0				4.3			1.4
CD8	Vβ12	Vβ5.2/3	Vβ6.7a	Vβ8	Vβ5.1	Vβ17	Vβ2	Vβ13.1	Vβ13.2	Vβ13.3	Vβ22	Vβ21	Vβ3
m + 3 SD	4.7	7.3	7.7	9.2	9.7	13.2	6.6	13.7	8.6	14.9	6.9	6.3	7.2
12	3.1	4.6	**9.2**	7.7	3.1	10.8	4.6	4.6	6.2		4.6	4.6	
13	0.7	4.7	0.7	2.7	2.0	5.3	2.0	10.7	0.7	10.7	4.0	2.7	
14	1.0	1.0	4.0	2.0	1.0	5.0	2.0	6.0	1.0	6.0	2.0	1.0	4.0
15	1.0	**13.0**	2.0	**12.0**	2.0	3.0	4.0	3.5	2.5	4.5	**11.5**	1.0	3.5
16	1.0	0.5	1.4	2.0	1.8	8.0	3.4	4.0	1.0	4.0	3.8	2.0	4.4
17	1.0	0.5	0.5	3.0	**22.0**	2.0	4.0	3.0	0.5	4.0	2.0	1.0	1.0
18	1.3	1.3	**37.5**		**20.9**	2.5				0.6			2.2

The numbers indicate percentages of CD4+ (or CD8+), CD3+ T cells that express the indicated Vβ. Values above the mean + 3 SD are boldfaced.

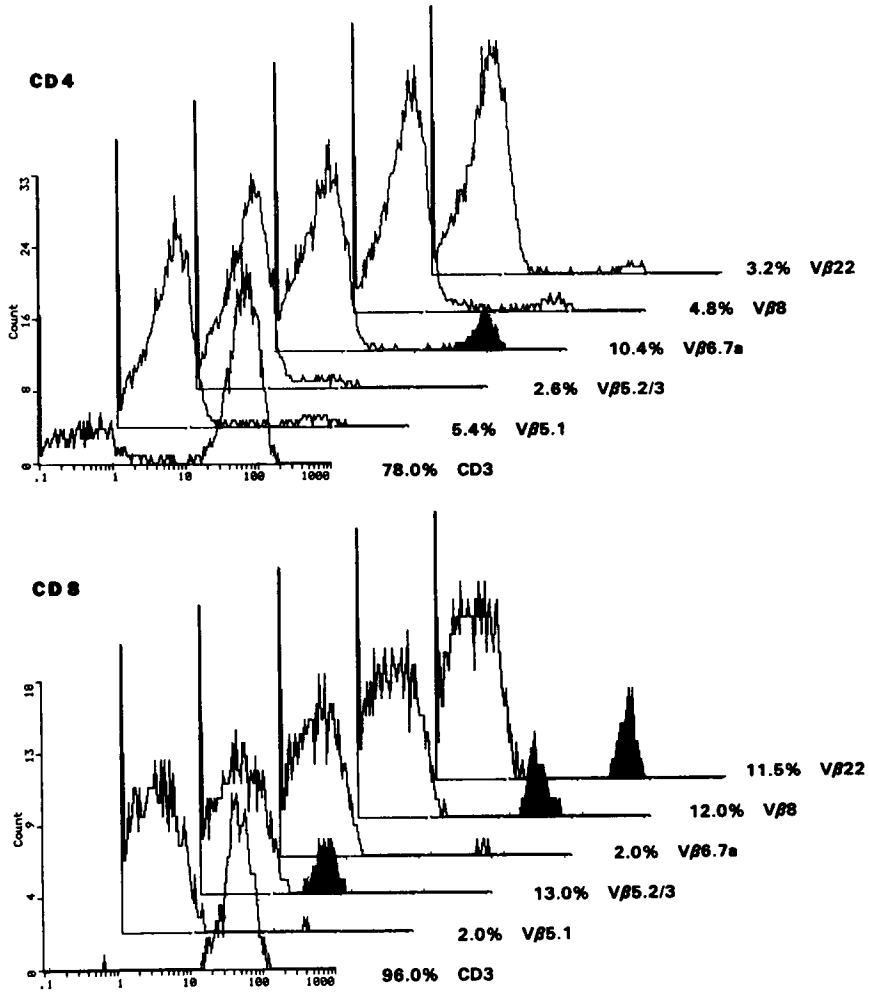
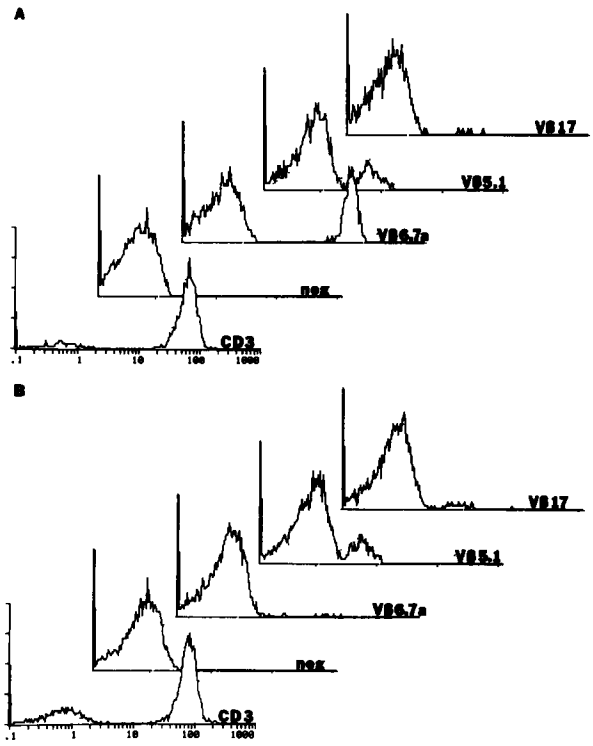


