Delayed recovery of CDR3 complexity of the T-cell receptor-β chain in recipients of allogeneic bone marrow transplants who had virus-associated interstitial pneumonia: Monitor of T-cell function by CDR3 spectratyping

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Background: In the T-cell receptor (TCR)-β chain, complementary-determining region 3 (CDR3) contains specific peptide sequences essential for recognition. Diversity of this region is considered to contribute to immunocompetence in humans.
Objective: The purpose of this study was to define the process of reconstitution of CDR3 complexity of the TCR-β chain after allogeneic bone marrow transplantation and to investigate the association between host immunocompetence and CDR3 complexity.
Methods: Diversity of the CDR3 region of the TCR-β chain was examined by CDR3 size distribution analysis with the use of an automated DNA sequencer.
Results: Reconstitution of the αβ T-cell repertoire and CDR3 diversity was incomplete for at least 2 months after bone marrow transplantation. Delayed reconstitution of T-cell diversity was more marked in immunocompromised hosts. Unlike the situation in patients who received allogeneic bone marrow grafts, the recovery of CDR3 complexity was almost perfect by 2 months after transplantation in patients who received allogeneic blood stem cells. Clonal expansion of αβ T cells after allogeneic bone marrow transplantation was readily detected by CDR3 size spectratyping analysis.
Conclusion: PCR-based CDR3 size spectratyping may be a useful tool for clinically monitoring immune reconstitution after allogeneic bone marrow transplantation. (J Allergy Clin Immunol 2000;106:S32-9.)

Key words: *Bone marrow transplantation, T cell, diversity, spectratyping*

Successful bone marrow transplantation requires the repopulation of the bone marrow by engrafted hematopoietic stem cells, the induction of tolerance to the recipient, and the reconstitution of the immune system. The initial 3 months after transplantation is the critical period for recipients of allogeneic bone marrow transplants, because they are susceptible to various opportunistic infections that result from defective T-cell immunity.[1,2] In general, a lower number of circulating CD4 cells, a decreased proliferative response, and decreased cytokine production by T lymphocytes in response to antigenic stimuli have been suggested as the causes of the immune dysfunction during the period of 3 to 6 months after transplantation.[3,4] However, the profile of immunologic recovery varies among patients and is also affected by the presence and treatment of acute graft-versus-host disease (GVHD).[5,6] Thus, it is important to develop a method for monitoring the immune status of recipients of bone marrow transplants.

Abbreviations used
CDR3: Complementary-determining region 3
GVHD: Graft-versus-host disease
TCR: T-cell receptor
TCRBV: TCR-β chain variable region

T-cell receptor (TCR) diversity is one of the mechanisms by which humans compete with various intracellular microorganisms for survival. Junctional diversity (involving coding sequences at the VJ, VD, and DJ junctions) contributes to the generation of the TCR diversity. The objectives of this study were to determine (1) whether reconstitution of TCR diversity was associated with the return of immunocompetence after allogeneic hematopoietic cell transplantation and (2) whether allogeneic peripheral blood stem-cell grafting was superior to bone marrow transplantation for the regeneration of T-cell diversity.

METHODS

Patients

The clinical characteristics of the patients are shown in Table I. All the patients were conditioned with myeloablative chemoradiotherapy, involving fractionated total body irradiation (12 Gy in 6 fractions) and cyclophosphamide (60 mg/kg/day for 2 days). This was followed by

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TABLE I. Clinical characteristics of the patients

Patient	Age (y)/ sex	Disease/ status	Graft source	Acute GVHD grade	Interstitial pneumonia	Outcome (mo after transplantation)	Cause of death
1	37/F	AML/CR1	BM	0	No (+30)	Alive	
2	16/F	ALL/CR1	BM	0	No (+48)	Alive	
3	43/F	ALL/CR1	BM	1	No (5)	Dead	Leukemia relapse
4	32/M	CML/CP1	BM	1	Yes (HSV) (+21)	Alive	
5	22/M	CML/CP1	BM	3	Yes (CMV) (+40)	Alive	
6	36/M	MDS/RAEB	BM	3	Yes (CMV) (4)	Dead	Respiratory failure
7	28/F	AML/relapse	PBSC	1	No (41)	Dead	Leukemia relapse
8	45/M	AML/relapse	PBSC	0	No (+24)	Alive	
9	16/M	ALL/relapse	PBSC	0	No (5)	Dead	Leukemia relapse

