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Replacement therapy with plasma-derived factor VIII concentrates induces skew in T-cell receptor usage and clonal expansion of CD8+ T-cell in HIV-seronegative hemophilia patients

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Summary

Replacement therapy with factor VIII (FVIII) products causes immune abnormalities in human immunodeficiency virus (HIV)-seronegative hemophilia patients. However, the question remains why an absolute increase in the number of CD8+ T-cells and diminished proliferation responses of lymphocytes to antigen stimulation in vitro occurs in HIV-seronegative hemophilia patients.

To examine whether the FVIII products induce skewing of T-cell receptor (TCR) repertoires, TCR variable region α-chain and β-chain repertoires were analyzed for peripheral blood mononuclear cells (PBMCs) from 15 hemophilia patients treated with heated and/or non-heated plasma-derived FVIII concentrates and 10 age-matched healthy adults. Also, T-cell clonality was compared between these groups using complementarity-determining region 3 (CDR3) size spectratyping. The skewing of TCR repertoires was significantly greater for hemophilia patients than healthy controls. The extent of T-cell clonality was greater for hemophilia patients than the controls, indicating that clonal T-cells frequently expanded in hemophilia patients. The skew in TCR usage and clonal expansion were primarily observed in patients treated with non-heated plasma-derived products.

The spectratyping and sequencing of CDR3 regions revealed that the clonal expansion of T-cells was observed for CD8+ T-cells, but not CD4+ T-cells.

These results suggest that extensive expansion of CD8+ T-cells is induced by some viruses other than HIV present in FVIII preparations, and the resulting accumulation of CD8+ T-cells is responsible for changes in peripheral T-cell population in HIV-seronegative hemophilia patients.

Keywords

Hemophilia, T lymphocytes, T cell receptors, clonal expansion

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Introduction

Hemophilia A is a hereditary X-linked bleeding disorder caused by factor VIII (FVIII) deficiency. Replacement therapy with FVIII concentrates is effective in clinical management of hemophilia patients (1). In recent years, very high-purity FVIII preparations such as recombinant and monoclonal-antibody-purified FVIII have been substituted for plasma-derived FVIII concentrates to reduce the risk of infection with virus, e.g. human immunodeficiency virus (HIV), hepatitis B, and hepatitis C. Although it has long been known that HIV infection modulates immune function, several abnormalities of immune parameters

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have been reported even in HIV-seronegative hemophilia patients (2).

The effects of plasma-derived FVIII concentrates on the immune system have been demonstrated by several researchers (3). It has been suggested that infection by viruses other than HIV, plasma proteins or allogeneic antigens co-transfused with the concentrates lead to the immune abnormalities (2, 4). Abnormal functions of B-cells including an increase in immunoglobulin levels and an increase in activated B-cells have been demonstrated (3, 5, 6). Abnormal functions of monocytes, i.e. down regulation of Fc-receptor and modulation of the phagocyte function, have also been reported (7, 8).

Many T-cell abnormalities have been reported, including a decreased number of CD4+ T-cells, an increased number of CD8+ T-cells, a decrease in CD4/CD8 ratio, and an increase in number of activated T lymphocytes (3,6,9-14). It is a well-known fact that HIV destroys CD4+ T-cells, resulting in a decreased number of CD4 T-cells in HIV-positive patients. However, the question remains why an absolute decrease in the number of CD4+ T-cells or an absolute increase in the number of CD8+ T-cells occurs in HIV-seronegative hemophilia patients. Moreover, diminished proliferation responses of lymphocytes to mitogen stimulation have been demonstrated in HIV-negative hemophilia patients *in vitro*. Recently, several studies have shown that transforming growth factor-β (TGF- β) contaminating plasma-derived FVIII concentrates have a modulating effect on the immune function (15,16). However, there is no convincing evidence that TGF-β or other plasma proteins cause alterations of T-cell population subsets.

T-cells play a major role in immunological function through antigen-recognition. Proliferation of T-cells specific for foreign or allogeneic antigen skews T cell receptor repertoires. In order to examine whether skewed TCR repertoires are observed in the peripheral blood of hemophilia patients, we analyzed TCR variable (V) region repertoires in hemophilia A patients treated with various combinations of FVIII preparations. In addition, we analyzed T-cell clonalities by CDR3 size spectratyping and sequencing of CDR3 regions.

We found that skew of TCR repertoires are significantly greater in hemophilia patients treated with heated and/or non-heated plasma-derived FVIII concentrates than in age-matched healthy donors. Furthermore, the clonal T-cell expansions are more frequently observed in the hemophilia patients. To reveal the immunophenotypical features of the clonal T-cells, we examined the surface markers of clonal T-cells.

Here we report the detection of skewing of TCR repertoires and clonal T-cell expansions with both CD8+CD28+ and CD8+CD28- T-cells, but not CD4+ T-cells. Identical T-cell clones appeared in both these T-cell populations, suggesting that a defective co-stimulation function affects proliferation of T-cells in response to antigens. These findings suggest that repeated infusion of FVIII concentrates causes clonal expansion of CD8+ T-cells and the resulting accumulation of CD8+CD28- T-cells in the peripheral blood of hemophilia patients. This study presents firm evidence of accumulation of CD8+CD28- T-cells causing immunological abnormalities in HIV-negative hemophilia patients.

Materials and methods

Patients

Peripheral blood mononuclear cells (PBMCs) were collected from 15 congenital hemophilia A patients who underwent replacement therapy at Nara Medical University. Patient details are shown in Table 1. The median age was 31 years (range: 11 – 50). All patients in this study were HIV-seronegative. Nine patients (60%) had factor VIII inhibitor (anti-FVIII antibody). The levels of inhibitor at the time of the study ranged from <0.3 to 764 Bethesda units (BU). Twelve patients (80%) had been treated with cryoprecipitates or non-heated plasma-derived factor VIII concentrates until the early 1980s. They have been also treated with heat-inactivated FVIII concentrates since. Seven of the 12 patients have been recently treated with very high-purity FVIII (monoclonal-antibody-purified FVIII or recombinant FVIII). The remaining 3 patients (20%) have been treated with heat-inactivated concentrates or very high-purity FVIII. PBMCs were also obtained from 20 healthy donors (median age 27, range 14 - 51) and used as normal control samples. All samples used in the present study were collected after informed consent had been obtained.

Flow cytometry and cell separations

PBMCs were isolated by the Ficoll gradient centrifugation method from heparinized blood. PBMCs were stained FITC-conjugated anti-CD4 antibody (BD Biosciences, CA), FITC-conjugated anti-CD3 antibody (BD Biosciences) and PE-conjugated anti-CD8 antibody (BD Biosciences). Cell fluorescence was measured with a 2-laser FACScalibur™ (BD Biosciences). Cell separations were performed using MACS beads technology (Miltenyi Biotec GmbH, Germany). CD4+ T-cells were separated from whole PBMC by positive selection with CD4 Microbeads (Miltenyi Biotec). After CD8+ T-cells had been positively isolated with CD8 Microbeads (Miltenyi Biotec), CD8+CD28+ T-cells were separated from CD8+CD28- T-cells by biotin anti-human CD28 antibody (BD Biosciences PharMingen, CA) and streptavidin microbeads (Miltenyi Biotec).

RNA isolation and adaptor-ligation PCR

The methods for isolation of RNA from PBMCs and adaptor ligation-mediated polymerase chain reaction (PCR) were previously reported (17, 18). In short, total RNA was extracted using Trizol LS reagent (Life Technologies, Grand Island, NY) according to the method of Chomczynski & Sacchi (19). The RNA was dissolved in 10 μL of distilled RNase-free water. One

Table 1: Hemophillia A patient details.

Patient No.	Age (year)	Virus infection*		Type**	Factors used for treatment#		
					intermediate-purity or high-purity factors		very high-purity factors
		Hepatitis B	Hepatitis C		non-heated	heat-inactivated	
3	31	+	+	I	Cryo, FVIII(3), PCC(2)	PCC(2)	MabFVIII, RFVII
4	15	+	-	I	-	PCC(2)	RFVIII(2), RFVII(2)
5	39	NA	+	I	Cryo, FVIII, FIX, PCC(2)	PCC(2)	-
8	34	+	+	S	Cryo, FVIII	FVIII	RFVIII(2)
13	11	vd	-	I	-	PCC(2)	MabFVIII, RFVIII(2), RFVII
14	16	-	-	S	-	FVIII(2), PCC(2)	MabFVIII, RFVIII, RFVII(2), PorFVIII
20	24	NA	+	S	FVIII		MabFVIII, RFVIII
22	40	+	+	S	Cryo, FVIII(3)	FVIII(3)	MabFVIII(2), RFVIII(3)
23	20	+	+	S/I	FVIII, PCC(2)	FVIII, PCC(2)	RFVIII
24	50	+	+	I	Cryo, FVIII(3)	FVIII, PCC(2)	RFVIII, RFVII(2)
25	38	+	+	I	Cryo, FVIII, FIX, PCC(3)	PCC(2)	RFVII(2), PorFVIII
26	36	+	+	I	Cryo, FVIII(2), FIX, PCC(3)	PCC(2)	RFVII(2)
30	31	+	+	S/I	Cryo, FVIII, FIX, PCC	FVIII, PCC(2)	MabFVIII(2), RFVIII
32	28	+	+	M	FVIII	FVIII(5)	MabFVIII
33	30	+	+	S	Cryo, FVIII(3)	FVIII(3)	MabFVIII, RFVIII

*: Anti-hepatitis B or C virus antibody, +: positive, -: negative, vd: vaccine done, NA: not available

**: Disease type: S: severe, M: Mild, I: mild with inhibitor, S/I: severe with inhibitor

#: Cryo: Cryoprecipitates, FVIII: plasma-derived factor FVIII concentrate, FIX: plasma derived factor IX concentrate, PCC: prothrombin coagulant complex (PCC) or activated PCC, MabFVIII: monoclonal-antibody-purified FVIII, RFVIII: recombinant factor VIII, RFVII: recombinant factor VII, PorFVIII: porcine FVIII. The number of kinds of preparations used for treatments was indicated by parenthesis.

microgram of total RNA was converted to double-stranded cDNA using Superscript cDNA synthesis kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions, except that a primer for cDNA synthesis (BSL-18) was used. The P10EA/P20EA adaptors were ligated to the 5' end of cDNA and this adaptor-ligated cDNA was cut with *Not* I restriction enzyme, followed by removal of excess linkers and small cDNA fragments by polyethylene glycol precipitation.

PCR and biotinylation of PCR products

PCR was performed in a 50 μL volume containing 1×PCR buffer (10 mM Tris, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 0.2 μM of each primer, 200 μM deoxyriobonucleotides (dNTP) and 1.25 units of Taq polymerase. The first PCR was performed by 20 cycles in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2 min using a one-twentieth volume of double stranded cDNA. The PCR mixture contained 0.2 μM of T-cell receptor α-chain constant region (TCRAC) or T-cell receptor β-chain constant region (TCRBC)-specific primers (CA1 or CB1) and P20EA. The second PCR was performed by 20 cycles under the same conditions using a one-tenth volume of the first PCR product and 1 μM nested primers of CA2 or CB2 and P20EA. A one-hundredth volume of the second PCR product was added to the biotinylated PCR mixture. PCR was performed by 20 cycles using both P20EA and 5'-biotinylated CA4 or CB4 primer under the conditions described above. Amplified PCR products were subjected to 1% agarose gel electrophoresis and ethidium bromide staining.

TCR repertoire analysis

TCRAV and TCRBV repertoires were analyzed by microplate hybridization assay (MHA). The MHA methods have been previously reported (17, 18). In short, 10 pmol of amino-modified oligonucleotides specific for TCRAV and TCRBV segments were immobilized onto carboxylate-modified 96-well microplates (C type, Sumitomo Bakelite, Tokyo, Japan) with water-soluble carbodiimide.