Figure 2. Fluorescence histograms from donor 15. In the top panel staining with the indicated Vβ antibodies is shown for the CD4 subset (analysis gates set on CD4-PE positive cells). In the bottom panel staining is shown for the CD8 subset. The highlighted peaks represent expanded subsets (see data in Table 2).



(9 of 17) represents a dominant Vβ6.7a clone, and a second group of clones (3 of 17) represents another clonal population of Vβ6.7a cells (Fig. 5). Both of these clones are not expanded among the CD4 cells from this donor (data not shown).

To further characterize the expanded Vβ6.7a subset in donor 18, two-color immunofluorescence was performed with the Vβ-specific mAb, OT145, directly labeled to FITC (Fig. 6). The Vβ6.7a cells were $CD11b^+$, in part $CD45RO^+$, and in part $CD45RA^+$, but $CD71^-$, $CD25^-$, $HLA\text{-}DR^-$, $CD57^-$, and $CD28^-$. There appeared to be two populations of $CD8\alpha^+$ cells (*arrows* in Fig. 6). The more prominent population stained intensely with the CD8α mAb consistent with prior data showing high levels of CD8α and low levels of CD8β expression on $CD8^+$ $CD28^-$ cells (21). Negatively selected $CD8^+$ cells from donor 18 failed to proliferate in vitro in response to the OT145 mAb but did respond to anti-CD3 (data not shown). Thus, the pheno-

Figure 3. Fluorescence histograms from donor 18. (*A*) Analysis of negatively selected CD8 cells, demonstrating an expanded subset of OT145-Vβ6.7a cells and of LC4-Vβ5.1 cells (see Table 2). (*B*) Reanalysis of the same CD8 cells after the OT145 cells were removed by positive selection with goat anti-mouse Ig–coated magnetic beads, demonstrating specific and nearly complete removal.