AML, Acute myelogenous leukemia; *CR1,* first complete remission; *BM,* bone marrow; *ALL,* acute lymphoblastic leukemia; *CML,* chronic myelogenous leukemia; *CP1,* first chronic phase; *HSV,* herpes simplex virus; *CMV,* cytomegalovirus; *MDS,* myelodysplastic syndrome; *RAEB,* refractory anemia with excess of blasts; *PBSC,* peripheral blood stem cell.

the infusion of allogeneic bone marrow (n = 6 patients) or peripheral blood stem-cell grafts (n = 3 patients) from HLA-matched donors. All the patients received cyclosporin A and short-term methotrexate for prophylaxis of acute GVHD.[7] Engraftment was achieved in all patients and was confirmed by the recovery of hematopoiesis and the presence of donor-derived sex chromosomes or mismatched antigens on red cells. The grading of acute GVHD was performed according to the criteria of Glucksberg et al.[8] Patients were monitored for cytomegalovirus infection by weekly cytomegalovirus antigenemia assays[9] from the day that the granulocyte count reached 500/μL until day 100 after transplantation. Recipients of unrelated donor bone marrow who were cytomegalovirus-seropositive received prophylactic ganciclovir (5 mg/kg/d, 3 days each week) from the day that the granulocyte count was greater than 1000/μL.[10]

Extraction of RNA and cDNA synthesis

Informed consent was obtained from the patients before blood and bone marrow samples were taken. PBMCs were isolated by the Ficoll/Hypaque (Pharmacia Biosystems, Piscataway, NJ) gradient centrifugation method from 10 mL of heparinized blood. Total RNA was extracted from PBMCs with an RNeasy kit (Qiagen, Hilden, Germany) and was used for first-strand cDNA synthesis with an oligo-dT primer (Amersham Pharmacia Biotech, Piscataway, NJ).

PCR amplification and complementary-determining region 3 size analysis of TCR-β chains

TCR-β chain complementary-determining region 3 (CDR3) size spectratyping was performed according to the method reported previously.[11-13] Aliquots of cDNAs prepared from blood and bone marrow samples were amplified with 1 of 35 V-β–specific oligonucleotides and the C-β primer. The sequences of the V-β and C-β primers are shown in Table II.[14] PCR amplification was performed for 40 cycles in a 20-μL reaction mixture containing 0.2 μmol/L of each primer and 0.5 U of Taq polymerase (TaKaRa, Osaka, Japan). The PCR conditions were denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes. The PCR buffer was 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.2 mmol/L of each deoxyribonucleoside triphosphate. After 40 PCR cycles, an additional extension was done at 72°C for 15 minutes.

Aliquots (4 μL) of the 35 unlabeled V-β–C-β PCR products were subjected to 1 cycle of elongation (runoff reaction) with an FAM-labeled nested C-β primer under the following conditions: denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 15 minutes. The reaction buffer was the same as that described earlier. The labeled PCR products were mixed with the size marker (GeneScan-500 TAMRA, Applied Biosystems, Warrington, UK) and were loaded onto 5% polyacrylamide sequencing gel for determination of size and fluorescence intensity with the use of an automated DNA sequencer (ABI 377; Applied Biosystems). Data were analyzed with GeneScan software (Applied Biosystems).

TCR-β chain variable region repertoire analysis

TCR-β chain variable region (TCRBV) repertoire analysis was performed by an adaptor ligation PCR-based microplate hybridization assay, as reported previously.[15] Briefly, the P10EA/P20EA adaptors were ligated to the 5′ end of complementary DNA prepared from PBMCs, and PCR was performed with a biotinylated TCR-β chain constant region-specific primer and a P20EA primer.[14] Biotinylated PCR products were hybridized with immobilized TCRBV-specific primers in 96-well microtiter plates. Then alkaline phosphatase-conjugated streptavidin was added to each well, and a colorimetric assay was performed.