Prehybridization and hybridization were performed in GMC buffer (0.5M Na_2HPO_4, pH7.0, 1 mM EDTA, 7% SDS and 1% BSA) at 47°C. Sixty microliters of denatured 5'-biotinylated PCR products mixed with an equivalent volume of 0.4 N NaOH/10 mM EDTA were added to 6 mL of GMC buffer. One hundred microliters of hybridization solution was used in each well of the microtiter plate which immobilized oligonucleotide probes specific for the V segment. After hybridization, the wells were washed 6 times with washing buffer (2×SSC, 0.1% SDS) at room temperature and then with VA (0.4× SSC, 0.1% SDS) or VB (0.6× SSC, 0.1% SDS) stringency washing buffer for 10 min at 37°C. Two hundred microliters of TB-TBS buffer [10 mM Tris-HCl, 0.5 M NaCl, pH7.4, 0.5% Tween 20 and 0.5% Blocking reagent (Boehringer Mannheim, Germany)] was used to block non-specific binding. One hundred microliters of a 1:1000-diluted, alkaline phosphatase-conjugated streptavidin (Life Technologies, Grand Island, NY) in TB-TBS was added

Table 2a: TCRAV repertoire in PBMCs from hemphilia patients*.

TCRAV probe	healthy controls	hemophilia patients (n=15)														
	mean ± sd	#3	#4	#5	#8	#13	#14	#20	#22	#23	#24	#25	#26	#30	#32	#33
VA1-1	1.0 ± 0.8	2.3	1.2	0.7	0.0	0.6	0.8	1.7	0.9	0.7	0.2	1.5	0.4	1.0	0.0	0.8
VA1-2	1.2 ± 0.5	0.6	1.0	2.2	1.1	1.3	1.4	0.9	1.5	1.1	0.9	0.8	1.4	1.2	1.6	1.7
VA1-3	1.4 ± 0.5	1.2	1.2	1.3	1.3	1.2	1.8	1.1	1.9	1.7	1.3	1.6	2.1	1.4	2.1	1.7
VA1-4	2.3 ± 0.9	2.5	2.4	3.3	4.1	2.3	2.2	1.7	2.5	3.0	2.2	2.6	2.2	1.6	4.7	2.9
VA2-1	3.1 ± 0.8	2.5	3.5	1.4	2.9	1.8	2.2	11.4	2.6	1.8	2.8	2.1	1.7	2.7	4.6	4.2
VA2-2	3.5 ± 1.0	3.1	4.0	4.7	4.0	4.3	3.9	2.3	2.6	4.2	2.3	4.6	5.6	2.2	4.6	1.1
VA2-3	4.5 ± 1.6	4.1	5.7	4.0	3.4	3.0	4.8	3.8	3.8	4.1	3.2	3.6	3.6	7.4	2.6	2.2
VA3-1	4.7 ± 1.0	7.4	3.2	8.1	3.8	2.5	6.1	5.3	4.8	5.5	20.7	4.1	5.0	3.4	3.3	4.9
VA4-1	2.4 ± 1.0	2.0	3.1	1.4	2.0	2.7	4.0	1.7	2.7	1.3	1.0	2.7	1.9	2.4	2.9	1.2
VA4-2	3.0 ± 0.9	2.1	3.7	2.5	4.0	3.8	3.7	2.3	3.4	3.1	0.5	4.7	4.0	2.6	1.7	1.8
VA5-1	1.9 ± 0.7	1.1	1.6	2.0	1.4	2.0	2.2	2.6	2.2	2.5	0.1	2.6	1.8	1.9	2.2	0.7
VA6-1	2.9 ± 1.3	2.0	2.0	2.4	1.8	2.3	2.9	2.0	1.1	4.7	9.5	3.9	0.5	2.2	0.0	9.6
VA7-1	1.7 ± 1.0	1.2	1.3	0.9	2.0	1.2	1.5	1.4	1.8	2.3	0.4	0.4	1.8	1.2	2.6	1.8
VA7-2	2.9 ± 0.9	1.7	2.5	2.7	4.2	8.9	4.5	3.0	2.3	3.6	4.4	2.9	7.9	1.9	2.3	1.8
VA8-1	6.0 ± 1.0	3.7	3.8	5.2	9.4	6.5	4.6	5.1	7.9	3.8	7.6	3.8	6.3	4.5	7.2	3.7
VA8-2	3.0 ± 0.8	2.1	3.4	1.8	7.1	2.9	3.2	2.2	2.8	4.2	4.3	4.8	2.5	2.5	1.2	1.8
VA9-1	1.0 ± 0.2	1.0	0.8	1.6	3.2	0.9	0.5	0.6	1.0	0.8	0.2	1.3	0.9	1.0	0.1	0.9
VA10-1	2.0 ± 1.2	2.4	1.9	5.5	2.5	2.0	1.6	1.1	0.6	1.1	3.0	2.8	1.8	1.1	0.1	1.6
VA11-1	4.2 ± 1.3	4.1	4.0	4.1	3.9	3.6	3.4	4.7	5.5	4.4	1.4	3.1	3.4	1.8	4.2	3.5
VA12-1	2.5 ± 1.0	3.9	2.2	1.9	1.8	2.0	2.9	2.3	1.9	3.0	2.5	3.0	6.2	4.7	1.3	3.4
VA13-1	1.2 ± 0.6	0.8	1.0	0.2	0.4	0.9	1.0	1.8	1.6	1.1	0.1	1.2	1.6	1.0	0.2	2.1
VA14-1	5.0 ± 1.5	3.2	4.7	7.1	3.7	6.0	3.6	3.3	4.1	6.0	3.5	4.6	3.1	3.7	4.7	8.6
VA15-1	1.2 ± 0.7	2.4	0.6	0.8	0.0	1.0	0.9	1.0	0.4	2.0	0.8	0.7	1.1	1.1	1.5	1.2
VA16-1	1.1 ± 0.6	1.1	0.9	1.3	0.7	1.0	0.6	2.8	1.4	1.7	0.2	0.6	0.6	0.8	2.5	1.3
VA17-1	2.0 ± 0.6	5.9	2.9	2.2	0.9	1.3	2.1	1.5	2.8	1.3	1.1	1.6	1.6	3.3	0.0	2.8
VA18-1	1.6 ± 0.7	1.0	1.4	1.4	2.0	1.3	2.3	1.2	1.7	1.1	2.4	1.3	1.4	1.3	1.5	1.3
VA19-1	1.4 ± 0.5	1.1	1.6	0.5	0.8	1.7	1.7	1.9	2.3	0.7	3.0	0.9	0.6	1.3	3.6	0.3
VA20-1	2.2 ± 0.8	2.8	2.7	3.0	2.4	1.9	1.8	2.6	2.5	2.9	1.1	1.7	0.9	1.8	0.8	3.3
VA21-1	4.1 ± 0.9	4.2	4.3	4.5	1.7	2.7	3.4	3.5	3.1	3.5	1.6	4.3	2.9	7.6	3.3	3.5
VA22-1	5.1 ± 1.0	2.4	3.9	5.5	5.7	5.4	4.6	5.4	6.7	2.5	7.4	4.7	4.1	4.4	5.2	3.6
VA23-1	6.4 ± 1.4	7.9	8.1	4.9	2.8	5.8	5.2	4.9	4.9	4.6	5.2	6.7	11.7	8.5	8.1	4.7
VA24-1	2.9 ± 0.9	4.5	3.0	2.3	1.5	3.9	3.0	2.9	3.2	7.2	2.1	4.2	1.3	2.9	0.0	2.7
VA25-1	2.1 ± 0.6	2.1	2.9	0.8	2.6	1.6	1.4	1.6	1.0	1.3	0.0	2.1	1.4	2.3	2.8	2.6
VA26-1	0.6 ± 0.4	0.6	0.6	0.3	0.2	1.6	1.1	0.2	0.1	0.4	0.1	0.9	0.2	0.3	0.0	0.2
VA27-1	0.9 ± 0.4	1.8	1.5	1.2	0.3	0.9	0.9	0.5	1.0	0.8	0.3	1.3	0.6	1.7	2.1	0.6
VA28-1	1.1 ± 0.4	0.6	0.8	1.0	1.7	1.5	0.9	1.0	1.3	1.7	0.0	1.2	1.3	0.7	1.4	0.6
VA29-1	0.6 ± 0.4	1.4	1.0	0.5	2.6	0.4	1.3	0.6	0.5	0.4	0.2	0.0	0.4	0.6	0.5	2.5
VA30-1	2.3 ± 0.9	1.7	2.5	2.9	2.1	3.1	2.0	1.5	3.4	1.2	0.9	1.9	1.4	2.2	8.4	2.8
VA31-1	1.8 ± 0.8	2.0	2.0	1.3	3.7	1.3	1.6	2.7	1.7	1.0	0.8	1.2	2.1	2.1	0.9	1.5
VA32-1	0.5 ± 0.5	0.3	0.4	0.5	0.0	0.5	1.2	0.6	0.7	0.5	0.3	0.8	0.4	0.4	2.7	0.4
VD1	0.3 ± 0.3	0.1	0.1	0.3	0.1	0.2	0.8	0.4	0.1	0.5	0.1	0.5	0.1	0.3	0.0	0.7
VD2	0.2 ± 0.2	0.6	0.3	0.3	0.0	0.2	0.2	0.7	0.2	0.3	0.1	0.2	0.1	0.4	0.1	0.6
VD3	0.1 ± 0.2	0.3	0.0	0.0	0.0	0.1	0.1	0.3	1.4	0.6	0.1	0.2	0.2	2.6	0.2	0.9

*V segments which significantly increased in each patient are underlined.

Table 2b: TCRBV repertoire in PBMCs from hemphilia patients.