A

n		Vβ	Dβ	Jβ	
		C A S S	P W T P E G S Y N S P L H F G		
1	Vβ5.2	TGTGCCAGCAGC	CCATGGACTCCGGAAGGG	TCCTATAATTCACCTCTCCACTTTGGG	Jβ1.6
		C A S S L	N P R	S Y E Q Y F G	
1	Vβ5.3	TGTGCCAGCAGCTTG	AATCCCCGA	TCCTACGAGCAGTACTTCGGG	Jβ2.7
		C A S S	L E S R G V	*	
1	Vβ5.3	TGTGCCAGCAGC	CTGGAAAGCCGGGGGGTAT	TACAATGAGCAGTTCTTCGGG	Jβ2.1
		C A S S L	K S	N T G E L F F G	
1	Vβ5.3	TGTGCCAGCAGCTTG	AAAAGC	AACACCGGGGAGCTGTTTTTTGGA	Jβ2.2
		C A S S L	T P G Q G	D T Q Y F G	
1	Vβ5.3	TGTGCCAGCAGCTTG	ACCCCGGGTCAGGG	AGATACGCAGTATTTTGGC	Jβ2.3
		C A S	A A Q T S G	Q E T Q Y F G	
1	Vβ5.2	TGTGCCAGC	GCCGCCCAGACTAGCGG	CCAAGAGACCCAGTACTTCGGG	Jβ2.5
		C A S R	G M G S L	Y E Q Y F G	
1	Vβ5.3	TGTGCCAGCAG	GGGAATGGGGTCTCT	CTACGAGCAGTACTTCGGG	Jβ2.7
		C A S S	N D G S	N E K L F F G	
1	Vβ5.3	TGTGCCAGCAGC	AATGACGGGAG	TAATGAAAAACTGTTTTTTGGC	Jβ1.4
		C A S S	P R D S	T Y E Q Y F G	
1	Vβ5.2	TGTGCCAGTAGC	CCCCGGGACAGTA	CCTACGAGCAGTACTTCGGG	Jβ2.7
		C A S S	P P N I	D N E Q F F G	
1	Vβ5.2	TGTGCCAGCAGC	CCTCCGAATATTG	ACAATGAGCAGTTCTTCGGG	Jβ2.1
		C A S S L	K L N	N Y G Y T F G	
1	Vβ5.2	TGTGCCAGCAGCTTG	AAACTGAAC	AACTATGGCTACACCTTCGGT	Jβ1.2
		C A S S L	I S R G N	Y E Q Y F G	
1	Vβ5.2	TGTGCCAGCAGCTTG	ATCTCCCGAGGGAA	CTACGAGCAGTACTTCGGG	Jβ2.7
		C A S S	G W T S P R	T Q Y F G	
1	Vβ5.2	TGTGCCAGCAGC	GGGTGGACTAGTCCTCG	GACCCAGTACTTCGGG	Jβ2.5
		C A S S	P K G V	Y E Q Y F G	
1	Vβ5.3	TGTGCCAGCAGC	CCGAAGGGAGT	CTACGAGCAGTACTTCGGG	Jβ2.7
		C A S S	W R D R G F	Y G Y T F G	
1	Vβ5.3	TGTGCCAGCAGCT	GGAGGGACAGGGGATT	CTATGGCTACACCTTCGGT	Jβ1.2
		C A S S	N D G S	*	
1	Vβ5.3	TGTGCCAGCAGC	CCGGATTCCGG	GAGACCCAGTACTTCGGG	Jβ2.5
		C A S S F	H	N E K L F F G	
1	Vβ5.2	TGTGCCAGCAGCTT	CCA	TAATGAAAAACTGTTTTTTGGC	Jβ1.4
		C A S S	R T D S	A Q Y F G	
1	Vβ5.3	TGTGCCAGCAGC	CGGACGGACAGTG	CGCAGTATTTTGGC	Jβ2.3
		C A S S L	R T S G A G	D T Q Y F G	
1	Vβ5.3	TGTGCCAGCAGCTTG	AGGACTAGCGGGGCGGG	AGATACGCAGTATTTTGGC	Jβ2.3
		C A S R	P A G	*	
1	Vβ5.2	TGTGCCAGCAG	GCCGGCGGGG	GAACACTGAAGCTTTCTTTGGA	Jβ2.1
		C A S S L	P P G Q G	Q F F G	
1	Vβ5.2	TGTGCCAGCAGCTT	ACCTCCGGGACAGGG	GCAGTTCTTCGGG	Jβ2.1
		C A S S L	R G	H E Q F F G	
1	Vβ5.3	TGTGCCAGCAGCTTG	AGAGGGC	ATGAGCAGTTCTTCGGG	Jβ2.1
		C A R S	R G S D R D F	D Q P Q H F G	
1	Vβ5.5	TGTGCCAGAAGC	AGGGGATCCGACAGAGATTTCG	ATCAGCCCCAGCATTTTGGT	Jβ1.5

