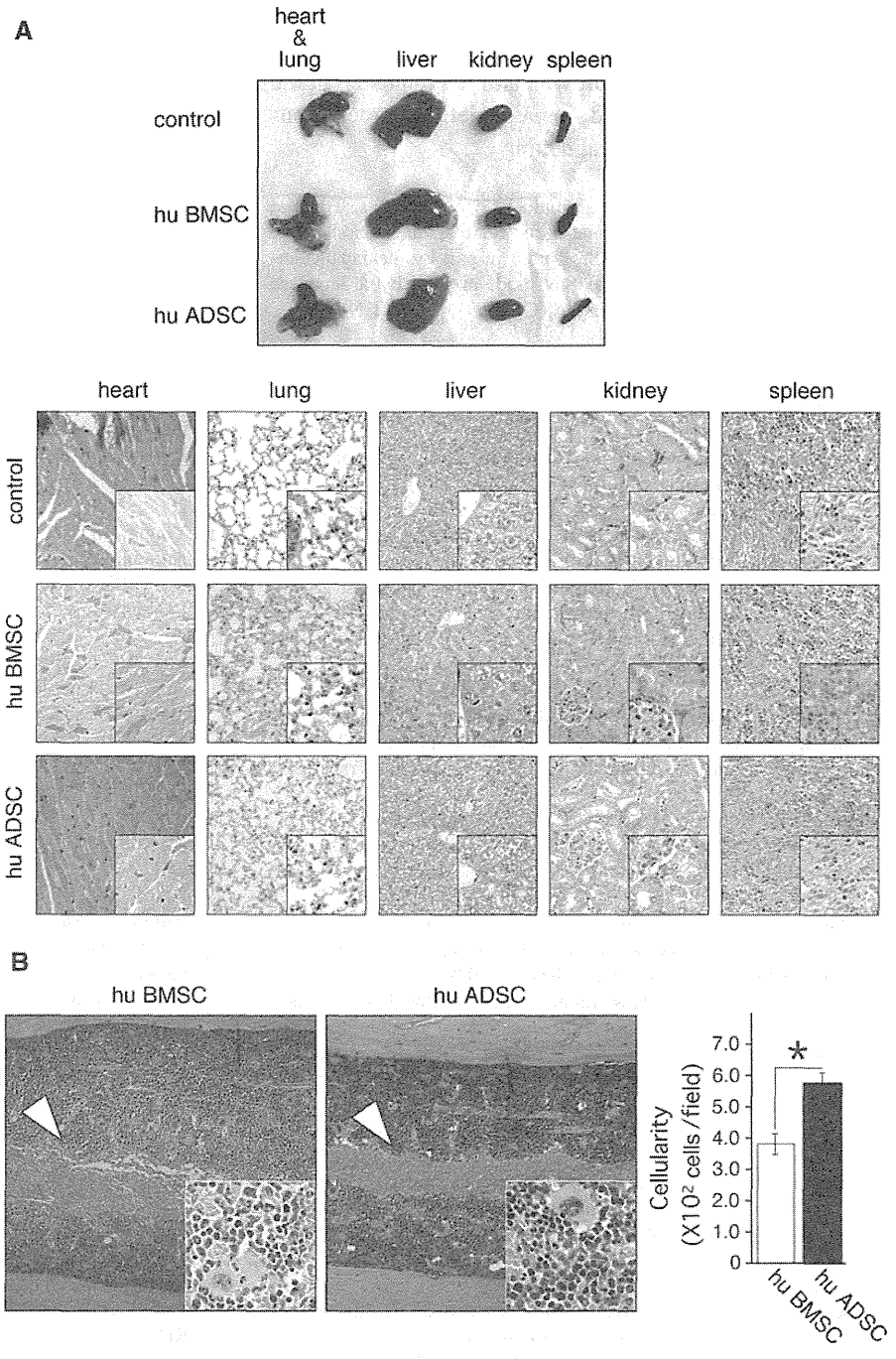

Fig. 2 Intra-bone Marrow Transplantation of human MSCs. Human (*hu*) BMSCs (1×10^5 cells), hu ADSCs (1×10^5 cells) or RPMI 1640 (control) were injected into the right tibiae of irradiated (3.0 Gy) 6- to 8-week-old NOD/SCID mice (one mouse per each MSC). All mice were humanely killed 5 weeks after injection, and the tibiae and major organs were excised for histological evaluation. **a** Representative gross morphology (*upper panel*) and microscopic histology (*lower panel* ×20; *inset* ×40) of heart, lung, liver, kidney, and spleen from control, BMSC- and ADSC-treated mice. **b** Representative microscopic histology of the BM stained with H&E stain (×10). *Inset* BM cellularity in mice that had received human BMSCs or ADSCs (×60). The cavities into which the 31-gauge needle had been inserted were filled with red blood cells (*arrowheads*). For quantitative analysis of BM cellularity, 4 fields were randomly selected and the number of nucleated cells was scored under a microscope. The mean nucleated cells per field ± SD is shown for each group. Statistical significance: $^*P < 0.05$

administration of 1×10^6 cells/kg body weight might be insufficient to restore a functional hematopoietic microenvironment. According to our recent findings, 1×10^5 ADSCs per mouse (about 25 g in weight) were required to facilitate hematopoiesis [13], which would be equivalent to 4×10^6 ADSCs per kilogram for adult humans. These data suggest that large quantities of infused cells are required for treatment. Thus, the rapid expansion capacity of ADSCs will also be advantageous for their clinical exploitation.

We observed no evidence of human ADSC-induced toxicity either during or after treatment. Histological evaluation 5 weeks after injection showed neither gross morphological nor microscopic changes in major organs of mice that received human ADSCs, which are sometimes a clue to the presence of latent adverse effects [22] (Fig. 2a). It has been reported that MSCs that persist in vivo may be tumorigenic [23]. However, we found that human ADSCs alone formed no colonies in semi-solid cultures (not

shown) and no tumors in vivo (Fig. 2). These data indicate the clinical safety of human ADSCs.

In summary, these data provide an important step in the regeneration of a perturbed hematopoietic microenvironment by ADSCs in a clinical setting.

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Escape of leukemia blasts from HLA-specific CTL pressure in a recipient of HLA one locus-mismatched bone marrow transplantation