TCRBV probe	healthy controls (n=20)	hemophilia patients (n=15)														
	mean ± sd	#3	#4	#5	#8	#13	#14	#20	#22	#23	#24	#25	#26	#30	#32	#33
VB1-1	2.1 ± 0.7	3.8	1.9	1.7	1.2	1.2	2.0	1.1	1.2	2.0	2.1	1.8	3.5	2.6	2.0	3.8
VB2-1	9.0 ± 1.9	16.5	8.9	13.2	8.4	9.0	8.3	6.5	10.4	12.8	5.4	11.8	9.7	4.7	7.2	6.1
VB3-1	5.9 ± 2.7	6.7	6.2	2.2	5.0	6.6	3.2	4.6	6.4	8.3	8.3	3.1	4.6	3.6	4.8	1.5
VB4-1	7.3 ± 2.1	6.1	6.3	6.4	4.5	8.5	7.7	6.1	8.3	12.8	8.7	12.1	7.5	7.4	6.7	6.1
VB5-1	5.7 ± 1.6	5.7	1.9	7.6	3.9	8.8	3.6	8.0	9.3	3.5	6.6	9.4	3.6	2.3	2.8	3.5
VB5-2	1.2 ± 0.4	0.8	0.5	1.0	0.7	1.1	1.5	0.8	0.8	1.6	0.9	1.4	0.7	1.5	1.9	0.9
VB5-3	0.7 ± 0.1	0.2	0.2	0.6	0.5	0.9	1.1	0.7	1.1	0.9	1.0	0.5	0.0	0.5	0.7	1.0
VB5-4	0.2 ± 0.1	0.0	0.2	0.3	0.1	0.2	0.6	0.0	0.3	0.1	0.3	0.3	0.3	0.3	0.5	0.3
VB5-5	1.2 ± 0.5	0.5	1.2	1.4	0.4	1.4	2.1	0.8	1.0	0.2	1.3	0.8	0.9	1.0	3.0	0.8
VB6-1	1.0 ± 0.4	0.9	0.7	1.0	1.4	1.1	1.7	0.8	0.7	0.9	0.6	0.5	0.8	1.0	0.4	0.8
VB6-2	1.5 ± 0.5	1.4	1.2	1.3	1.3	1.2	1.8	1.4	1.3	1.3	1.1	1.3	1.3	1.6	0.9	1.2
VB6-3	1.1 ± 0.3	1.0	1.3	1.1	0.5	1.1	1.1	1.6	0.8	0.8	1.3	1.1	1.3	0.9	0.5	1.5
VB6-4	7.2 ± 1.9	5.0	7.8	7.4	12.4	6.7	4.8	9.4	6.4	5.9	6.8	5.4	4.3	5.7	6.4	6.3
VB6-5	2.2 ± 0.7	2.2	6.2	1.8	1.0	1.5	2.5	2.9	2.4	1.3	1.4	2.2	1.9	3.6	2.0	2.1
VB7-1	3.8 ± 1.3	2.8	7.6	5.9	1.6	2.1	7.6	2.4	4.1	2.4	1.5	2.1	5.0	4.1	2.3	7.9
VB8-1	5.2 ± 1.6	5.3	5.0	5.9	7.3	3.9	3.2	3.9	5.9	2.6	1.2	4.2	4.5	5.5	3.4	4.7
VB8-2	0.8 ± 0.3	0.0	0.8	1.1	0.0	0.9	0.8	0.5	0.6	0.3	0.6	0.4	1.4	0.7	0.7	0.5
VB9-1	3.0 ± 1.4	2.7	2.2	2.2	1.7	3.1	3.6	2.5	2.2	1.7	6.5	2.9	2.5	2.8	2.7	3.0
VB10-1	1.8 ± 0.5	0.9	0.9	1.7	1.7	2.1	1.8	0.7	2.2	1.6	3.1	2.2	2.1	0.9	1.4	0.7
VB11-1	1.3 ± 0.4	1.0	1.0	1.2	0.8	1.2	2.6	0.9	1.2	1.7	2.0	2.8	2.8	1.1	1.6	1.6
VB12-1	3.6 ± 0.8	4.6	3.6	2.8	3.0	3.3	4.2	3.3	2.8	3.0	3.0	3.4	4.2	3.5	4.2	3.1
VB13-1	5.4 ± 1.0	5.1	5.0	2.4	4.1	4.7	5.0	3.9	5.0	4.6	3.3	3.2	2.5	8.5	4.3	3.6
VB13-2	2.3 ± 0.8	1.4	4.2	3.3	0.7	2.2	3.6	0.7	3.1	1.8	5.5	2.0	2.1	1.9	4.0	4.2
VB13-3	2.1 ± 0.9	2.4	1.8	1.6	1.3	1.5	2.0	2.5	1.8	1.8	1.5	2.0	1.0	3.2	1.8	4.0
VB13-4	1.5 ± 0.7	1.5	1.3	1.1	0.8	3.3	5.3	1.8	1.0	2.2	4.5	1.3	2.1	1.5	2.3	1.5
VB14-1	2.8 ± 0.9	3.3	3.1	1.8	2.1	2.9	2.1	4.1	1.4	1.9	1.5	2.2	2.1	4.0	2.6	3.9
VB15-1	1.8 ± 0.6	1.7	2.1	1.9	19.1	1.7	0.9	2.9	1.0	0.8	2.6	2.0	8.1	1.1	2.0	2.2
VB16-1	1.8 ± 0.8	0.8	1.1	1.4	2.9	1.7	1.5	6.8	2.5	2.1	1.6	2.9	2.5	2.0	1.6	1.7
VB17-1	4.7 ± 1.7	4.3	5.9	4.0	3.3	4.6	2.4	4.7	3.5	5.4	3.7	2.7	3.0	5.5	6.7	5.2
VB18-1	2.9 ± 0.6	2.4	2.7	2.6	1.4	2.6	3.1	4.7	4.2	3.9	2.5	3.3	2.0	3.3	5.2	2.6
VB19-1	1.4 ± 0.8	0.7	0.8	1.3	0.9	1.2	2.4	1.2	1.3	2.7	2.0	1.7	1.0	1.4	1.7	2.3
VB20-1	1.0 ± 0.4	0.8	0.7	0.9	0.4	0.8	1.3	0.8	0.6	0.9	1.3	0.7	1.2	0.6	1.5	2.0
VB21-1	1.8 ± 0.4	1.3	1.5	3.6	2.2	1.8	1.4	1.3	1.4	1.7	1.8	1.3	2.2	1.3	3.1	1.3
VB22-1	0.7 ± 0.3	0.4	0.6	0.4	0.3	0.7	0.3	0.4	0.5	0.5	0.4	0.5	0.3	1.5	1.0	1.4
VB23-1	2.4 ± 1.0	3.4	2.0	2.4	2.0	2.1	1.3	1.6	1.9	2.1	3.3	3.0	5.2	3.7	3.7	1.1
VB24-1	1.6 ± 0.5	2.7	1.6	3.2	1.2	2.2	1.3	2.9	1.4	1.2	0.9	1.1	1.2	5.3	2.5	3.0
VB25-1	0.2 ± 0.2	0.0	0.1	0.2	0.0	0.0	0.2	0.6	0.0	0.7	0.2	0.4	0.6	0.0	0.0	2.6

V segments which significantly increased in each patient are underlined.

and incubated at 37°C for 30 min. The plates were washed 6 times in T-TBS (10 mM Tris-HCl, 0.5 M NaCl, pH7.4, 0.5% Tween 20). One hundred microliters of substrate solution (4 mg/mL p-nitrophenylphosphate, Sigma, in 20% diethanolamine, pH9.8) was added for colour development and then absorbance was determined at 405 nm.

Repertoire skewing and delta score

The delta score was used as an indicator to evaluate the extent of skewing of TCR repertoires. The delta score indicates the sum of absolute differences between the frequency of respective V segments in individual patients and the mean frequency in 20 healthy donors. A low delta score indicates similarity between patients and healthy donors, whereas a high score indicates dissimilarity. The delta score was used in our previous report (18). We use the term "skewing of repertoire" to describe changes at the level of V gene usage. On the other hand, we use the term "clonality" for changes at the level of clonotype usage obtained with the CDR3 size spectratyping method.

CDR3 size spectratyping

PCR for CDR3 size spectratyping was performed by 30 cycles in a 20-μL volume under the same conditions as described above. PCR was performed with 1 μL of the second PCR product, 0.1 μM of 5'-Cy5 CA2/CB2, and 0.1 μM primer specific for each variable segment. The oligonucleotide probes for hybridization were used as primers specific for each variable segment (17). Five microliters of 1:20 or 1:50 diluted PCR product in dye solution (95% formamide, 10 mM EDTA and 0.1% blue dextrane) was analyzed in 6% denatured acrylamide gel with an ALFred sequence analyzer (Pharmacia Biotech, Uppsala, Sweden). The data obtained were transferred to Fragment manager software (Pharmacia Biotech). As a control, PBMCs from 10 healthy donors were also analyzed for the peak patterns, revealing multiple peaks as Gaussian patterns with 3-nucleotide intervals in each V segment.

Sequencing analysis

PCR was performed with a forward primer specific for the TCR variable-region and a reverse primer specific for the constant-region under conditions described above. Primers used in this study were as follows: VB2-1: TCATCAACCATGCAAGCCTGA, VB7-1: CTGAATGCCCCAACAGCTCT, VB8-1: TTTACTTTAACAACAACGTTCCG, VB11-1: ATAAGGACGGAGCATTTTCCC, VB17-1: CACAGATAGTAAATGACTTTCAG, VB21-1: TCTGCAGAGAGGCTCAAAGG, VB23-1: AATCTTGGGGCAGAAAGTCGA, VB24-1: GCCCCAAAGCTGCTGTTCC, CB4: ACACCAGTGTGGCCTTTTGGGTG. After the PCR products were eluted from agarose gel, the PCR products were cloned into pGEM-T vector using TA-cloning kit (pGEM-T system, Promega, MI). DH5α competent cells (Life Technologies, Grand Island, NY) were transformed with the recombinant plasmid DNA. Sequencing reactions were performed with a BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, CA) and analyzed by ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Statistical analysis

Data were statistically analyzed by Wilcoxon rank-sum test (20).

Results

All patients were HIV-seronegative. Anti-hepatitis C antibodies were detected in all of the 12 patients who had been treated with cryoprecipitates or non-heated plasma-derived FVIII concentrates until the early 1980s, but not in the 3 patients who had

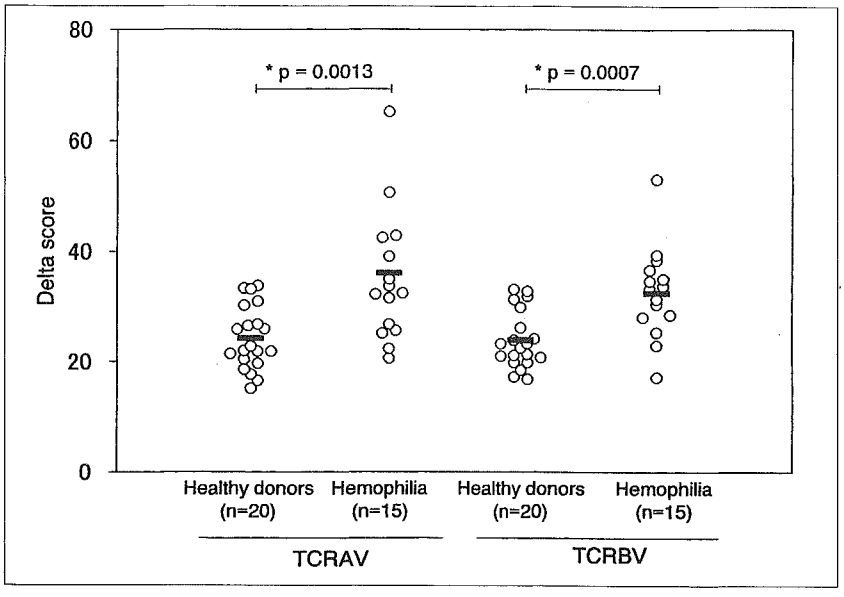

Figure 1: Comparison of the extent of skew in TCRAV and TCRBV repertoires between hemophilia patients and healthy controls. The delta score was used as an indicator to evaluate the extent of skew of TCR repertoires as described in materials and methods. The delta score of TCRAV and TCRBV was significantly greater in the hemophilia patients (n = 15) than in the healthy donors (n = 20). Each open circle indicates a single patient. Horizontal bars indicate mean delta scores.

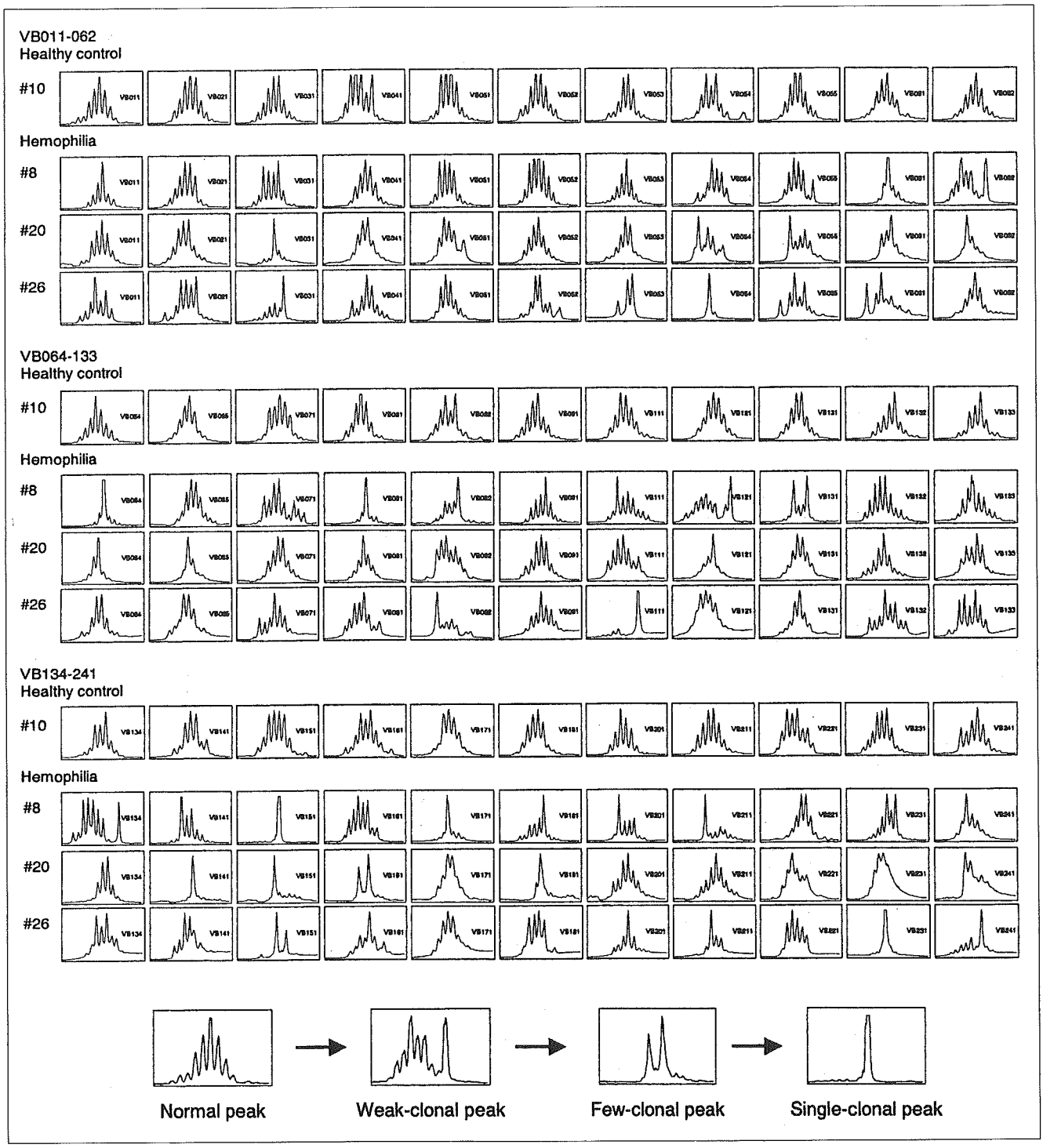

Figure 2: CDR3 size spectratyping profiles of TCRBV
Representative results are shown. The peak patterns obtained by each primer specific for BV segments are displayed in boxes. The results from a healthy control (#10) and three hemophiliacs (#8, #20 and #26) are shown. Examples of normal, weak-clonal, few-clonal and single-clonal peak pattern were shown in the bottom of figure.