B

n		Vβ	Dβ	Jβ	
		C A S	A A Q T S G	Q E T Q Y F G	
12	Vβ5.2	TGTGCCAGC	GCCGCCCAGACTAGCGG	CCAAGAGACCCAGTACTTCGGG	Jβ2.5
		C A S	S S G G A L	N E Q F F G	
1	Vβ5.2	TGTGCCAGC	TCTAGCGGGGGGGCTCT	CAATGAGCAGTTCTTCGGG	Jβ2.1
		C A S S L	V G G	E T Q Y F G	
1	Vβ5.2	TGTGCCAGCAGCTTG	GTAGGGGG	AGAGACCCAGTACTTCGGG	Jβ2.5
		C A S	I G	A D T Q Y F G	
1	Vβ5.3	TGTGCCAGCA	TAGGGG	CAGATACGCAGTATTTTGGC	Jβ2.3
		C A S S L	F P V G S	D T Q Y F G	
1	Vβ5.2	TGTGCCAGCAGCTTG	TTCCCCGTGGGATCC	GATACGCAGTATTTTGGC	Jβ2.3
		C A S S L	E D G G L K F	T D T Q Y F G	
1	Vβ5.2	TGTGCCAGCAGCTTG	GAGGACGGGGGCCTAAAATTC	ACAGATACGCAGTATTTTGGC	Jβ2.3
		C A S R	G Q G	S Y N E Q F F G	
1	Vβ5.2	TGTGCCAGCAG	GGGACAGGGT	TCCTACAATGAGCAGTTCTTCGGG	Jβ2.1
		C A	S S Q R G L P G	E T Q Y F G	
1	Vβ5.2	TGTGC	TTCGAGCCAGCGGGGGGTTACCGGGG	GAGACCCAGTACTTCGGG	Jβ2.5
		C A S S	S S G T P G D	Y E Q Y F G	
1	Vβ5.3	TGTGCCAGCAGCT	CCTCCGGGACTCCCGGTGA	CTACGAGCAGTACTTCGGG	Jβ2.7

n		Vβ	Dβ	Jβ	
		C A S	E D P P H	D E Q F F G	
9	Vβ6.7a	TGTGCCAGC	GAGGACCCCCCCCACG	ATGAGCAGTTCTTCGGG	Jβ2.1
		C A S S L	V T G Q S	S P L H F G	
3	Vβ6.7a	TGTGCCAGCAGCTTAG	TGACAGGGCAGAG	TTCACCCCTCCACTTTGGG	Jβ1.6
		C A S S	R A D	N Y G Y T F G	
1	Vβ6.7a	TGTGCCAGCAGC	CGGGCCGAC	AACTATGGCTACACCTTCGGT	Jβ1.2
		C A S S L	A G G A S	G N T I Y F G	
1	Vβ6.7a	TGTGCCAGCAGCTT	GGCAGGGGGCGC	CTCTGGAAACACCATATATTTTGGA	Jβ1.3
		C A S S	R G D	N Y G *	
1	Vβ6.7a	TGTGCCAGCAGC	CGGGGGGAC	AACTATGGCTA	CCTTCGGT Jβ1.2
		C A	A G P G G R *		
1	Vβ6.4	TGTGC	GGCAGCCGCGGGAGGGCGGT	TACGAGCAGTACTTCGGG	Jβ2.7
		C A S S	A V A	N Q P Q H F G	
1	Vβ6.4	TGTGCCAGCAGC	GCCGTTGCA	AATCAGCCCCAGCATTTTGGT	Jβ1.5

Figure 5. Sequence analysis of CD8+ OT145+ cells from donor 18 demonstrating a major dominant Vβ6.7a clone (sequenced nine times) and a minor Vβ6.7a clone (sequenced three times). Sequences that match genomic Dβ1 or Dβ2 are underlined. The asterisk denotes a frame shift. Possible P nucleotides are in bold.