Tomonori Kato [1], Seitaro Terakura [1], Makoto Murata *, Kyoko Sugimoto, Miho Murase, Chisako Iriyama, Akihiro Tomita, Akihiro Abe, Momoko Suzuki, Tetsuya Nishida, Tomoki Naoe

Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

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ABSTRACT

A case of leukemia escape from an HLA-specific cytotoxic T lymphocyte (CTL) response in a recipient of bone marrow transplantation is presented. Only the expression of HLA-B51, which was a mismatched HLA locus in the graft-versus-host direction, was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts. All CTL clones, that were isolated from the recipient's blood when acute graft-versus-host disease developed, recognized the mismatched B*51:01 molecule in a peptide-dependent manner. The pre-transplant leukemia blasts were lysed by CTL clones, whereas the post-transplant leukemia blasts were not lysed by any CTL clones. The IFN-γ ELISPOT assay revealed that B*51:01-reactive T lymphocytes accounted for the majority of the total alloreactive T lymphocytes in the blood just before leukemia relapse. These data suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA molecule can lead to clinical relapse after bone marrow transplantation.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative for leukemia by virtue of the immune reaction mediated by donor T lymphocytes, termed the graft-versus-leukemia (GVL) effect [1]. For HSCT recipients from HLA-matched donors, the GVL effect can be triggered by minor histocompatibility antigens [2–4], and several studies using sequential flow cytometric analysis with tetramers have clearly demonstrated that minor histocompatibility antigen-specific T lymphocytes increase in frequency in the recipient's blood before and during clinical regression of leukemia [5–10]. On the other hand, for HLA-mismatched HSCT recipients, extremely limited biological studies have demonstrated that the GVL effect can be mediated by mismatched HLA-specific donor T lymphocytes [11].

Allogeneic HSCT is a well-established immunotherapy for leukemia, but, unfortunately, some recipients relapse after transplantation. It is difficult to evaluate the role of individual factors in relapse. Nevertheless, it is reasonable to assume that the selective pressure exerted by donor T lymphocytes can lead to the outgrowth of pre-existing leukemia variants that have lost expression of gene products such as HLA molecules. Some studies have demonstrated loss of the mismatched HLA haplotype in the leukemia blasts of HSCT recipients as a consequence of loss of heterozygosity in chromosome 6 [12–14]. However, the mechanisms involved in leukemia relapse after HLA locus-mismatched HSCT remain largely uninvestigated.