been treated with only heat-inactivated FVIII preparations. The clonality analyses were performed with PBMCs from 10 healthy donors and used as normal controls. The ages of the 10 healthy donors ranged from 17 to 51 (median: 26). There was no significant difference in age between the hemophilia patients and the healthy donors.

TCRAV and TCRBV repertoires were analyzed in PBMCs from the 15 patients by MHA. We defined the increase as signif-

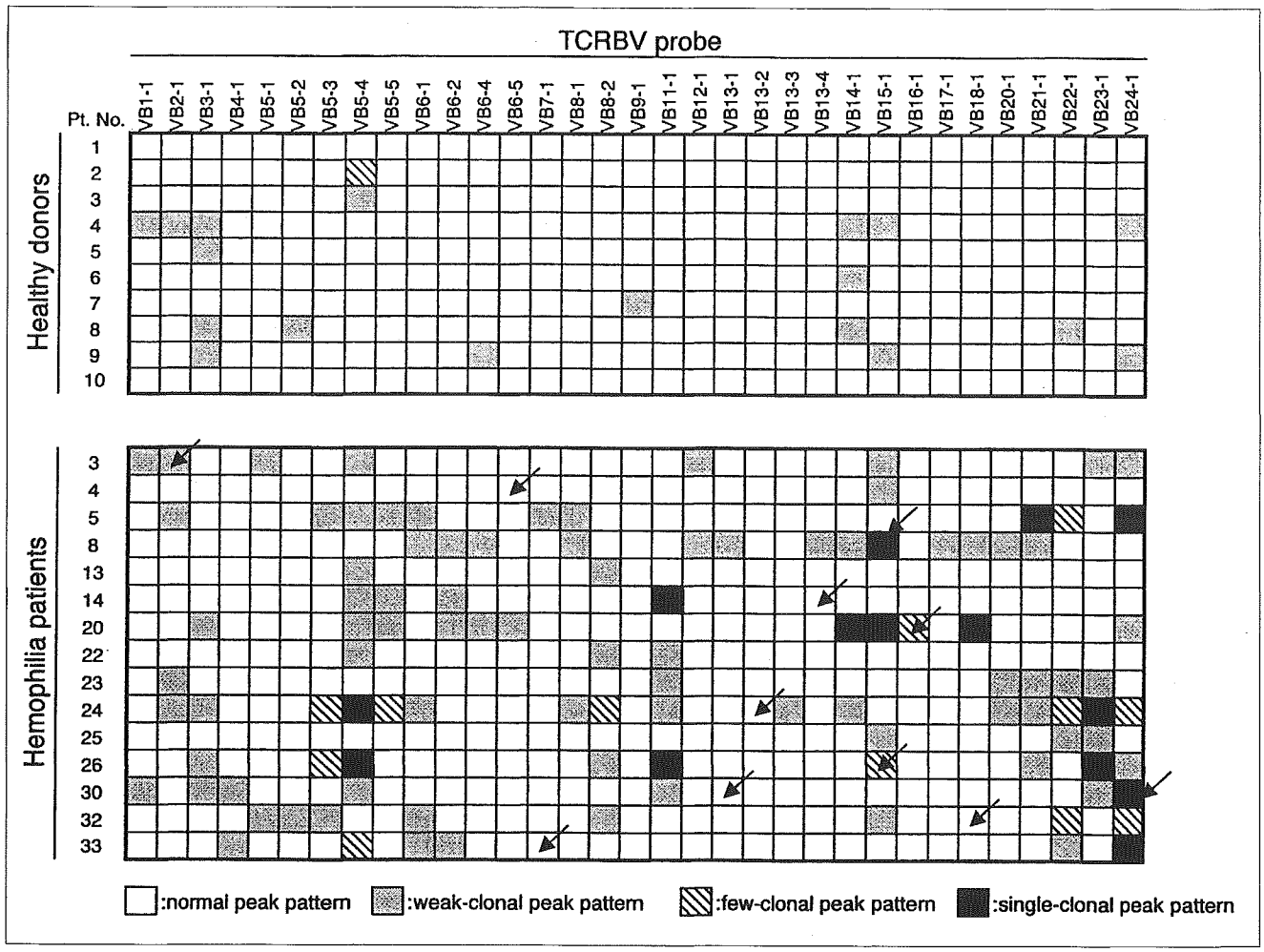

Figure 3: Summary of CDR3 size spectratyping results
The results of 825 analyses using PBMCs from 10 healthy donors and 15 hemophilia patients are shown (upper: healthy adults, n = 10, bottom: hemophilia patients, n = 15). For comparison, the patterns obtained by CDR3 size spectratyping were classified into four groups based on the number of major and minor peaks: normal pattern (open box), weak-clonal peak pattern (light dark box), few-clonal peak pattern (middle dark box), and single-clonal peak pattern (dark box). The normal pattern displays multiple peaks with a Gaussian pattern with 3-nucleotide intervals. When a weak clonal T-cell expansion occurs, a single peak appears with multiple low peaks, and this suggests a moderate level of clonality (weak-clonal peak pattern). A few dominant peaks suggest intermediate levels of clonality (few-clonal peaks pattern). A single peak suggests the highest level of clonality (single-clonal peaks pattern). Interpretable results were not obtained for VB10-1 and VB19-1 because these segments are probably pseudogenes. The results obtained for VB25-1 were excluded because a skewed peak pattern was often detected in the healthy donors due to extremely low frequencies. The analysis results were obtained from 33 BV segments but these 4 segments were summarized. The arrows indicate 11 BV segments in which skewing of repertoire at the level of V gene usage was observed.

icant, when (1) the percentage was greater than the mean percentage + 3 standard deviations of 20 healthy controls, and (2) the absolute percentage was >5%. The expression level was defined as unchanged unless the numbers fulfilled these two criteria. There were significant increases in the usage of TCRAV and TCRBV repertoires in the hemophilia patients (Table 2A and Table 2B). Significant increases in the frequencies of one or more TCRAV and TCRBV segments were detected in 11 (73%) and 10 (67%) of the 15 hemophilia patients, respectively. There was no restricted usage of TCRAV and TCRBV among these patients.

The average numbers of AV and BV segments, the frequencies of which significantly increased above the control levels, were 1.00 ± 0.85 (mean ± SD) and 0.73 ± 0.59 per patient, respectively. This result suggests that the skew in the usage of TCR repertoires was greater in hemophilia patients treated with plasma-derived FVIII than in healthy donors.

We compared the extent of the skew in the usage of TCR repertoires with the delta score as an indicator (18). There was a significant correlation between the delta scores of TCRAV and those of TCRBV in each donor. The delta scores of TCRAV and TCRBV ranged from 15.1 to 33.4 (mean: 25.1) for TCRAV rep-

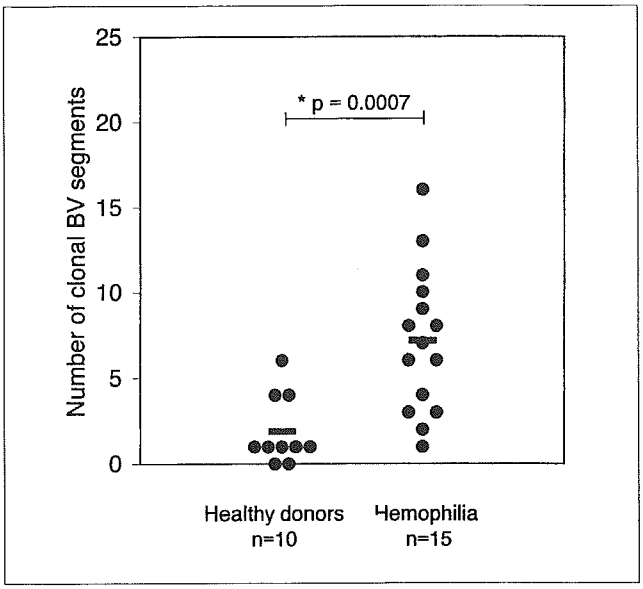

Figure 4: Comparison of the number of TCRBV demonstrating the clonal expansion T-cell expansion patterns among healthy donors and hemophilia patients.
Clonal expansion of T-cells was more frequently observed in hemophilia patients treated with plasma-derived FVIII concentrates (n = 15) than in healthy donors (n = 10). Each closed dot indicates a single patient. Horizontal bars indicate the mean number.

ertoires and from 17.2 to 33.1 (mean: 24.1) for TCRBV in 20 healthy donors, respectively. The delta scores of TCRAV and TCRBV were significantly higher in the hemophilia patients than in the healthy donors, respectively [35.1 ± 11.8 vs. 25.1 ± 7.8 (p = 0.0013) for TCRAV, 32.5 ± 8.2 vs. 24.1 ± 5.5 (p = 0.0007), for TCRBV] (Fig. 1). This result suggests that TCR repertoires were highly skewed in hemophilia patients treated with unpurified preparations derived from plasma concentrates.

We tried to determine whether the skew in the usage of TCR repertoires was due to clonal T-cell expansion. T-cell clonality was analyzed by CDR3 size spectratyping with primers specific for the 37 BV segments, with the representative results shown in Fig. 2. In a healthy donor, normal patterns were detected in all the variable segments. In patients #8, #20 and #26 treated with plasma-derived FVIII concentrates, clonal peak patterns including the weak-clonal peak, few-clonal peak and single-clonal peak patterns were found in a large number of variable segments. Figure 3 summarizes the analyses using PBMCs from 10 healthy donors and 15 hemophilia patients. The normal pattern was observed in the majority of the analyses using PBMCs from the normal adults. In contrast, abnormal patterns of a single or a few dominant peaks were observed in multiple variable segments using PBMCs from the hemophilia patients. Evidence of clonal expansion was observed in 107 (21.6%) of the 495 analyses of hemophilia patients, but in only 19 (5.8%) of the 330 analyses of healthy donors (Fig. 3). The number of TCRBV with clonal T-cell expansion patterns was significantly higher for the hemophilia patients than the healthy donors (7.1 ± 4.2 vs. 1.9 ± 2.0 per patients, p = 0.0007) (Fig. 4). To examine the impact of heat pasteurization on T-cell clonality, we compared the number of TCRBV demonstrating the clonal T-cell expansion patterns between three patients (#4, #13, and #14) treated only with the heat-inactivated preparations and 12 patients treated with cryoprecipitates or non-heated plasma-derived concentrates. There was a significant difference in the level of T-cell clonality between these two patient groups (2.3 ± 1.5 vs. 8.3 ± 3.8 per patient, p = 0.02). We also examined the relationship between the number of BV segments demonstrating the clonal T-cell expansion patterns and several parameters, such as the presence of inhibitor and disease type. However, there were no relationships between the clonal changes and the presence of inhibitor or the disease type. These results suggest that T-cell clonality is related to the purity of FVIII products used for replacement therapy but not to aging and other factors.