RESULTS

CDR3 size spectratyping for the determination of T-cell repertoire diversity and the detection of clonal expansion after bone marrow transplantation

Diversity of coding sequences at the VJ, VD, and DJ junctions contributes to the generation of TCR diversi-

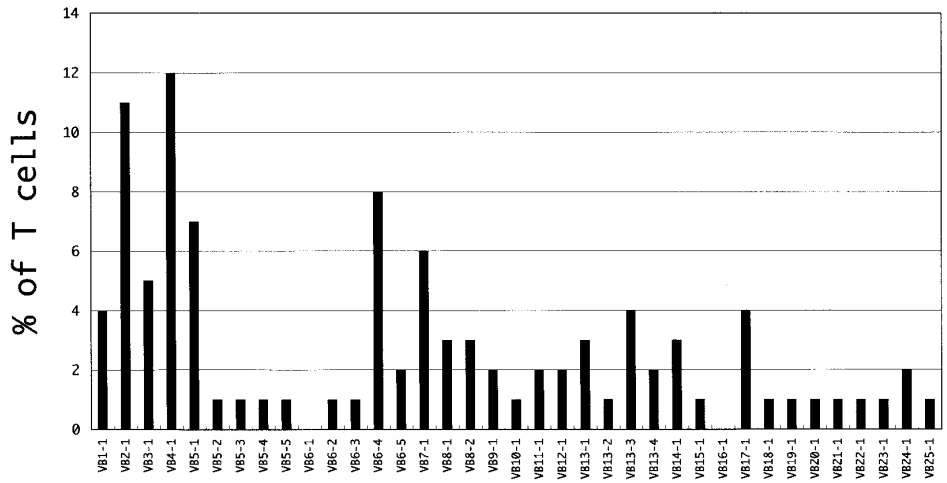

FIG 1. TCRBV repertoire analysis in the bone marrow donor for patient 3.

TABLE II. Nucleotide sequences of the TCRBV and TCRBC primers

Primer	Sequence(5′-3′)	Target segment*
VB1-1	ACAACAGTTCCCTGACTTGCA	BV1S1
VB2-1	TCATCAACCATGCAAGCCTGA	BV2S1
VB3-1	GTCTCTAGAGAGAAGAAGGAG	BV3S1
VB4-1	TTGACAAGTTTCCCATCAGCC	BV4S1, BV4S2
VB5-1	CTTCAGTGAGACACAGAGAAA	BV5S1
VB5-2	ATTATGAGGAGGAAGAGAGACA	BV5S2
VB5-4	TGACGAGGGTGAAGAGAGAAA	BV5S8
VB5-5	ATTATAGGGAGGAAGAGAATGG	BV5S6
VB6-1	ACGATCGGTTCTTTGCAGTCA	BV6S1
VB6-2	TCAACTAGACAAATCGGGGCT	BV6S2
VB6-3	CCAACAAGACAAATCAGGGCT	BV6S3
VB6-4	AAGGCTGCTCAGTGATCGGT	BV6S4
VB6-5	TCGCTTCTCTGCAGAGAGGA	BV6S5
VB7-1	CTGAATGCCCCAACAGCTCT	BV7S1, BV7S2, BV7S3
VB8-1	TTTACTTTAACAACAACGTTCCG	BV8S1, BV8S2
VB8-2	AAGGACTGGAGTTGCTGGCT	BV8S3
VB9-1	AAACAGTTCCAAATCGCTTCTC	BV9S1, BV9S2
VB10-1	CCAAAAACTCATCCTGTACCTT	BV10S1
VB11-1	TCAACAGTCTCCAGAATAAGGA	BV11S1
VB12-1	CAAAGGAGAAGTCTCAGATGG	BV12S1, BV12S2, BV12S4
VB13-1	CAATGGCTACAATGTCTCCAG	BV13S1, BV13S8
VB13-2	GGTCCCTGATGGCTACAATG	BV13S2
VB13-3	TGATGGTTATAGTGTCTCCAG	BV13S5
VB13-4	CCGAATGGCTACAACGTCTC	BV13S6
VB14-1	GTCTCTCGAAAAGAGAAGAGG	BV14S1
VB15-1	AGTGTCTCTCGACAGGCACA	BV15S1, BV15S2
VB16-1	GTGAAAGAGTCTAAACAGGAT	BV16S1
VB17-1	CACAGATAGTAAATGACTTTCAG	BV17S1
VB18-1	GATGAGTCAGGAATGCCAAAG	BV18S1
VB20-1	TCTGAGGTGCCCCAGAATCT	BV20S1
VB21-1	TCTGCAGAGAGGCTCAAAGG	BV21S1, BV21S3, BV21S4
VB22-1	TTCAGTGACTATCATTCTGAACT	BV23S1
VB23-1	AATCTTGGGGCAGAAAGTCGA	BV22S1
VB24-1	CCAGGAGGCCGAACACTTC	BV24S1
VB25-1	AACAGGTATGCCCAAGGAAAG	BV25S1
CB1	GAACTGGACTTGACAGCGGAACT	TCRBC
FAM-CB3	FAM-ACTGTGCACCTCCTTCCCATTCA	TCRBC