This paper presents a case of selective HLA down-regulation in post-transplant leukemia blasts but not in pre-transplant blasts of a recipient who received bone marrow transplantation from an HLA one locus-mismatched donor. All cytotoxic T lymphocyte (CTL) clones that were isolated from the recipient's blood during acute graft-versus-host disease (GVHD) demonstrated cytotoxicity specific for the mismatched HLA-B molecule, lysed pre-transplant blasts but not post-transplant blasts, and persisted in the patient's blood until leukemia relapse. These results suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA allele can lead to clinical relapse.

2. Patient, materials and methods

2.1. Patient

A 24-year-old man with primary refractory T lymphoblastic leukemia/lymphoma received allogeneic bone marrow transplantation without ex vivo T lymphocyte depletion from his mother. Because the patient had neither a sibling nor an HLA-matched unrelated donor, his mother was chosen as an alternative donor. PCR sequencing-based typing for HLA alleles of the patient and mother revealed one HLA-B allele mismatch in

* Corresponding author. Fax: +81 52 744 2157.
E-mail address: mmurata@med.nagoya-u.ac.jp (M. Murata).
[1] These authors contributed equally.

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Table 1
HLA types of the patient and donor.

	A	B	C	DRB1	DQB1	DPB1
Patient	1101/2402	5401/5101	0102/–	0901/–	0303/–	0501/–
Donor	1101/2402	5401/5201	0102/1202	0901/1502	0303/0601	0501/–

The mismatched HLA allele in the graft-versus-host direction is underlined.

the graft-versus-host direction (Table 1). The preparative regimen consisted of 180 mg/m^2 melphalan and 12 Gy total body irradiation. GVHD prophylaxis consisted of 0.03 mg/kg tacrolimus and short-term methotrexate. Neutrophil engraftment (neutrophil count $\geqslant 0.5 \times 10^9$/l) was achieved 14 days after transplantation with full donor-type chimera. The patient developed severe acute GVHD involving the skin, gut, and liver on day 46 (maximum stage: skin 3, gut 2, and liver 1; maximum grade: III on day 53), evaluated according to previously published criteria [15]. Acute GVHD was temporarily controlled by additional immunosuppressants, but it was incurable and transitioned to chronic GVHD. On day 261, the patient relapsed with ascites, a hydrocele, and a subpapillary tumor. Leukemia blasts in the ascites fluid were confirmed by cytological examination. Immunosuppressant therapy was required to control GVHD until his death on day 279.

2.2. Cell culture

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the recipient on day 56, when severe acute GVHD developed, were stimulated in vitro with aliquots of γ-irradiated PBMCs that had been obtained from the recipient pre-transplant and cryopreserved. After three weekly stimulations, the CTL clones were isolated from the polyclonal T lymphocyte culture by limiting dilution. The CTLs were expanded by stimulation every 14 days with 30 ng/ml OKT3 monoclonal antibody (Janssen Pharmaceutical), using unrelated allogeneic γ-irradiated (25 Gy) PBMCs and γ-irradiated (75 Gy) EB virus-transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture medium consisted of RPMI-1640-HEPES (Sigma–Aldrich) containing 10% pooled, heat-inactivated human serum, and recombinant human IL-2 (R&D Systems). The T lymphocytes were used in assays 14 days after stimulation or 1 day after thawing of a frozen aliquot. All samples were collected after written informed consent had been obtained. B-LCLs were maintained in RPMI-1640-HEPES with 10% FBS. COS cells were maintained in DMEM (Sigma–Aldrich) with 10% FBS.