Three patterns suggesting clonal expansion (weak-clonal peak pattern, few-clonal peak pattern and single-clonal peak pattern) were observed for 5 (45%) of the 11 BV segments, which was significantly greater than the controls. These results strongly suggest that the skew in the usage of TCR repertoires is due to the clonal expansion of T-cells. A significant increase in the frequencies was observed in 5 (4%) of the 126 analyses in which clonal expansion was suggested by CDR3 size spectratyping. We believe that the levels of clonal expansion in these segments were not high enough to change the percentage. There was a significant correlation between the levels of clonal T-cell expansion and the delta scores. This result strongly suggests that the skew in the usage of TCR repertoires was caused by clonal expansion of T-cells. The clonal patterns demonstrating clonal expansion were detected in almost all the variable segments, but more frequently in VB5-4 (10 of the 15 patients, 67%), VB15-1 (7/15, 47%), VB24-1 (8/15, 53%). This result suggests that clonally expanding T-cells do not use restricted TCR repertoires.

We observed an increase in the percent frequencies of CD8+ T-cells (42.2% for patient 8, 42.2% for patient 30) and a decrease in the CD4/CD8 ratio (0.9 for patient 8, 0.8 for patient 30) with flow cytometry. This suggested the relation of the clonal T-cells observed with CDR3 size spectratyping to CD8+ T-cells.

To elucidate the phenotypical features of the clonal T-cells, we examined TCRAV and TCRBV repertoires with peripheral blood mononuclear cell (PBMC), CD4+ T-cells, CD8+CD28+ T-cells, and CD8+CD28- T-cells separated from the same hemophilia patient. For Patient 23, we analyzed TCR repertoires within CD4+ T-cells and CD8+ T-cells separated from PBMC after 1 year. The levels of expression in VA2-1, 6-1, 7-2, 12-1, 16-1 and 21-1 were definitely higher in CD8+ T-cells than in CD4+ T-cells (Fig. 5A). The expression levels of VB2-1, 4-1, 6-4, 7-1, 11-1, 13-1, 17-1 and 23-1 were also higher in CD8+ T-cells than in CD4+ T-cells (Fig. 5B). These results indicated that there

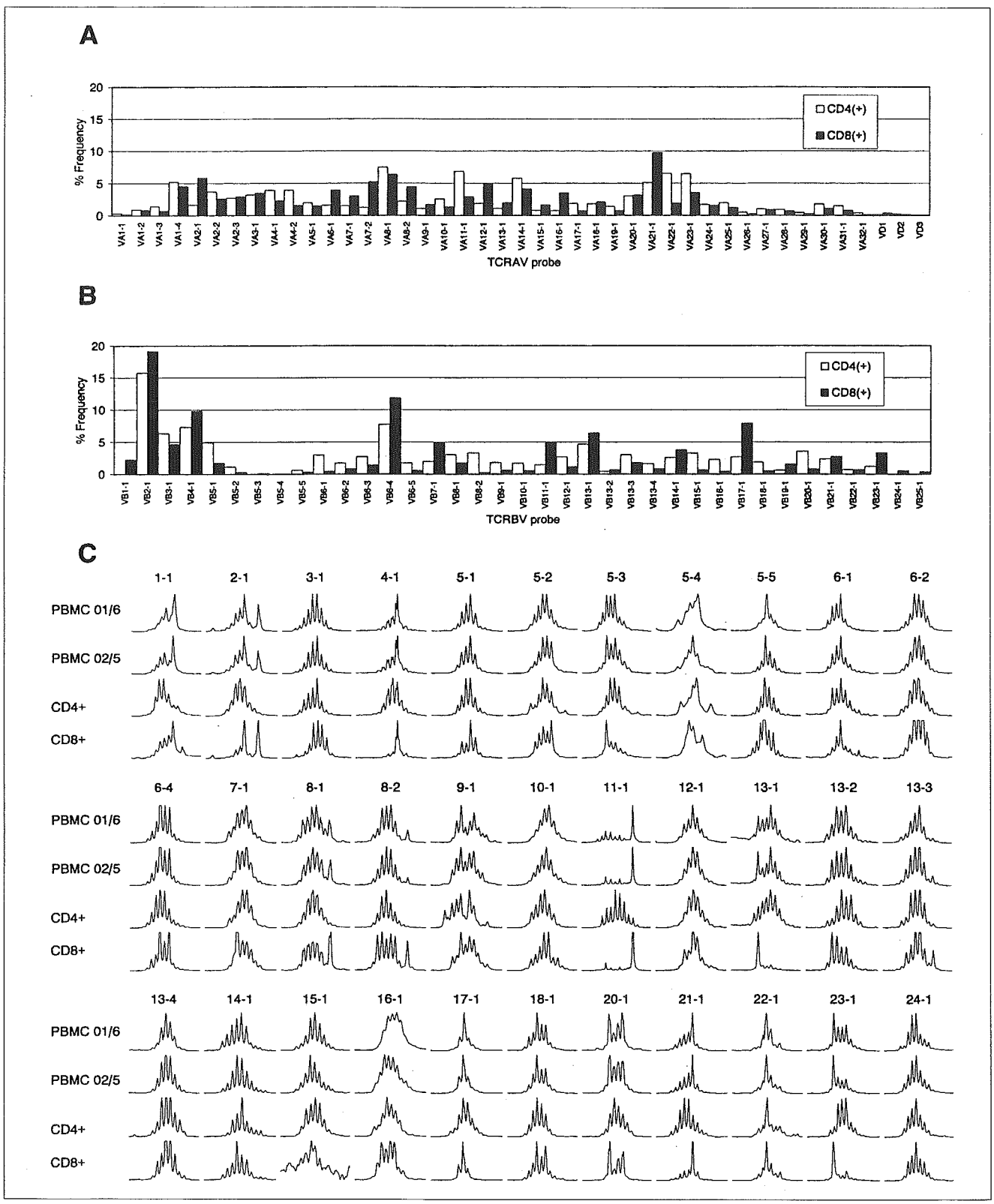

Figure 5: TCRAV (A) and TCRBV (B) repertoires, and CDR3 size spectratyping profiles (C) in whole PBMC, CD4+ T-cells and CD8+ T-cells from hemophilia Patient 23. Each plot represents the results obtained with T-cell fractions separated from whole PBMC of Patient 23. PBMC was collected twice from Patient 23 at an interval of one year (01/6 and 02/5). CD4+ and CD8+ T-cells were separated from whole PBMC (02/5) after one year. Each bar indicates percent frequencies of expression in individual AV (A) and BV (B) segment. The T-cell clonality was examined in each T-cell fraction with CDR3 size spectratyping method (C). The peak patterns obtained with each primer specific for BV segments are displayed in line.

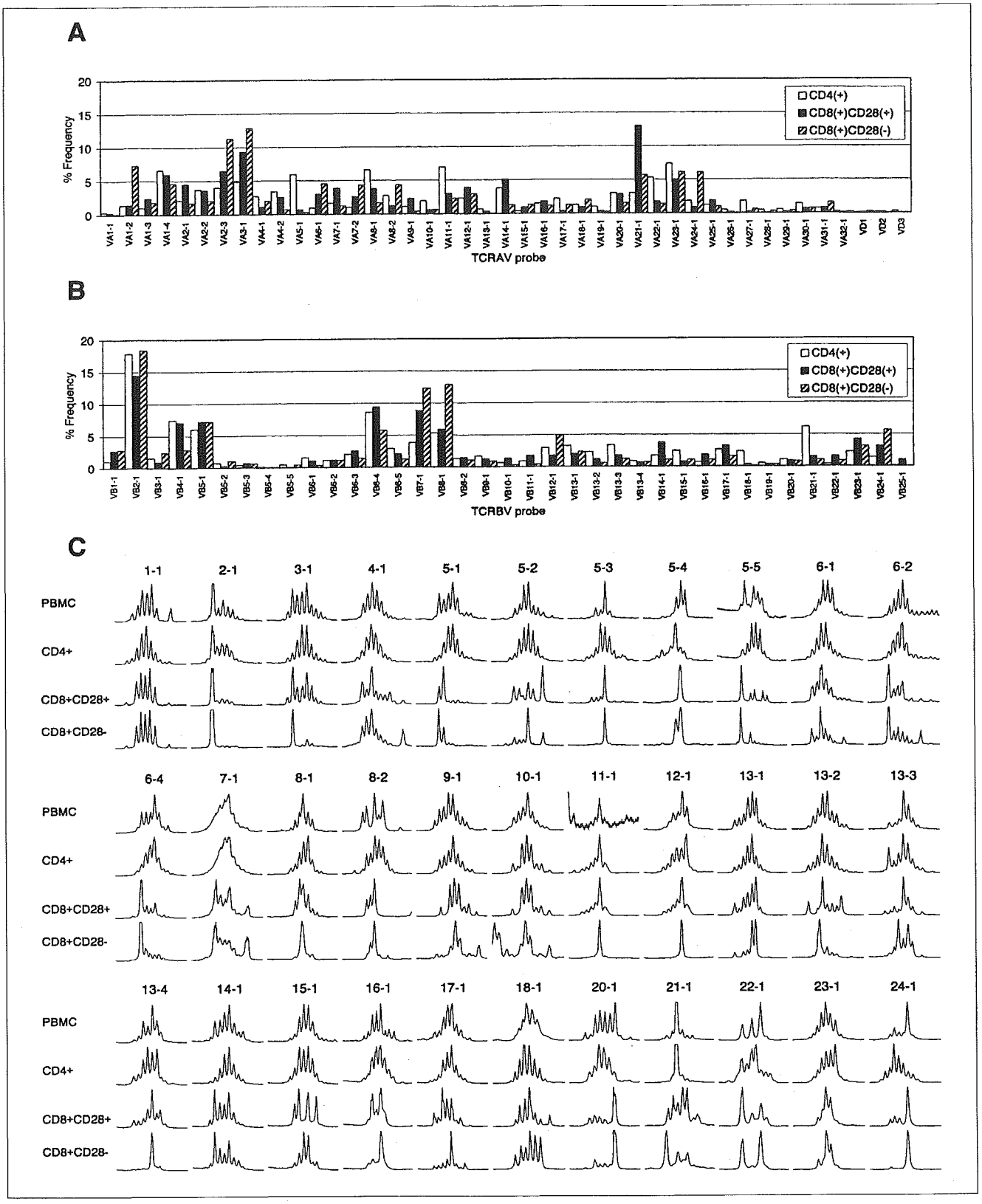

Figure 6: TCRAV (A) and TCRBV (B) repertoires CDR3 size spectratyping profiles (C) in whole PBMC, CD4+, CD8+CD28+ and CD8+CD28- T-cells from hemophilia Patient 5. Each plot represents the results obtained with T-cell fractions separated from whole PBMC from Patient 5. Each bar indicates percent frequencies of expression in individual AV (A) and BV (B) segment. The T-cell clonality was examined in each T-cell fractions with CDR3 size spectratyping method (C). The peak patterns obtained with each primer specific for BV segments are displayed in line.

Table 3: Frequency of occurrence of T-cell clones among PBMC, CD4+, CD8+CD28+, CD8+CD28-subpopulations.