*TCRBV gene segments are described according to the nomenclature reported by Concannon P, Robinson MA. Human T-cell receptor gene nomenclature. Ann NY Acad Sci 1995;756:124-9.

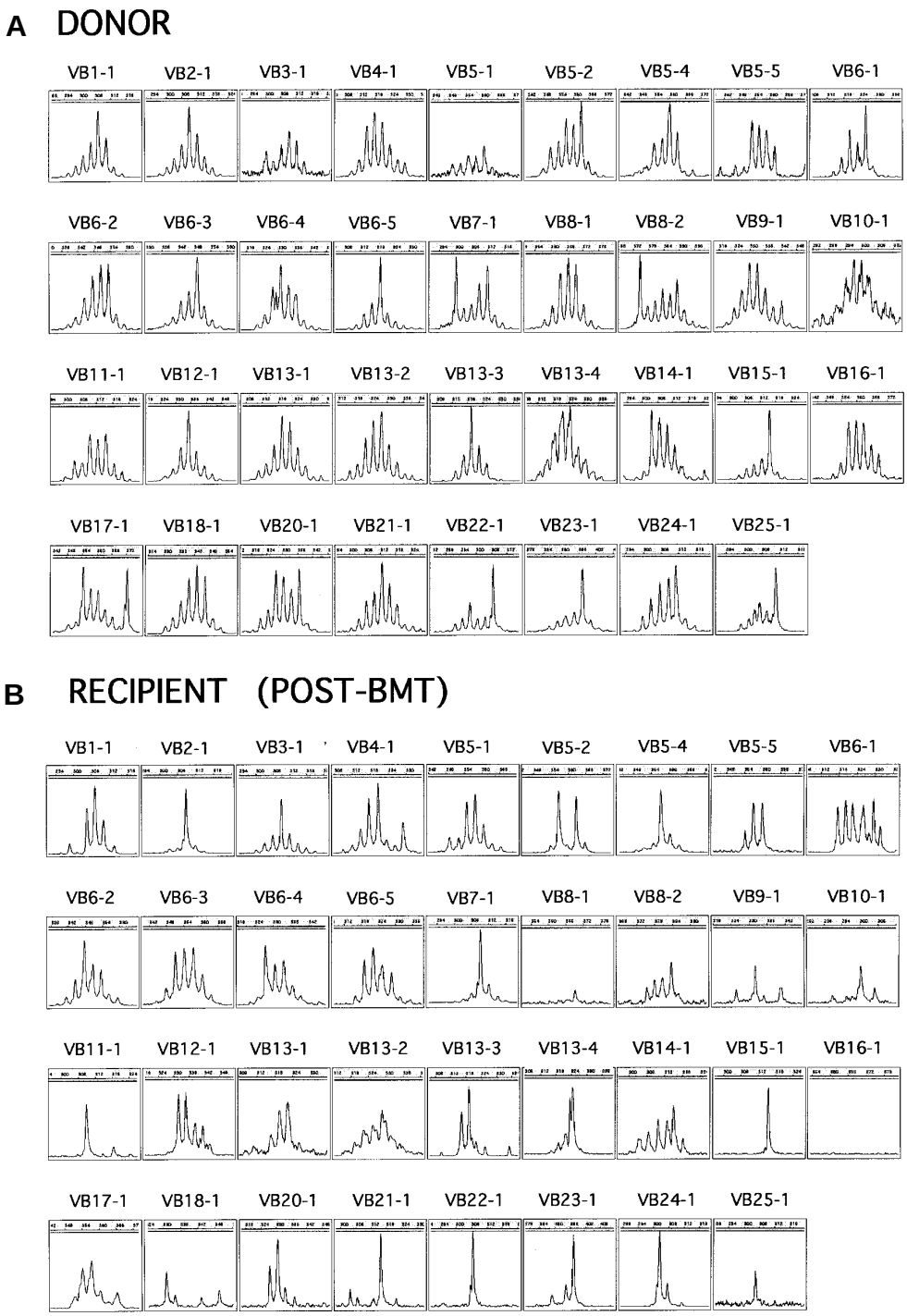

FIG 2. CDR3 size distribution analysis of TCR-β chains in a donor (**A**; patient 3) and recipient (**B**) pair. Note that VB6-1 and VB16-1 show a Gaussian-like distribution in this assay, although T cells that carry these TCRs are undetectable among donor lymphocytes by TCRBV repertoire analysis (as shown in Fig 1). The recipient was examined 2 months after the transplantation. *BMT*, Bone marrow transplantation.