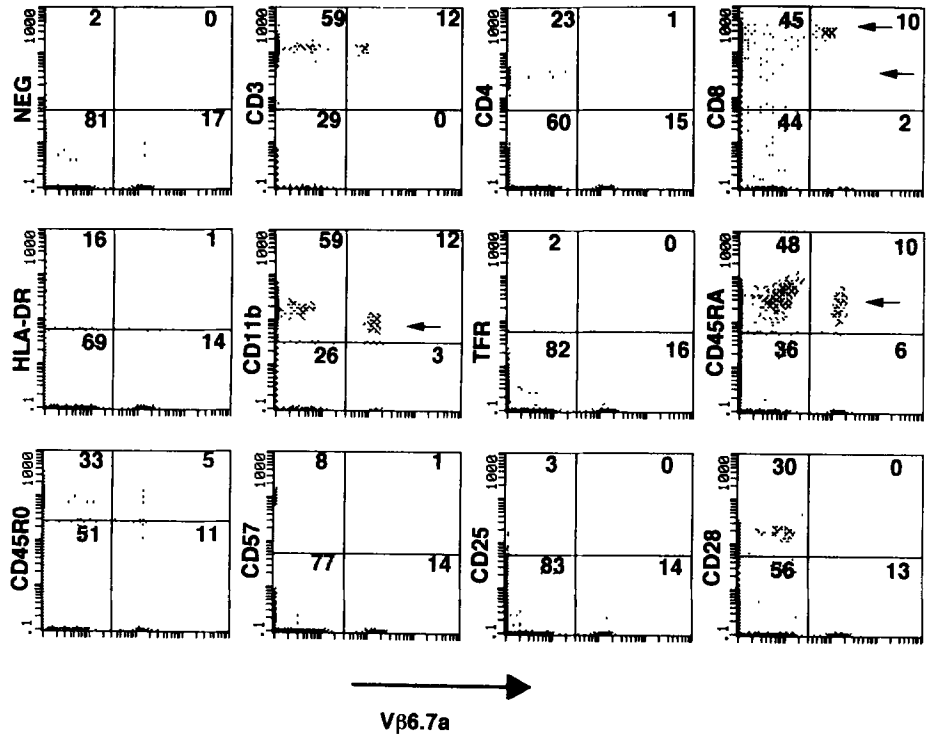


Figure 6. Two-color immunofluorescence of peripheral blood lymphocytes from donor 18. OT145 (anti-Vβ6.7a) was directly labeled with FITC. All other mAbs (indicated in each panel) were indirectly labeled with PE. The percentages of cells are indicated in each quadrant. Arrows point to the two CD8+ Vβ6.7a+ populations, to the CD11b+ Vβ6.7a+ cells and to the CD45RA+ Vβ6.7a+ cells. Hardly any Vβ6.7a cells were CD28+.

type of the expanded Vβ6.7a cells of donor 18 resembles that of CD8+, CD28− T cells, which coexpress CD11b, use CD8αα preferentially, and lack proliferative responses to CD3/TCR stimuli (21).

To determine whether the expanded CD8+ clones were commonly CD28−, five random elderly subjects were screened with 13 Vβ-specific antibodies. In three of five subjects expanded Vβ subsets were detected in CD8 cells isolated by negative selection (donors S, P, and GR in Table 3). In each case the expanded Vβ subsets had the CD8+ CD28− phenotype as determined by two-color flow cytometry with a PE-conjugated CD28 mAb (Table 3). As an in-

Figure 4. Sequence analysis of (*A*) CD4 Vβ5.2/3 cells and (*B*) CD8 Vβ5.2/3 cells from donor 15. In *A* the top 13 sequences are derived from fresh CD4 cells, while the bottom 10 sequences are from CD4 cells cultured with IL-2 for 6 d. In *B* half of the sequences come from fresh CD8 cells and half from IL2 cultured cells. The dominant Vβ5.2 clone was sequenced six times from fresh cells and six times from cultured cells. The dominant CD8 clone was also found in a single sequence from the CD4 subset (*6th sequence from the top in A*), presumably because of contamination of the CD4 subset with some of these expanded CD8 cells. Sequences that match genomic Dβ1 or Dβ2 are underlined. The asterisk denotes a frame shift. Possible P nucleotides are in bold.