2.3. Flow cytometric analysis

Leukemia blasts were incubated at 37 °C for 30 min with anti-HLA-A24/A23 (One lambda), anti-HLA-A11/A1/A26 (One lambda), and anti-HLA-B51/B52/B49/B56 (One lambda) antibodies to detect A24, A11, and B51, respectively, of patient cells followed by incubation at 37 °C for 15 min with fluorescein isothiocyanate-conjugated antimouse IgM (Beckman Coulter). To detect HLA-DR9 of patient cells, leukemia blasts were incubated at 37 °C for 30 min with fluorescein isothiocyanate-conjugated anti-HLA-DR antibody (BD Pharmingen). Antibody to detect HLA-B54 without cross-reaction to B51 was not available. After washing, the cells were analyzed by a BD FACSAria (BD Biosciences). Leukemia blasts were sorted by BD FACSAria with anti-CD7 (BD Biosciences) and anti-CD10 (eBiosciences) antibodies from pre-transplant bone marrow and post-transplant ascites fluid samples. The purities of pre-transplant and post-transplant blasts were ~62% and ~99%, respectively. CTL clones were analyzed using three-color flow cytometry for expression of CD3, CD4, and CD8 using phycoerythrin-cyanin 5.1-conjugated anti-CD3 (Beckman Coulter), phycoerythrin-conjugated anti-CD4 (BD Biosciences), and fluorescein isothiocyanate-conjugated anti-CD8 (BD Biosciences) antibodies.

2.4. Chromium release assay

Leukemia blasts and B-LCLs were used as target cells in a cytotoxicity assay. Leukemia blasts and B-LCLs were labeled for 2 h with ^{51}Cr. After washing, the cells were dispensed at 2×10^3 cells/well into triplicate cultures in 96-well plates and incubated for 4 h at 37 °C with CTL clones at various E:T ratios. Percent-specific lysis was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] ×100.

2.5. Determination of T cell receptor (TCR)-Vβ gene usage and nucleotide sequences

TCR Vβ usage was assessed by RT-PCR using primers covering the entire families of functional TCR Vβ chains [17–19]. Briefly, total RNA was extracted from individual CTL clones, and cDNA was synthesized using SuperScript III RT (Invitrogen). RT-PCR reactions were carried out with the appropriate Vβ sense primers specific for different Vβ families and a primer specific for the constant region of TCR-β. Subsequently, the complementarity determining region 3(CDR3) of each positive PCR product was sequenced with corresponding antisense primer. TCR Vβ gene usage was determined by the international ImMunoGeneTics information system (IMGT) software, IMGT/V-QUEST (http://www.imgt.org/).

2.6. HLA-B cDNA constructs

Total RNA was extracted from the patient and donor B-LCLs and converted into cDNA. Constructs containing the full-length *HLA-B*51:01*, *B*52:01*, and *B*54:01* cDNA were generated from the cDNA by PCR and cloned into the pEAK10 expression vector (Edge BioSystems). Two mutated *HLA-B*51:01* cDNA constructs, in which amino acid at position 63 or 67 was substituted with the corresponding amino acid in *B*52:01*, and two more mutated *HLA-B*51:01* cDNA constructs, in which the amino acid at position 194 or 199 was substituted with the corresponding amino acid in *B*44:03*, were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

2.7. Transfection of B-LCLs and COS cells with HLA cDNA

B-LCL (5×10^6) were transfected by electroporation (200 V, 500 μFD) in 200 μl of potassium-PBS with the 15 μg of pEAK10 plasmid encoding *HLA-B*51:01* cDNA and selected with puromycin (Edge BioSystems), beginning 48 h after transfection. Three days after selection, they were used as targets in a chromium release assay. COS cells (5×10^3) were plated in individual wells of 96-well flat-bottom plates and transfected with 100 ng of the pEAK10 plasmid encoding *HLA-B*51:01*, *HLA-B*52:01*, *HLA-B*54:01*, or mutated *HLA-B*51:01* cDNA using the FuGENE 6 Transfection Reagent (Roche).

2.8. CTL stimulation assay

COS transfectants (5×10^3) were cocultured with CTL clones (2×10^4) in individual wells of 96-well flat-bottom plates for 24 h at 37 °C, and IFN-γ production was measured in the supernatant using ELISA (Endogen).

2.9. Enzyme-linked immunospot (ELISPOT) assay

T lymphocytes were isolated from recipient's PBMCs by negative depletion using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and used as responder T cells. Responder T cells at a concentration of 2×10^5 per well were plated in individual wells of the 96-well MultiScreen-IP filter plates (Millipore) coated with anti-human interferon (IFN)-γ antibody (5 μg/ml; Mabtech) and tested in triplicate against a total of 2×10^5 stimulator cells: patient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. The plates were incubated for 24 h at 37°C, washed, and incubated with biotinylated anti-human IFN-γ antibody (1 μg/ml; Mabtech) for 2 h at room temperature. After addition of streptavidin (Fitzgerald Industries International) to the wells, the plates were developed with a 3-amino-9-ethylcarbazol substrate kit (Vector Laboratories). Spots were counted using a microscope, and mean numbers were calculated from triplicate wells after subtraction of the number of spots obtained with medium alone.