ty. We used CDR3 size spectratyping of TCR-β chains to analyze the diversity of αβ T cells in recipients of bone marrow transplants. With this method of analysis, the diversity of the T-cell repertoire in clinical samples can be estimated with a high sensitivity, and clonal expansion of T cells can be easily detected. Representative results are shown in Figs 1 and 2. T cells that carry VB6-1 and VB16-1 were undetectable among donor lymphocytes by TCRBV repertoire analysis based on the adaptor-ligation PCR and microplate

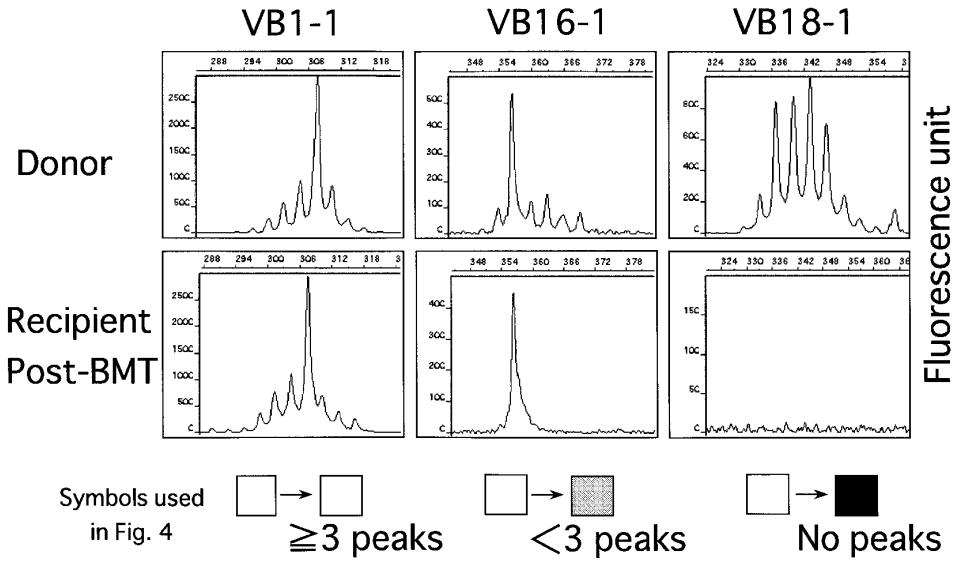

FIG 3. CDR3 size distribution patterns. VB1-1 shows polyclonality in both donor and recipient, although VB16-1 shows oligoclonality in the recipient and VB18-1 is not detected in the recipient. *BMT*, Bone marrow transplantation.