Table 3. *Vβ Repertoire Among CD8 Subsets*

	Vβ1	Vβ5.2/3	Vβ8	Vβ13.1	Vβ13.2	Vβ17	Vβ22
Donor S							
CD3+	0.7	0.3	6.2	6.8	2.3	5.8	2.7
CD3+8+	2.0	1.4	**21.0**	7.6	3.5	6.8	2.9
CD3+8+28+	0.9	0.9	2.8	6.0	3.0	7.2	2.8
CD3+8+28−	3.8	2.5	**31.3**	12.5	3.8	5.0	5.0
Donor P							
CD3+	0.7	0.3	3.1	5.5	1.6	5.1	1.4
CD3+8+	0.3	0.3	0.8	**15.3**	6.4	3.0	0.5
CD3+8+28+	0.5	0.0	0.5	0.5	1.5	5.0	1.5
CD3+8+28−	0.3	0.3	0.3	**16.6**	6.0	2.7	0.0
Donor GR							
CD3+	1.5	4.3	3.1	3.8	2.2	5.4	3.8
CD3+8+	**6.1**	2.4	2.7	2.0	2.0	**13.4**	4.6
CD3+8+28+	2.5	4.0	4.0	5.0	1.0	4.0	3.5
CD3+8+28−	**12.9**	1.7	0.0	0.8	0.8	**18.3**	4.2
Donor GA							
CD3+	1.6	5.8	3.3	6.7	2.2	6.7	3.3
CD3+8+	0.7	2.0	4.0	5.0	2.0	7.2	2.3
CD3+8+28+	1.1	0.0	4.6	5.4	2.1	6.8	3.6
CD3+8+28−	1.0	0.8	1.7	0.0	1.7	4.2	0.0
Donor C							
CD3+	0.4	1.8	4.6	6.8	0.7	6.4	3.8
CD3+8+	2.0	2.4	2.0	7.5	0.6	2.4	1.4
CD3+8+28+	1.7	2.8	1.7	6.1	0.6	3.3	1.1
CD3+8+28−	2.0	1.6	2.4	11.6	0.4	0.4	0.0

Vβ percentages represent fractions of total $CD3^+$ cells, of $CD3^+8^+$ cells, of $CD3^+8^+28^+$ cells, or of $CD3^+8^+28^-$ cells. Purified CD8 populations (containing <2% CD4 cells) were analyzed by two-color flow cytometry. The percentages of $CD8^+CD3^+$ cells over total $CD3^+$ cells were for *donor S*, 28.9%; *donor P*, 23.1%; *donor GR*, 16.9%; *donor GA*, 21.6%; and *donor C*, 16.0%. The distribution of CD28 among the CD8 cells of the five donors was for *donor S*: 85/15 ($CD28^+/CD28^-$); *donor P*: 40/60; *donor GR*: 45/55; *donor GA*: 70/30; and *donor C*: 42/58. Vβ subsets that are clearly expanded in the CD8 subset are boldfaced. Results with six additional Vβ-specific antibodies did not show any further expansions of Vβ subsets (data not shown).

ternal control, other nonexpanded Vβ subsets were usually equally represented or decreased among $CD8^+$ $CD28^-$ cells as compared with $CD8^+$ $CD28^+$ cells.

Discussion

Herein we show that normal elderly donors frequently have expanded Vβ subsets and that these are primarily found among $CD8^+$ $CD28^-$ cells. In two donors the expanded subsets were analyzed by RT-PCR and sequencing, demonstrating that they represent clonally expanded populations of T cells.

Clonal expansions of TCR-α/β T cells in man are frequently observed at the tissue sites of chronic inflammatory disorders including rheumatoid arthritis (6, 7), multiple sclerosis (3–5), and leprosy reversal reactions (13). Similar studies have shown oligoclonality in the γ/δ T cell subset (9–11), which was studied in multiple sclerosis and leishmaniasis. We have followed a 72-yr-old patient with immune thrombocytopenia and a TCR-α/β clone (Vβ6.7a) in the peripheral blood represented by a dominant TCR β chain rearrangement. The clonal cells are $CD8^+$, represent about 20–30% of all CD3 cells and have remained present over several years without malignant transformation (12). The presence of clonal T cell populations in normal individuals should result in constraint in the interpretation of clonal T cell populations in disease states. Without functional data on antigen/MHC reactivity the presence of a clone of T cells at the site of an immune response is not necessarily linked to the pathogenesis of the disease.