		Frequency*					CDR3 Region				
Patient	BV probe	PBMC 1/6	PBMC 2/5	CD4+	CD8+	BV Seg.	V Seq.	N (D-N-D)	J seq.	BJ Seg.	BC Seg.
23	VB2-1	1/11	3/12	0/11	4/11	BV2S1	YICSAR	ETRGF	QETQYFG	BJ2S5	CB2
		0/11	1/12	0/11	3/11	BV2S1	YICSAR	GQGSLWPD	TDTQYFG	BJ2S3	CB2
	BV11-1	4/11	5/11	0/12	9/10	BV11S1	YLCASSE	SFGKGD	SNQPQHFG	BJ1S5	CB1
		0/11	1/11	0/12	1/10	BV11S1	YLCASS	VGRMD	YNEQFFG	BJ2S1	CB2
	VB13-1	1/12	2/12	0/11	5/12	BV13S6	YFCA	TNGD	SYEQYFG	BJ2S7	CB2
	VB17-1	7/12	4/10	0/11	6/10	BV17S1	YLCASSI	IG	SYNEQFFG	BJ2S1	CB2
		1/12	1/10	0/11	1/10	BV17S1	YLCASS	RRTGLLH	TQYFG	BJ2S3	CB2
		1/12	0/10	0/11	1/10	BV17S1	YLCASSI	ALAH	NEQFFG	BJ2S1	CB1
		1/12	1/10	0/11	0/10	BV17S1	YLCASS	TGPP	YGYTFG	BJ1S2	CB1
	VB21-1	2/5	0/5	0/6	3/8	BV21S3	YLCASSL	YDGTGG	YEQYFG	BJ2S7	CB2
	VB23-1	0/12	5/11	0/11	5/12	BV23S1	YFCASS	AIE	TEAFFG	BJ1S1	CB1
		PBMC	CD4(+)	CD8(+) CD28(+)	CD8(+) CD28(-)						
5	VB2-1	4/7	0/8	9/11	10/10	BV2S1	YICSA	SIGGG	EQYFG	BJ2S7	CB2
		1/7	0/8	1/11	0/10	BV2S1	YICSA	SLGGP	EQFFG	BJ2S1	CB2
	VB7-1	2/4	0/11	4/11	8/11	BV7S3	YLCASSQ	GTSGA	YEQYFG	BJ2S7	CB2
		0/4	0/11	2/11	1/11	BV7S2	YLCASSQ	EPWTSGAL	EQFFG	BJ2S1	CB2
	VB8-1	0/8	0/10	2/9	6/10	BV8S1	YFCAS	TPEGGG	DTQYFG	BJ2S3	CB2
		1/8	0/10	1/9	1/10	BV8S1	YFCASS	PKRQV	QETQYFG	BJ2S5	CB2
	VB24-1	2/6	0/5	1/4	2/10	BV24S1	YLCATSR	VGTEW	SYNEQFFG	BJ2S1	CB2
		0/6	0/5	1/4	4/10	BV24S1	YLCATSR	VGTAF	SYNEQFFG	BJ2S1	CB2

*: The number of cDNA clones bearing identical sequence of CDR3 region/the number of cDNA clones analysed

were definite differences of TCRAV and TCRBV repertoires between CD4+ T-cells and CD8+ T-cells. Furthermore, we examined T-cell clonality within these T-cells by using the CDR3 size spectratyping method (Fig. 5C). Overall, the spectratyping patterns for PBMC were similar to those obtained after 1 year. In both PBMC samples, weak clonal peaks were detected in many BV segments, i.e. VB1-1, 2-1, 11-1, 20-1, 21-1, 22-1, and 23-1. This indicated that T-cells clonally expanded in PBMC and these clonal T-cells persisted in the periphery over 1 year. These clonal peaks, detected in whole PBMC, were detected within CD8+ T-cells, whereas polyclonal peak patterns were obtained for CD4+ T-cells. These clonal peaks were identical in size between PBMC and CD8+ T-cells. The extents of clonality were obviously higher in CD8+ T-cells than in PBMC. This indicated that the skewing of TCR repertoires and the occurrences of the clonal T-cells were due to clonal expansion of CD8+ T-cell, but not of CD4+ T-cells.

For Patient 5, CD8+ T-cells were separated into CD8+CD28+ and CD8+CD28- T-cells using microbeads technology. Subsequently, TCRAV and TCRBV repertoires were analyzed for CD4+, CD8+CD28+ T-cells and CD8+CD28- T-cells. For TCRAV, the frequencies of VA1-2, 2-3, 3-1, 8-2, 24-1 were higher in CD8+CD28- T-cells than in CD4+ and CD8+CD28+ T-cells (Fig. 6A). For TCRBV, the frequencies of 7-1, 8-1, 12-1 and 24-1 were higher in CD8+CD28- T-cells than in CD4+, CD8+CD28+ T-cells (Fig. 6B). Clonality analyses were performed with PBMC, CD4+, CD8+CD28+ and CD8+CD28- T-cells obtained from the same patient (Fig. 6C). In PBMC, clonal peaks were detected in many BV segments, i.e. VB2-1, 5-3, 5-4, 5-5, 7-1, 8-1, 21-1, 22-1, 24-1. There were similarities of CDR3 size spectratyping patterns between CD8+CD28+ T-cells and CD8+CD28- T-cells. In addition, peaks of identical size were detected among PBMC, CD8+CD28+ T-cells and CD8+CD28- T-cells. The extent of clonality was distinctly greater in CD8+CD28- T-cells than in CD8+CD28+ T-cells. Higher levels of expression in CD8+CD28- T-cells were observed for VB2-1, 7-1, 8-1, 12-1 and 24-1 compared with CD8+CD28+ T-cells. The extents of clonality in these BV segments were higher in CD8+CD28- than in CD8+CD28+ T-cells. This finding indicated that clonal expansion of CD8+CD28- T-cells causes skewing of TCR repertoires observed in PBMC of hemophilia patients.

To confirm whether clonal T-cells are identical at the sequence levels between whole PBMC and CD8+ T-cells, nucleotide sequences of CDR3 regions were determined among multiple cDNA clones that carried BV segments demonstrating clonal patterns (Table 3). For VB2-1, 11-1, 13-1, 17-1 and 21-1 of Patient 23, identical cDNA clones were obtained from two PBMC samples. This indicated persistent peripheral circulation of the clonal T-cells over about a year. In addition, the clonal T-

cell clones were more frequently detected within CD8+ T-cells than in PBMC, but not at all in CD4+ T-cells. For VB2-1, 7-1, 8-1 and 24-1 of Patient 5, identical amino acid sequences of CDR3 regions were obtained from multiple cDNA clones separately isolated from whole PBMC, CD8+CD28+ T-cells and CD8+CD28- T-cells. The frequency in the number of cDNA clones was higher for CD8+CD28- T-cells than CD8+CD28+ T-cells or whole PBMC. This indicated that identical T-cell clones existed within both CD8+CD28+ and CD8+CD28- T-cell populations, and that the majority of clonal T-cells were included in CD8+CD28- T-cell populations.

Discussion

Several immune abnormalities have been demonstrated in HIV-seronegative hemophilia patients treated with plasma-derived factor VIII (FVIII) (2, 6, 9, 14). However, the mechanism and causative agent(s) involved remain unclear. Abnormalities have been demonstrated by immunophenotyping with monoclonal antibodies such as anti-CD4, CD8 and DR (14, 21), IgM and IgG levels (14), or lymphocyte proliferation assay after stimulation by antibody or mitogens (14). The changes in numbers of CD4+ and CD8+ T-cells, a decrease in the CD4/CD8 ratio, and an increase in the number of activated T lymphocytes have been reported (6,9-12, 14). These studies demonstrated an abnormal number of T-cell population subsets, but not changes at clonal levels in the T-cell population.

In the present study, we first showed skew in the usage of TCR repertoires and clonal T-cell expansion in HIV-negative hemophilia patients. Delta scores, which signify the extent of skew in a TCR repertoire, were significantly greater for patients treated with the plasma-derived FVIII concentrates than for age-matched, healthy donors. This demonstrates that the skew in TCR repertoires was induced by the FVIII concentrates, but not by aging. It also suggests that the FVIII preparations may contain non-FVIII factor(s) that can induce skewing in TCR repertoires. The skewing was detected in both TCRAV and TCRBV repertoires, and thus may not be caused by some superantigen which induces polyclonal proliferation of T-cells bearing certain BV segments. Clonal T-cell expansion was observed in most of the BV segments, the frequencies of which significantly increased. This finding suggests that these T-cell clones expanded enough to cause skewing of the TCR repertoires. However, no restricted usage of TCRAV and TCRBV repertoires was found in the hemophilia patients in the present study. Various segments were used by clonally expanding T-cells in peripheral blood. T-cells may have used distinct TCRBV repertoires in each patient since the HLA varied among the patients.

The possibility exists that virus contaminating the FVIII concentrates induced clonal expansion of T-cells. We demonstrated that clonal T-cells were more frequently observed in CD8+ T-cells than in CD4+ T-cells. The phenotypic feature of the clonal T-cells is CD8+ T-cells. CD8+ T-cells recognize endogenous self-antigen or viral antigens present in the context of MHC class I by antigen-presenting cells and play an essential role in protection against viral infection. This strongly suggests that virus contaminating the FVIII concentrates induces clonal expansion of T-cells. Infection by virus, e.g. hepatitis B (HBV), hepatitis C (HCV) and parvovirus, has been associated with immune abnormalities in hemophiliacs (22-24). The rates of positive results in serologic tests for HBV and HCV were higher in patients treated with the FVIII concentrates (Table 1). This points to the possibility that infection by these hepatitis viruses causes clonal expansion of T-cells. Recently, clonal expansion of T-cells has been detected with CDR3 size spectratyping analysis in liver specimens of patients with chronic hepatitis C (25), offering support for the possibility of HCV inducing the clonal expansion of T-cells found in the present study. All patients treated with the non-heated concentrates were HCV-positive, whereas none of 3 patients (#4, #13, and #14) treated with only heat-inactivated preparations was. The low level of T-cell clonality in patients treated with the heat-inactivated concentrates seems to imply that virus contamination in the concentrates can induce clonal expansion of T-cells in hemophilia patients. For definite evidence of HCV infection leading to clonal expansion of T-cells, there is a need to analyze T-cell clonality in the peripheral blood of HCV-positive normal adults without hemophilia and to examine antigen specificity of the clonal T-cells. On the other hand, low levels of T-cell clonality were detected in three other HCV positive patients. Further analyses were needed to search for the cause of the clonal expansion.

The lymphocyte proliferation response to mitogens diminishes in the presence of factor concentrates in vitro (8). Long-term administration of FVIII concentrations causes inhibition of the release of IL-2 by T lymphocytes (26). The immunosuppressive effect is due to contamination with transforming growth factor-β (TGF-β) (15,16,27). TGF-β exerts immunosuppressive effects by inhibition of interleukin 2 (IL-2) production or enhances secretion of IL-4 and IL-10 (26,28). The immune function may also affect other protein contaminates, including IgG aggregates, immune complexes, β2-microglobulin, fibronectin, and orosomucoid (9). TGF-β or some plasma proteins have a modulating effect on the immune function. However, there is no convincing evidence that TGF-β causes a decrease of the CD4/CD8 ratios, which is a major feature of immune abnormalities. Thus, it does not seem likely that TGF-β specifically stimulated a limited number of T-cells and caused the clonal expansion of T-cells that we observed. One of the causative factors may be allogeneic proteins. T lymphocytes can recognize allogeneic peptide antigens presented on major histocompatibility antigen (MHC) molecules and proliferate to react against non-self antigens.

The influence of age on T-cell clonality can be excluded, at least for the patients examined in this study, because the appear-

ance of clonal T-cells was not frequent in the age-matched healthy donors. There was no difference in the levels of T cell clonality between healthy donors and hemophilia patients treated with very high-purity FVIII (monoclonal-antibody-purified FVIII or recombinant FVIII), although they were younger than the healthy donors used in this study (data not shown). This suggests that clonal expansion of T-cells is associated with the purity of FVIII preparations used for replacement therapy.

What needs to be examined is whether clonal T-cell expansion is observed in hemophilia patients after long-term administration of recombinant and/or purified FVIII. The repeated injection of pure FVIII may have the potential to induce activation of T-cells. It is known that anti-FVIII antibody (inhibitor) is often detected in hemophilia patients. However, we could not find a significant difference in the number of clonal BV segments demonstrating clonal patterns between patients with and without inhibitor. We found that clonal T-cells are largely composed of CD8+ T-cells. Thus, clonal expansion of CD8+ T-cells may be induced by viral antigens rather than FVIII. This suggests that factor VIII per se is not responsible for the clonal expansion of T-cells that we observed.