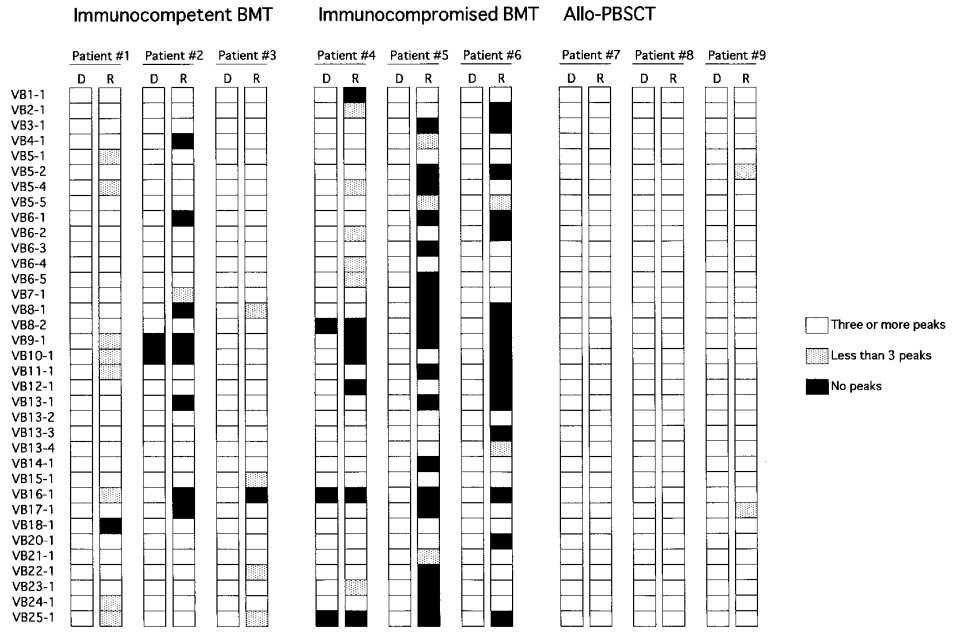

FIG 4. CDR3 size distribution patterns in immunocompetent and immunocompromized hosts after allogeneic bone marrow and blood stem-cell transplantation. Blood samples were obtained from the patients at 8 to 10 weeks after transplantation. *D,* Donor; *R,* recipient; *BMT*, bone marrow transplantation; *PBSCT,* peripheral blood stem-cell transplantation.

hybridization assay (Fig 1). However, PCR-based CDR3 size spectratyping revealed the presence of T cells that carry VB6-1 and VB16-1 with a typical gaussian distribution (Fig 2, *A*). Thus, PCR-based CDR3 size spectratyping was suitable for the determination of the diversity of T cells present at low frequency. After transplantation in this patient, the recovery of junctional diversity was incomplete for multiple TCRBV subfamilies, including VB2-1, VB5-2, VB8-1, VB11-1, VB15-1, VB16-1, VB18-1, VB22-1, VB24-1, and VB25-1 (Fig 2, *B*). CDR3 size spectratyping also revealed that the repopulated T cells that carry VB15-1 were highly clonal (Fig 2, *B*). Therefore, clonal expansion of αβ T cells was readily detected by CDR3 size spectratyping in conjunction with TCRBV repertoire analysis.