3. Results

3.1. Selective down-regulation of HLA-B locus in post-transplant leukemia blasts

To determine whether expressions of some HLA loci in post-transplant relapsed leukemia blasts were down-regulated or lost, flow cytometric analysis was performed for HLA-A*24:02, A*11:01, B*51:01, and DR*09:01 using anti-HLA-A24/A23, -HLA-A11/A1/A26, -HLA-B51/B52/B49/B56, and -pan HLA-DR antibodies, respectively. The expression of B*51:01 was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts, whereas expressions of A*24:02, A*11:01, and DR*09:01 were the same or higher in post-transplant blasts than in pre-transplant blasts (Fig. 1). These data led us to question whether B*51:01-selective pressure mediated by donor T lymphocytes was present in the patient post-transplant.

3.2. Isolation of alloreactive CTL clones

Ten CTL clones, termed TK1 to TK10, were isolated from the peripheral blood of the recipient during acute GVHD. In a cytotoxicity assay, all isolated clones lysed recipient B-LCL but failed to lyse donor B-LCL (Fig. 2), demonstrating that all clones were alloreactive. Flow cytometric analysis revealed that all CTL clones

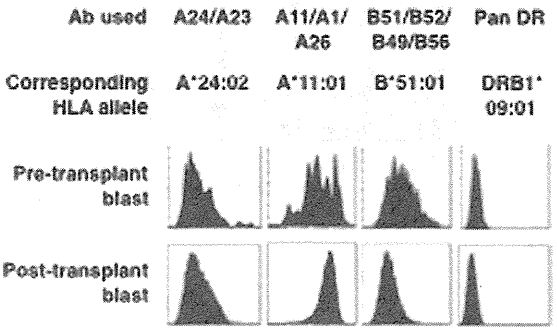

Fig. 1. HLA expression on leukemia blasts. Pre-transplant and post-transplant leukemia blasts were stained with anti-HLA-A24/A23, anti-HLA-A11/A1/A26, anti-HLA-B51/B52/B49/B56, and anti-HLA-pan DR antibodies to detect A*24:02, A*11:01, B*51:01, and DRB1*09:01, respectively. Data are representative of four experiments.

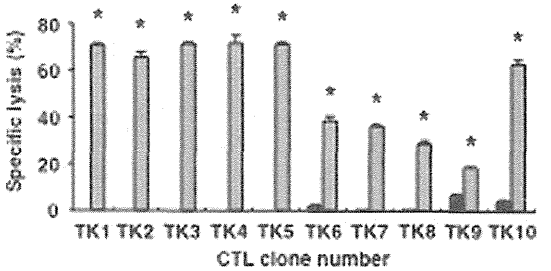

Fig. 2. Cytotoxicities of CTL clones against B-LCLs. B-LCLs that originated from the recipient (gray) and the donor (black) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at an E:T ratio of 10:1. *Significant difference ($p < 0.0001$: Student's *t*-test) in the lysis of recipient B-LCL compared with donor B-LCL. Data are representative of three experiments.

Table 2
Clonotypes of isolated CTL clones.

CTL	TCR Vβ	Nucleotide and deduced amino acid sequences of complementarity determining region 3																	
TK1	Vβ6.5	GCC	AGC	AGT	CCC	GGG	ACT	AGC	GGA	ACC	TAC	GAG	CAG	TAC	TTC				
		A	S	S	P	G	T	S	G	T	Y	E	Q	Y	F				
TK2	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT						
		S	Q	G	P	A	V	T	G	E	L	F	F						
TK3	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT						
		S	Q	G	P	A	V	T	G	E	L	F	F						
TK4	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK5	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK6	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK7	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK8	ND																			
TK9	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK10	Vβ2	GCC	AGC	AGT	GAC	TCT	ATC	GCG	GAT	GAG	CAG	TTC	TTC							
		A	S	S	D	S	I	A	D	E	Q	F	F							

ND, not detected.