Our results demonstrate that clonal CD8+ T-cells accumulate in the peripheral blood of hemophilia patients, in particular patients treated with non-heated preparations. The CD8+ T-cell, which is known to be a cytotoxic T-cell or suppressor T-cell, recognizes endogenous self-antigen presented in the context of MHC class I by antigen presenting cells and plays an essential role in host defense. CD8+ T lymphocytes may clonally expand by stimulation with antigens specific for viruses other than HIV including FVIII concentrates. Consequently, the CD4/CD8 ratio should be decreased in HIV-negative hemophilia patients.

The majority of clonal CD8+ T-cells lack expression of the co-stimulatory molecule CD28. The CD8+CD28- T-cells have been characterized by a low proliferative capacity to conventional stimulation in vitro (29, 30). The CD8+CD28- T-cells have been demonstrated to have shorter telomeres than CD8+CD28+ T-cells (31). The frequency of the CD8+CD28- T-cells increases with human aging (32). Persistent stimulation with antigens down-regulates CD28 surface expression in vitro (33). Sequence analysis of CDR3 reveals that identical T-cell clones exist within both CD8+CD28+ and CD8+CD28- T-cell populations. This suggests that CD8+CD28- T-cells are generated from chronically stimulated CD8+CD28+ T-cells. It has been reported that CD8+ memory cytotoxic lymphocytes that respond to human cytomegalovirus (CMV) or HIV clonally expand, and these T-cells exist in a CD8+CD28- T-cell subset (34-38). Clonal accumulation of a poorly responsive T-cell population may lead to immunosuppression in HIV-negative hemophilia patients.

In conclusion, we demonstrated that clonal expansion of CD8+ T-cells is frequently observed in HIV-negative hemophilia patients treated with plasma-derived FVIII preparations for replacement therapy. The majority of clonal CD8+ T-cells lacks CD28 molecule. This suggests that viruses other than HIV contained in FVIII preparations, in particular non-heated concentrates, induces activation of CD8+CD28+ T-cells to down-regulate the CD28 molecule. The resulting clonal accumulation of CD8+CD28- T-cells may reflect a decreased CD4/CD8 ratio, which leads to a defective immune response to antigens in HIV-negative hemophilia patients. Our results showed a high prevalence of virus infection in hemophilia patients with clonal expansion, suggesting that a virus such as HCV is a causative factor that induces T-cell clonal expansion. These findings strongly support the use of very high-purity FVIII preparations for replacement therapy in hemophilia patients.

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血栓止血誌 14(4)：337～344, 2003

◆症　例◆

血友病ハイレスポンダーインヒビター保有患者に対する凝固因子製剤によるインヒビターの中和と持続輸注療法

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ハイレスポンダーインヒビター保有血友病Aおよび血友病B患者に起きた3回の重症出血に対し，凝固因子製剤の大量投与でインヒビターを中和し，引き続き持続投与によって充分な血漿凝固因子活性を維持し，止血に成功した．症例1は第VIII因子インヒビター保有血友病A患者．38歳時に小脳出血で緊急入院した．2.1ベセスダ単位（BU）/m*l*の第VIII因子インヒビターに対し，第VIII因子製剤5,000 Uのボーラス投与に続き，3.8 U/kg/hrの持続投与を行った．血漿第VIII因子活性は4日間0.9～1.44 U/m*l*を維持し血腫は縮小した．40歳時には，外傷性頭頂葉皮質出血をきたし，バイパス療法を行ったが，新たに右硬膜下血腫を認めたためインヒビター中和療法を施行した．10 BU/m*l*の第VIII因子インヒビターに対し，第VIII因子製剤12,000 Uのボーラス投与に続き，4～6 U/kg/hrの持続投与を行った．血漿第VIII因子活性は，5日間0.54～2.04 U/m*l*を維持し血腫は縮小した．症例2は4歳の第IX因子インヒビター保有血友病B患者．右足背部の打撲により大きな皮下血腫を生じた．連日のバイパス療法でも改善せず，インヒビター中和療法に変更した．2.1 BU/m*l*の第IX因子インヒビターに対し，第IX因子製剤2,000 Uのボーラス投与に続き，13 U/kg/hrの持続投与を開始した．血漿第IX因子活性は1.0～1.3 U/m*l*を維持し，血腫は速やかに縮小した．

Key words: hemophilia, high responder, inhibitor, continuous infusion, intracranial hemorrhage

緒　言

血友病のインヒビターは，補充療法の反復経過中に，第VIII因子もしくは第IX因子に対する同種抗体として発生する[1]．重症型血友病Aでは，約30%の症例で第VIII因子インヒビターを発生する．その約半数は，自然経過中もしくは免疫寛容療法で消失するが，残りはその後も長期にわたり存続し，通常の凝固因子補充療法では止血効果は不良となる[2]．当該凝固因子製剤の投与によりインヒビターが10ベセスダ単位（BU）/m*l*以上に上昇するハイレスポンダー[3]症例の止血治療は一般的にバイパス療法が行われる[4)~6)]．バイパス療法には，プロトロンビン複合体製剤（prothrombin complex concentrate：PCC），活性型プロトロンビン複合体製剤（activated prothrombin complex concentrate：APCC），遺伝子組換え活性型第VII因子製剤が用いられている．しかしどのバイパス製剤も，インヒビター保有患者の止血治療に常に確実な効果を期待できるものではない．今回われわれは，血友病Aおよび血友病Bのハイ

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レスポンダーインヒビター症例の重症出血に対して，凝固因子製剤の大量投与によるインヒビターの中和と持続輸注療法を行った．その結果，十分な血漿凝固因子活性が数日間維持され，良好な止血効果が得られたので報告する．

方　法

インヒビターの中和に必要な凝固因子量はベセスダ法の原理に基づいて決定した．インヒビター力価1 BU/m*l*の血漿1 m*l*は，試験管内で0.5 Uの当該凝固因子を失活させる．したがってtBU/m*l*のインヒビターを中和するのに必要な凝固因子量は理論上以下のようになる．

凝固因子量（U）＝循環血漿量（m*l*）×インヒビター力価t(BU/m*l*)× 0.5

≒40×体重（kg）×インヒビター力価t(BU/m*l*)× 0.5

≒20×体重（kg）×インヒビター力価t(BU/m*l*)

さらに凝固因子活性を0.8～1.0 U/m*l*に上昇させるために，第VIII因子製剤の場合40～50 U/kgを，第IX因子製剤の場合80～100 U/kgの因子量を加えボーラス投与し，引き続いて持続投与[3]を行った．持続投与中は血栓性静脈炎の予防と輸注回路の閉塞防止のためにヘパリンを10 U/m*l*の濃度で製剤中に添加した．

症　例　1

1986年（27歳時）以来0.5～500 BU/m*l*の第VIII因子インヒビターを保有する血友病A症例である．1997年38歳時，頭痛と意識障害を主訴に来院した．頭部CT画像上，小脳出血と診断し（**Fig. 1**），入院加療となった．入院時の体重は52 kg，血漿第VIII因子活性は0.01 U/m*l*未満，第VIII因子インヒビターは2.1 BU/m*l*を認めた．重篤出血例であったため，止血療法は第VIII因子製剤大量投与によるインヒビター中和療法を選択した．遺伝子組換え第VIII因子製剤（リコネイト®，バクスター社）5,000 U（≒20×52 kg×2.1 BU/m*l*＋50 U/kg）のボーラス投与に引き続き，3.8 U/kg/hrで持続投与を開始した．血漿第VIII因子活性は4日間0.9～1.44 U/m*l*を維持した．しかし，持続投与開始第5日に，第VIII因子活性は0.057 U/m*l*，第6日には0.027 U/m*l*と急激に低下し，第VIII因子インヒビターは7.5 BU/m*l*に上昇した（**Fig. 2**）．頭部CT撮影で小脳血腫の縮小が認められたため，持続投与は6日間で終了した．投与開始第13日に第VIII因子インヒビターは522 BU/m*l*まで上昇した．その後，小脳血腫は消失し，リハビリテーションを行い後遺症も残さず，在院日数137日で退院した．インヒビター力価は，7週後に最高値5,700 BUまで上昇した．その後は低下し，19週後には200 BUとなった．その間新しい出血はなかった．

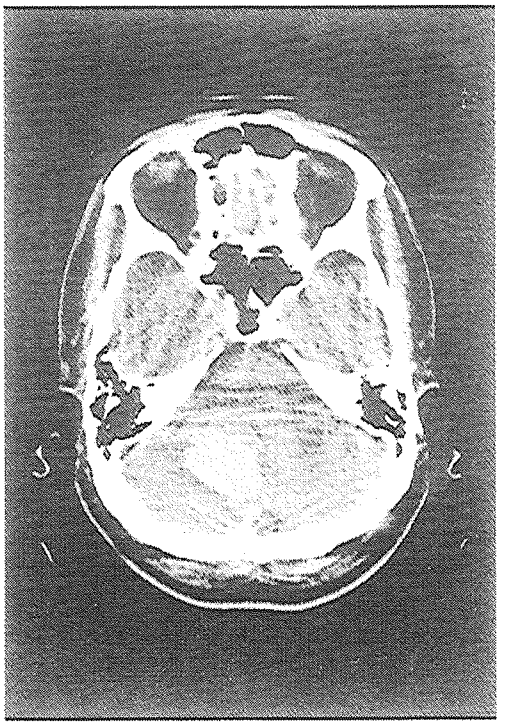

Fig. 1 Brain CT scan in patient 1 reveals intracerebellar hemorrhage in episode 1.

本症例はさらに1999年（40歳時），転倒して左前額部を打撲し，右頭頂葉の皮質出血（**Fig. 3**）で入院となった．患者は入院時，意識清明．体重は50 kg，第VIII因子活性は0.01 U/m*l*，

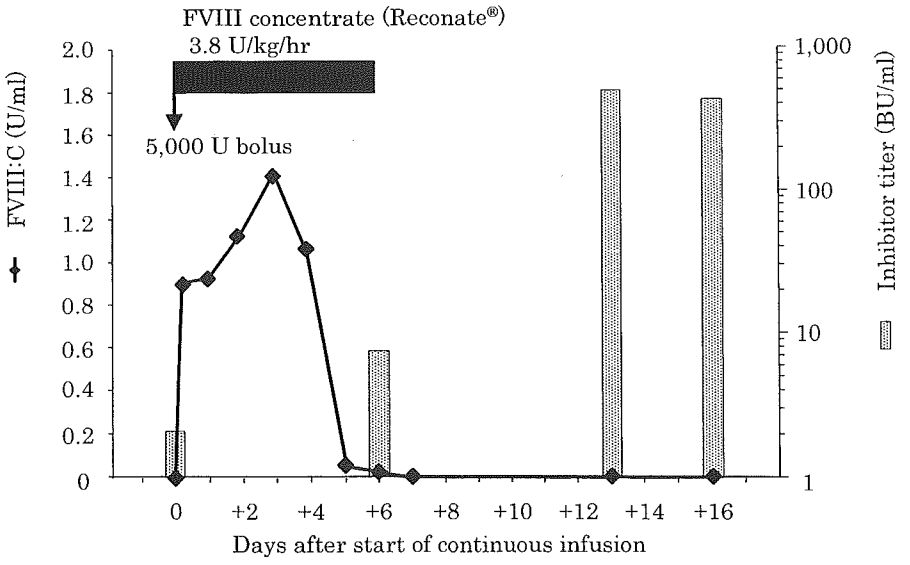

Fig. 2 Course of therapy with continuous infusion of factor VIII for hemophilia A with factor VIII inhibitor: episode 1, patient 1.