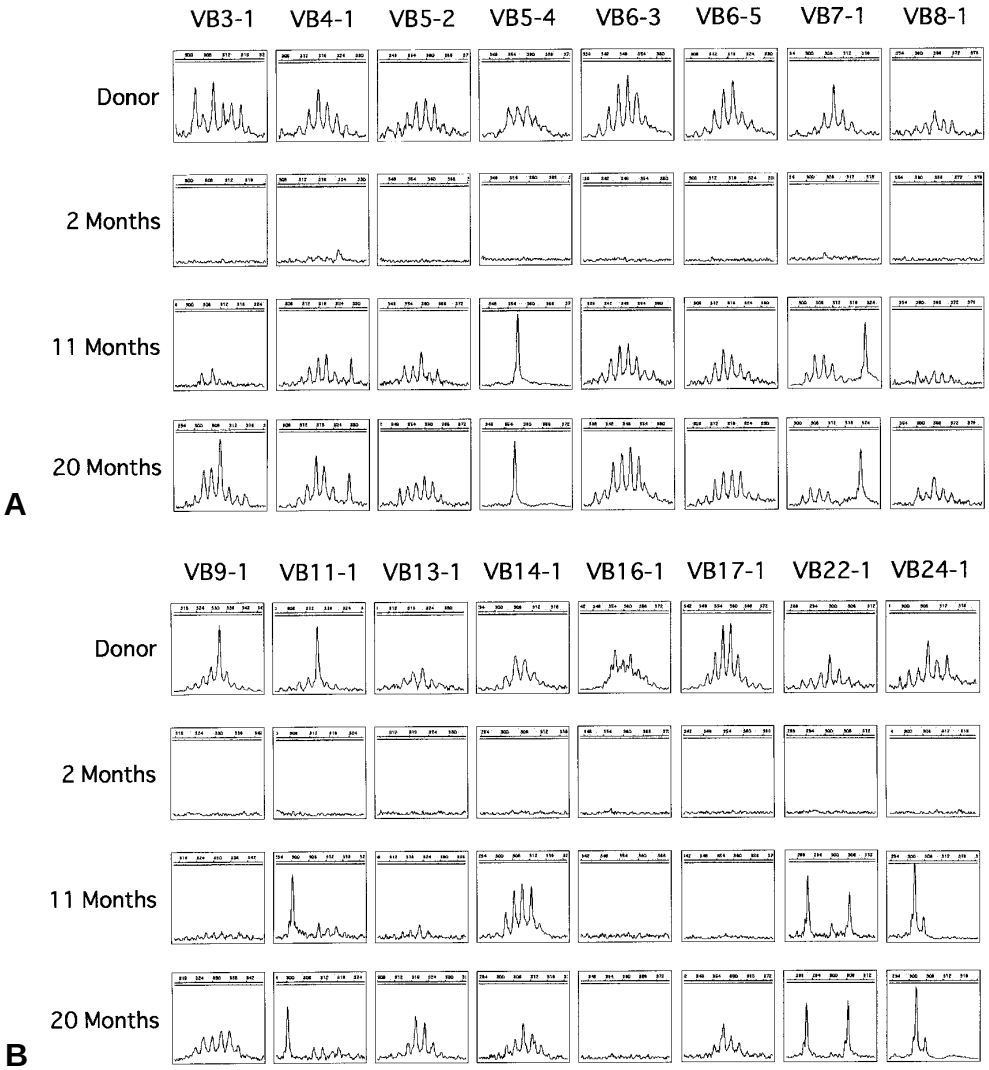

FIG 5. Reconstitution of defective TCRBV subfamilies in patient 5, who survived cytomegalovirus-associated interstitial pneumonia.

Description of CDR3 complexity

The typical CDR3 size distribution pattern of V-β–C-β PCR products obtained from healthy PBMCs showed a Gaussian-like distribution with multiple peaks. After bone marrow transplantation, monoclonal/oligoclonal peaks were observed in certain V-β subfamilies that differed among the patients, and sometimes a few V-β-C-β transcripts were undetectable in certain patients (Fig 3).