In addition, the clonal populations in normal subjects appear to persist over time and show no signs of transformation to malignancy (12, 22, 23). They may represent rela-

tively large populations of clonal T cells easily detected by Southern blotting or PCR techniques. Analysis of T cell clonality by these techniques is used clinically as an aid in the diagnosis of malignancy (14). Caution must therefore be advised in the interpretation of the presence of clonal T cell populations.

Prior studies on patients with expansions of large granular lymphocytes (LGL), also called Tγ-lymphoproliferative disease, have demonstrated frequent clonal expansions mostly among TCR-α/β $CD8^+$ T cells (24, 25). These patients are often anemic, have splenomegaly, and abnormal white blood cell counts. They may also have an associated autoimmune disease. None of the normal donors investigated herein had signs of this disorder and their peripheral blood counts and peripheral blood smears were considered normal. Nevertheless, one may speculate that patients with Tγ-lymphoproliferative disease have exaggerated CD8 immune responses that are similar to the clonal expansions among normal subjects described herein.

Previous studies demonstrate that T cells from normal individuals may contain expanded clones, which can be detected if special subpopulations are examined. For instance, human intestinal intraepithelial lymphocytes (IEL) are frequently oligoclonal (26–28), presumably due to chronic stimulation by food and microbial antigens in the gut. Human IEL are primarily $CD8^+$ TCR-α/β T lymphocytes. A subset of human $CD8^+$ IEL are known to express $CD8\alpha\alpha$ homodimers rather than the more usual $CD8\alpha\beta$ heterodimers (reference 26, personal communication from P. Poussier), but CD28 expression by these cells has not yet been studied. Human IEL do not proliferate in vitro in response to TCR/CD3 stimuli (29), although this may depend on the availability of integrins expressed uniquely by intestinal APCs (30). Thus, there are several similarities between $CD8^+$ IEL and the $CD8^+$ $CD28^-$ subset in peripheral blood (21). The latter is absent in cord blood but accumulates variably in adults, in which it may represent 1–15% of PBMCs (14). In view of the results on subject 18 (Figs. 5, 6) and the results with three of five random elderly subjects (Table 3), it is the $CD8^+$ $CD28^-$ subset in the blood that is frequently oligoclonal. It is possible that this PBL subset represents CD8 cells derived from the IEL population, consistent with the absence of a thymus in elderly individuals and the known potential of the gut to serve as a thymic equivalent for maturation of α/β T cells (31).

Two further small subsets of normal adult T cells have been shown to be oligoclonal. $CD4^-8^-$ α/β T cells, which are capable of recognizing bacterial antigens, are often oligoclonal (22, 32) and the expanded clones may persist for several years (22). Lastly, $V\delta1^+$ γ/δ T cells are polyclonal at birth, but become oligoclonal in adults (33). The latter subset are also the predominant γ/δ T cells in the gut (34).

The presence of clonal expansions primarily among $CD8^+$ $CD28^-$ cells, suggests that these cells are specific for MHC class I–restricted antigens in vivo. Since these are commonly intracellular antigens of viral origin, we postulate that the expanded clones represent cytotoxic T cells with specificity for antigens of ubiquitous viruses (EBV, CMV, influenza, etc.). $CD8^+$ $CD28^-$ cells have been shown to lack proliferative responses to CD3/TCR stimuli and display high CTL activity in a redirected cytotoxicity assay anti-CD3–coated target cells (21). Therefore it is possible that repeated exposure to MHC class I–restricted antigens results in clonal accumulation of endstage mature CTL. Dramatic accumulation of CD8 cells has been observed in vivo with mice immunized with human class I transfected cells, in which reactive CD8 clones may represent over 50% of T lymphocytes (35) and show clonally restricted diversity of TCR chains (36). Human CTL lines derived in vitro with influenza peptide presented by HLA-A2 are also oligoclonal and use a restricted TCR repertoire (37). An alternative hypothesis is that the expanded CD8 clones represent autoreactive cells, just like the benign monoclonal gammapathies that frequently represent autoreactive specificities (2). Autoreactive T cells may be increased in frequency in old mice (38) and it has been suggested that the increased expression of HLA class I that occurs with age may lead to autoreactivity (39). Both hypotheses can be tested using the expanded $V\beta$ T cell populations described herein.

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