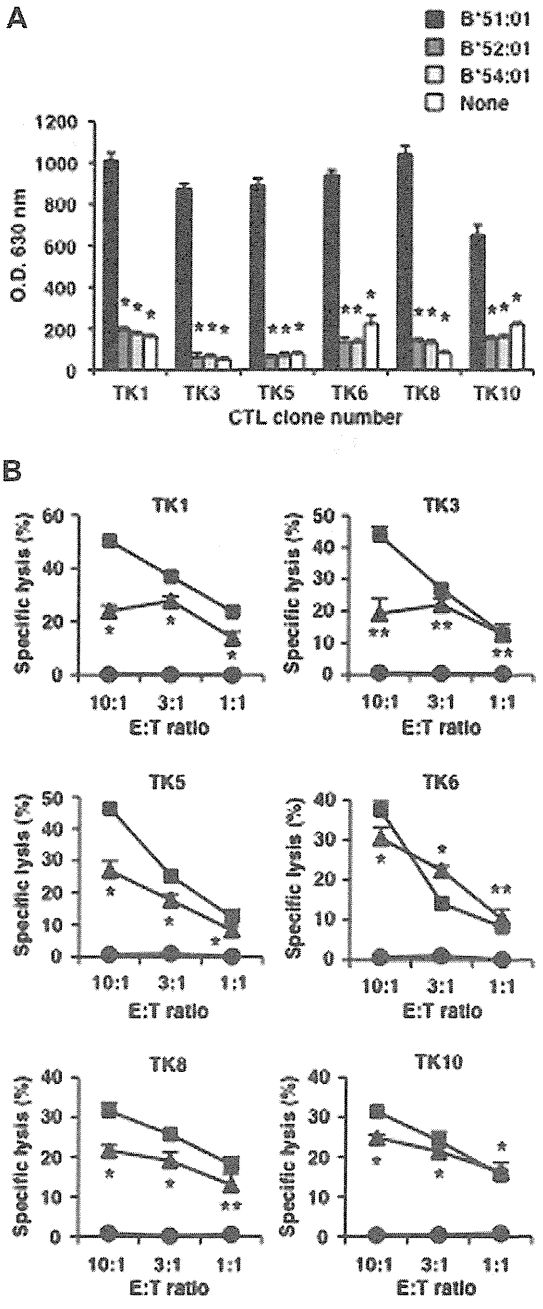

Fig. 3. Recognition of the HLA-B*51:01 molecule by CTLs. (A) COS cells were transfected with a plasmid encoding *B*51:01* cDNA, *B*52:01* cDNA, *B*54:01* cDNA, or no cDNA cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.01$: Student's *t*-test) in the IFN-γ production stimulated by *B*52:01* cDNA, *B*54:01* cDNA or no cDNA compared with *B*51:01* cDNA. Data are representative of three experiments. (B) Recipient B-LCL (square), donor B-LCL (circle), and donor B-LCL transfected with *HLA-B*51:01* cDNA (triangle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Significant difference (*$p < 0.01$; **$p < 0.05$) in the lysis of *B*51:01*-transfected donor B-LCL compared with donor B-LCL (negative control). Data are representative of three experiments.

were CD3+/CD4−/CD8+ (data not shown). The nucleotide sequences of the uniquely rearranged *TCR Vβ* gene of each clone were determined by direct DNA sequencing of the amplified PCR products of TCR (Table 2). The TK2 and TK3 clones had the same nucleotide sequences in the CDR3 regions of their TCR Vβ20, suggesting that these CTLs originated from a single clone. Similarly, TK4 and TK5, as well as TK6, TK7, and TK9, had the same nucleotide sequences in the CDR3 regions of their TCR Vβ19*1 and Vβ12, respectively, suggesting that each group also originated from a single clone. Thus, the 10 isolated alloreactive CTL clones appeared to have been derived from six independent clones.