Impaired reconstitution of T-cell repertoire diversity in immunocompromised recipients who underwent bone marrow transplantation

Virus-associated interstitial pneumonia is one of the major obstacles to successful allogeneic bone marrow transplantation and usually occurs within 3 months after transplantation.[16,17] We performed CDR3 size distribution analysis in 3 patients with interstitial pneumonia (cytomegalovirus, 2 patients; herpes simplex virus, 1 patient) after allogeneic bone marrow transplantation. In these severely immunocompromised patients, multiple TCRBV subfamilies were not reconstituted by 2 months after transplantation (Fig 4). In contrast, defective reconstitution of TCRBV subfamilies was far less prominent in the 3 immunocompetent patients who underwent bone marrow transplantation. In 1 patient with cytomegalovirus-associated pneumonia who eventually recovered, CDR3 size distribution patterns were followed for 20 months (Fig 5). Although most of the TCRBV subfamilies that had been defective at 2 months after transplantation were reconstituted by 20 months, certain TCRBV subfamilies still showed clonal reconstitution.

Rapid recovery of T-cell repertoire diversity in recipients of allogeneic peripheral blood stem-cell transplants

Unlike the situation in patients who received allogeneic bone marrow grafts, the recovery of CDR3 complexity was almost perfect by 2 months after transplantation in patients who received allogeneic blood stem cells (Fig 4). This result suggests a potential advantage in the transplantation of blood stem cells rather than bone marrow cells for obtaining T-cell regeneration after grafting.

DISCUSSION

Not only sufficient T cells but also T-cell repertoire diversity are required for the maintenance of immunocompetence. Conditioning chemoradiotherapy depletes T-cell populations in recipients of bone marrow transplants; therefore, adequate T-cell regeneration is essential for successful transplantation. Theoretically, 3 pathways of T-cell regeneration can be postulated after bone marrow transplantation: thymus-dependent and thymus-independent extrathymic pathways of T-cell differentiation of T-cell progenitors and peripheral expansion of mature T cells in the graft. Thymus-dependent T-cell regeneration is more efficient than the thymus-independent pathway in terms of the restoration of T-cell numbers and T-cell repertoire diversity.[18,19] In adults, the thymic tissue is mostly atrophic, which suggests that extrathymic differentiation of T-cell progenitors and peripheral expansion of mature T cells are the principal mechanisms of T-cell regeneration in adult patients. Mackall et al[20] reported that, when the number of T cells in the graft was limited, skewing of the V-β repertoire was observed in mice. This finding has recently been confirmed in humans. Patients who receive highly purified hematopoietic progenitor cell grafts show decreased diversity of V-β TCR expression that is associated with a decrease in the absolute number of circulating T cells.[21]

The present study demonstrated that, during the initial 2 months after transplantation, even immunocompetent recipients who did not experience the development of any opportunistic infection showed a contracted CDR3 spectratype in certain V-β subfamilies. In contrast, it has been reported that immunocompetent recipients of bone marrow transplants show normal repertoire complexity for all V-β subfamilies.[22] This discrepancy may be explained by the larger number of V-β subfamilies that we could examine by CDR3 size spectratyping.

Recovery of CDR3 complexity was rapid in patients who received allogeneic blood stem-cell grafts that contained a 10-fold higher number of T cells than bone marrow. These results suggest that T-cell repertoire diversity is generated rapidly when a sufficient number of T cells are present in the graft. Patients with virus-associated interstitial pneumonia showed marked defects in T-cell repertoire diversity. In 1 patient, recovery was shown to be associated with the restoration of the T-cell repertoire.

Small et al[23] have recently reported that adoptive immunotherapy with a small number of donor T lymphocytes can restore circulating T-cell numbers and antigen-specific T-cell responses in patients who receive T-cell–depleted allogeneic bone marrow, accompanied by the resolution of virus-associated opportunistic infection. Based on our results, incomplete recovery of T-cell repertoire diversity appears to be associated with fatal opportunistic infection, and CDR3 size spectratyping may be useful for identifying patients who would benefit from adoptive transfer of donor lymphocytes.

In conclusion, CDR3 size spectratyping is a powerful tool for the detection of clonal expansion of T cells and the monitoring of immune reconstitution after human allogeneic bone marrow transplantation.

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