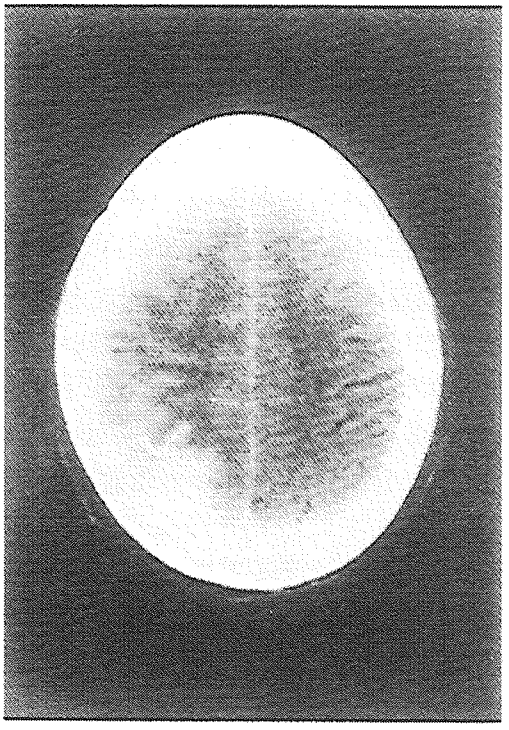

Fig. 3 Brain CT scan in patient 1 reveals traumatic intracerebral hemorrhage in the parietal lobe in episode 2.

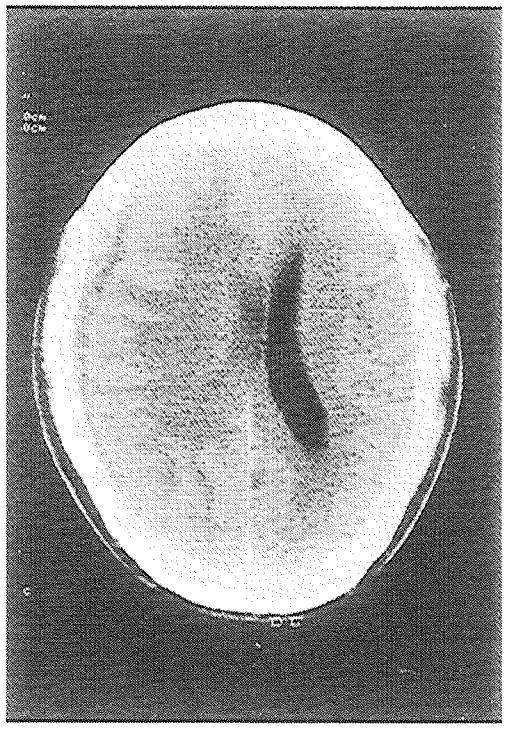

Fig. 4 Brain CT scan in patient 1 reveals another hemorrhage in the right side of subdural space in episode 2.

第 VIII 因子インヒビターは 11.5 BU/m*l* を認めた．PCC（プロプレックス®ST，バクスター社）80 U/kg によるバイパス療法を 5 日間施行した．その後の頭部 CT 撮影では頭頂葉血腫の増大はなく，頭痛は軽快傾向にあった．しかし，入院第 13 病日の CT 撮影で，頭頂葉血腫は吸収傾向であったにもかかわらず，新たに右硬膜下血腫（**Fig. 4**）および脳浮腫が認められた．本

注）本論文に提示した症例 1 と 2 の止血治療時期には，遺伝子組換え活性型第 VII 因子製剤は本邦では発売されていなかった．

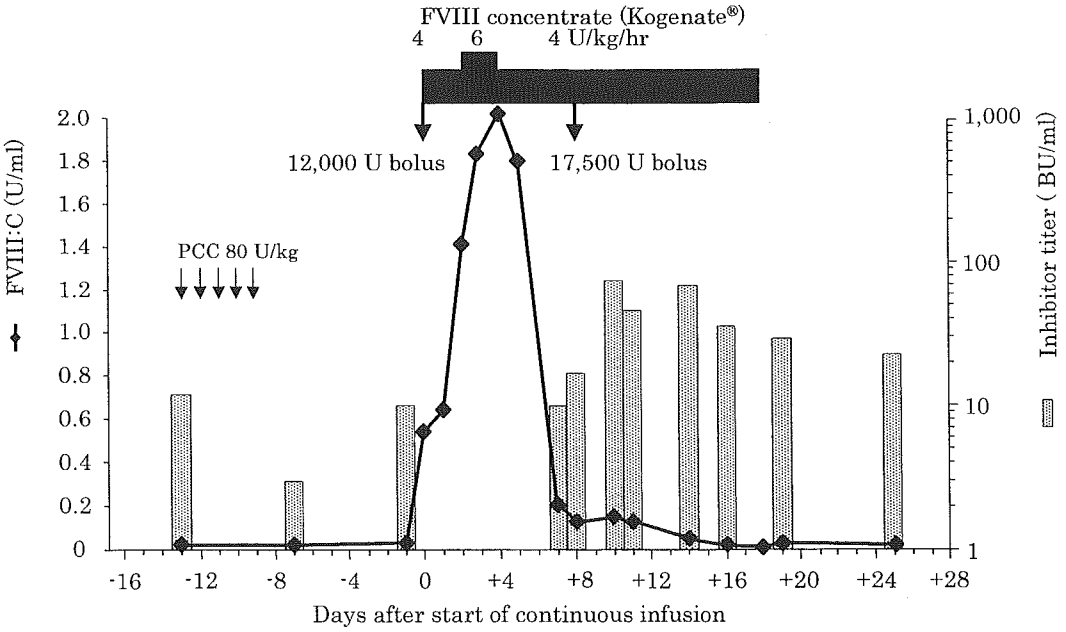

Fig. 5 Course of therapy with continuous infusion of factor VIII for hemophilia A with factor VIII inhibitor: episode 2, patient 1.
PCC: prothrombin complex concentrate, Proplex ®ST

症例は，以前の出血時にAPCCの投与歴があったが，PCCを上回る明確な止血効果は認めなかった．そこで，第VIII因子製剤の大量投与によるインヒビター中和療法を開始した．この時点の第VIII因子インヒビターは10 BU/m*l*．遺伝子組換え第VIII因子製剤（コージネイト®，バイエル社）12,000U（≒20×50kg×10BU/m*l* 40 U/kg）のボーラス投与に引き続き，4 U/kg/hrの持続投与を開始した（**Fig. 5**）．血漿第VIII因子活性は0.54 U/m*l*に上昇した．持続投与開始第3日より6 U/kg/hrに増量し，活性は1.41～2.04 U/m*l*に増加した．投与開始第5日より4 U/kg/hrに戻したところ，活性は1.8 U/m*l*を示した．投与開始第7日に活性が0.13 U/m*l*に急落し，10 BU/m*l*の第VIII因子インヒビターが検出された．頭部CT上，硬膜下血腫は吸収傾向にあったが，まだ脳浮腫像を認めたため，第VIII因子製剤の持続投与は4 U/kg/hrで継続した．血漿残存第VIII因子活性は依然として0.1 U/m*l*前後を維持していた．投与開始第8日に16 BU/m*l*のインヒビターに対し，再度コージネイト® 17,500 Uのボーラス投与による中和を試みたが，投与3時間後の血漿第VIII因子活性の上昇は得られなかった．持続投与開始第18日には，血漿第VIII因子活性が0.012 U/m*l*まで低下したため，持続投与を中止した．その後，血腫は吸収消失して在院日数41日で，後遺症もなく退院となった．インヒビター力価は，持続投与開始第10日に67 BUを示した後漸減し，13週後には8 BUとなった．その間新しい出血はなかった．

症 例 2

1994年（1歳2カ月時）以来，1～80 BU/m*l*の第IX因子インヒビターを保有する血友病B症例．1997年（4歳時），ペットボトルを右足背に落とし皮下血腫を形成した．本症例は軽度の出血症状に対しては，PCCによるバイパス療法で止血効果が得られていた．そこでプロプレックス®ST 75 U/kgを1回投与したところ，一時軽快した．8日後，同部位に軽度の腫脹が残るため再度75 U/kgを投与した．受傷21日後，同部位の再出血による腫脹，疼痛が増強したため，プロプレックス®ST 75～100 U/kgを6日間連日投与した．しかし連日のPCC投与でも血腫

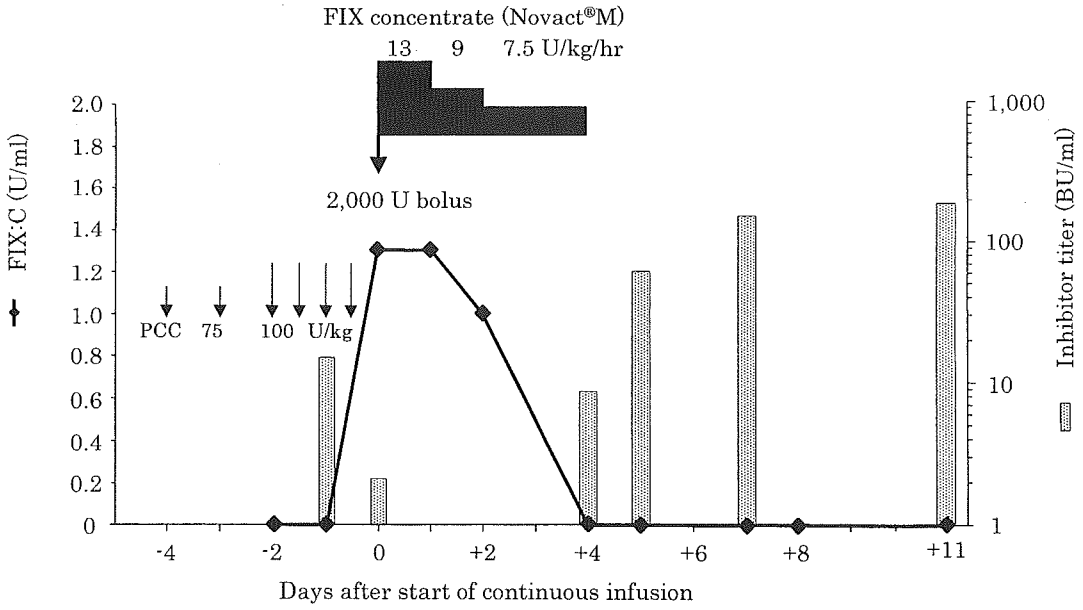

Fig. 6　Course of therapy with continuous infusion of factor IX in patient 2 with hemophilia B with factor VIII inhibitor.
PCC : prothrombin complex concentrate, Proplex ®ST

の改善が見られなかった．本症例は，過去にAPCCの投与に際して，喘鳴と嘔気の即時性アレルギー反応があったため，その使用は躊躇された．そこで，入院下で第IX因子製剤大量投与によるインヒビターの中和を試みた．入院時の体重は16 kg，第IX因子インヒビターは2.1 BU/m*l*を認めた．モノクローナル抗体精製第IX因子製剤（ノバクト®M，化血研）2,000 U（≒20×16 kg×2.1 BU＋80 U/kg）をボーラス投与し，続いて13 U/kg/hrで持続投与を開始した（**Fig. 6**）．血漿第IX因子活性は1.3 U/m*l*まで上昇し，血腫は速やかに縮小した．持続投与開始第4日には，活性が0.01 U/m*l*未満に低下し，インヒビター力価は8.6 BU/m*l*に上昇したため投与を中止した．投与開始第11日に，192 BU/m*l*まで上昇したが，患者は第16日に軽快退院となった．その後のインヒビター力価は，8週後に100 BU，18週後には30 BUに低下した．その間は新しい出血は認めなかった．

考　察

インヒビター保有血友病患者に対する凝固因子大量中和療法の報告は，欧米で少数みられる程度である[4)~7)]．Blattらは，体重70 kgの第VIII因子インヒビター患者に対して5,000～10,000 Uのボーラス投与後に300～1,000 U/hrの持続投与を行っている[4)]．Kasperは，第VIII因子インヒビターに対する中和療法の初期投与量を，抗体中和に必要な第VIII因子量（インヒビター力価（BU/m*l*）当たり20 U/kg）と第VIII因子活性上昇を期待する量を加えて総量としている[8)]．最初に提示した症例1の38歳時のエピソードでは，小脳出血に対して第VIII因子製剤によるインヒビター中和と持続輸注療法を選択した．Kasperの方法に準じて，インヒビター中和量を計算し，さらに血漿第VIII因子活性を1 U/m*l*に上昇させる単位量の製剤を加えてボーラス投与を行い，その活性を維持するように持続投与を行った．その結果，ほぼ期待通りの血漿第VIII因子活性の上昇が確認され，十分な止血効果が得られた．しかし，投与開始第6日には既往免疫反応によりインヒビターは再上昇し，それに伴い血漿第VIII因子活性は急激に低下した．

症例1の38歳時に行った中和療法では，既往