3.3. CTL clones recognized the HLA-B*51:01 molecule

To evaluate the possibility that isolated CTL clones recognize the HLA-B*51:01 molecule, COS cells were first transfected with an *HLA-B*51:01*, *−B*52:01*, or *−B*54:01* cDNA construct, COS transfectants were cocultured with six independent CTL clones, and then the production of IFN-γ in the supernatant was measured. The COS cells transfected with *HLA-B*51:01* clearly stimulated IFN-γ production by six independent CTL clones, whereas neither *B*52:01* nor *B*54:01* stimulated them (Fig. 3A). Then, donor B-LCL were transfected with an *HLA-B*51:01* cDNA construct and used as target cells in a cytotoxicity assay. The donor B-LCL transfected with *HLA-B*51:01* were lysed by six CTL clones (Fig. 3B), indicating that all clones recognized the mismatched HLA-B*51:01 molecule as an alloantigen. On the other hand, these data suggest that the CTL response toward the HLA-B*51:01 molecule accounted for the majority of the recipient's CTL alloresponse during acute GVHD.

3.4. Recognition of HLA molecules by CTL clones was peptide-dependent

Various forms of T lymphocyte recognition of the allogeneic major histocompatibility antigen, ranging from peptide-dependent to peptide-independent, have been demonstrated [20]. To confirm peptide dependency in CTL recognition, examinations were focused on the difference in the amino acid sequences of the recipient B*51:01 and the donor B*52:01. They differed in two amino acids at positions 63 and 67 (Fig. 4A), which constitute peptide binding pockets A and/or B [21,22]. In particular, B-pocket has a critical role in peptide binding to HLA-B*51:01 molecules [23], and substitution of a single amino acid constituting peptide binding pocket can affect peptide binding [24]. Two mutated *B*51:01* cDNA constructs, *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser*, in which individual amino acids were substituted with the corresponding amino acid in B*52:01 (Fig. 4A), were generated, as well as two more mutated *B*51:01* cDNA constructs, *B*51:01-Val194Ile* and *B*51:01-Ala199Val*, in which individual amino acids exist in *B*44:02* and other B alleles and localize outside the positions constituting peptide binding pockets. COS cells were then transfected with each wild or mutated cDNA construct and examined in the CTL stimulation assay. IFN-γ production of the TK3 clone was significantly decreased when stimulated by the *B*51:01-Phe67Ser* mutant in comparison with the wild-type *B*51:01* construct (Fig. 4B). IFN-γ production of all other CTL clones, TK1, TK5, TK6, TK8, and TK10, was significantly decreased when stimulated by *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser* mutants in comparison with the wild-type *B*51:01* construct (Fig. 4B). However, both *B*51:01-Val194Ile* and *B*51:01-Ala199Val* mutants stimulated all CTL clones to the same degree as the wild-type *B*51:01* construct. Thus, these data suggest that recognition of the HLA-B*51:01 molecule by CTL clones was peptide-dependent.

Furthermore, CTL clones should recognize certain peptides other than leukemia antigens, presented by HLA-B*51:01 molecules, because *B*51:01*-transfected COS cells, which are derived from monkey kidney cells, stimulated IFN-γ production of CTLs

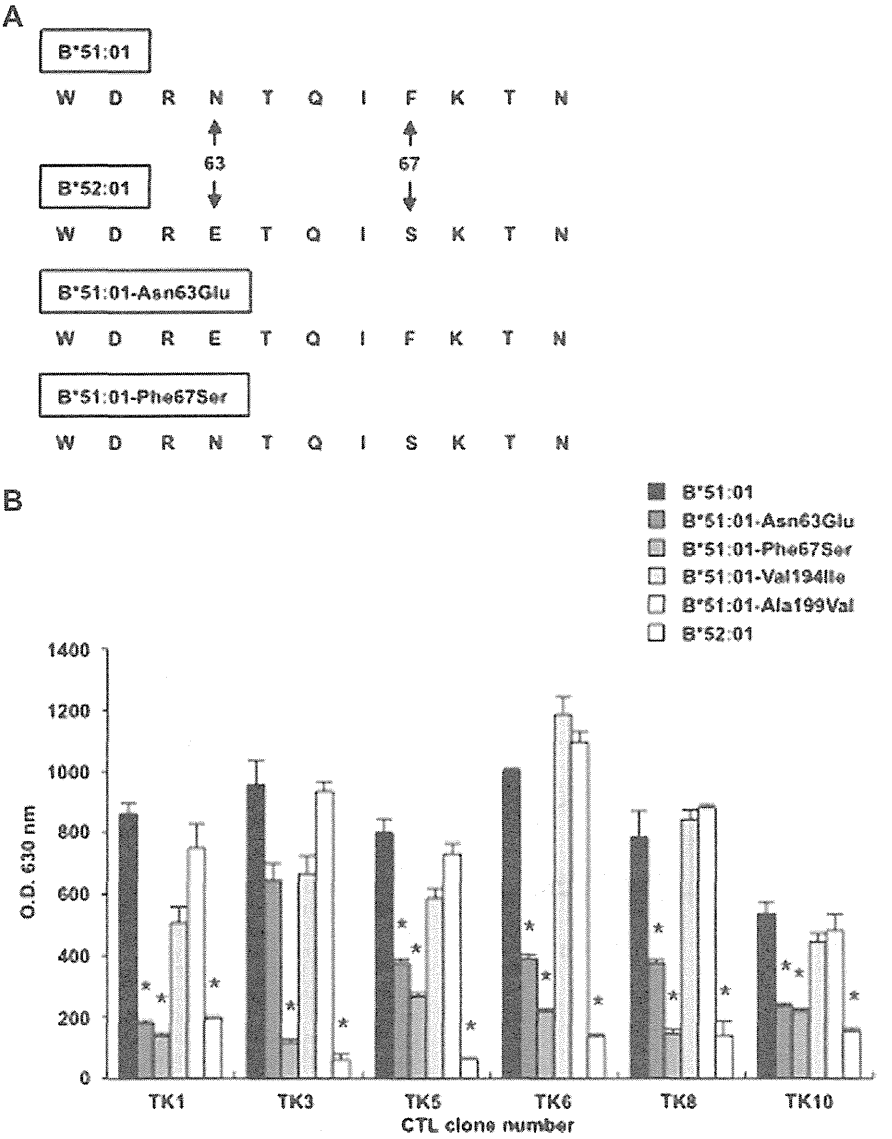

Fig. 4. Recognition of the HLA-B*51:01 molecule by CTLs is peptide-dependent. (A) The amino acid sequences at position 60 to 70 of the *B*51:01*, *B*52:01*, *B*51:01-Asn63Glu*, and *B*51:01-Phe67Ser* cDNAs are shown. Asn at position 63 was substituted with the corresponding amino acid in B*52:01, Glu, in the *B*51:01-Asn63Glu* mutant. Phe at position 67 was substituted with the corresponding amino acid in B*52:01, Ser, in the *B*51:01-Phe67Ser* mutant. (B) COS cells were transfected with a plasmid encoding *B*51:01*, *B*51:01-Asn63Glu*, *B*51:01-Phe67Ser*, *B*51:01-Val194Ile*, *B*51:01-Ala199Val* or *B*52:01* cDNA construct, cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.05$: Student's *t*-test) in the IFN-γ production stimulated by each mutant or *B*52:01* cDNA construct compared with the wild-type *B*51:01* cDNA construct. Data are representative of three experiments.

(Fig. 3A), and *B*51:01*-transfected donor B-LCL, which are derived from B lymphocytes, were lysed by CTLs (Fig. 3B).

3.5. Leukemia blasts escaped from immunological pressure by HLA-B-specific CTLs

Whether the leukemia blasts escaped from the cytotoxicity of HLA-B*51:01-specific CTL clones was then examined. Pre-transplant and post-transplant leukemia blasts were purified by fluorescence-activated cell sorter (purity, ~62% and ~99%, respectively), and a cytotoxicity assay was performed only for the TK1 CTL clone because of the limited number of cryopreserved blasts. Weak but clear lysis of pre-transplant leukemia blasts by the TK1 CTL clone was observed, whereas post-transplant leukemia blasts were not lysed (Fig. 5A). All other CTL clones (TK3, TK5, TK6, TK8, and TK10) also did not lyse post-transplant leukemia blasts (Fig. 5B).

In addition, whether HLA-B*51:01-specific CTL pressure persisted until leukemia relapse was examined. The IFN-γ ELISPOT assay was performed to detect HLA-B*51:01-reactive CTLs in patient blood on day 232, 1 month before clinical leukemia relapse (Fig. 6). IFN-γ-producing B*51:01-reactive T lymphocytes were detected at a level nearly equal to the level of recipient B-LCL-reactive CTLs, that is, the total CTL alloresponse.

4. Discussion

The mechanism of leukemia relapse in this recipient can be explained as follows. CTLs specific for HLA-B*51:01 molecule/

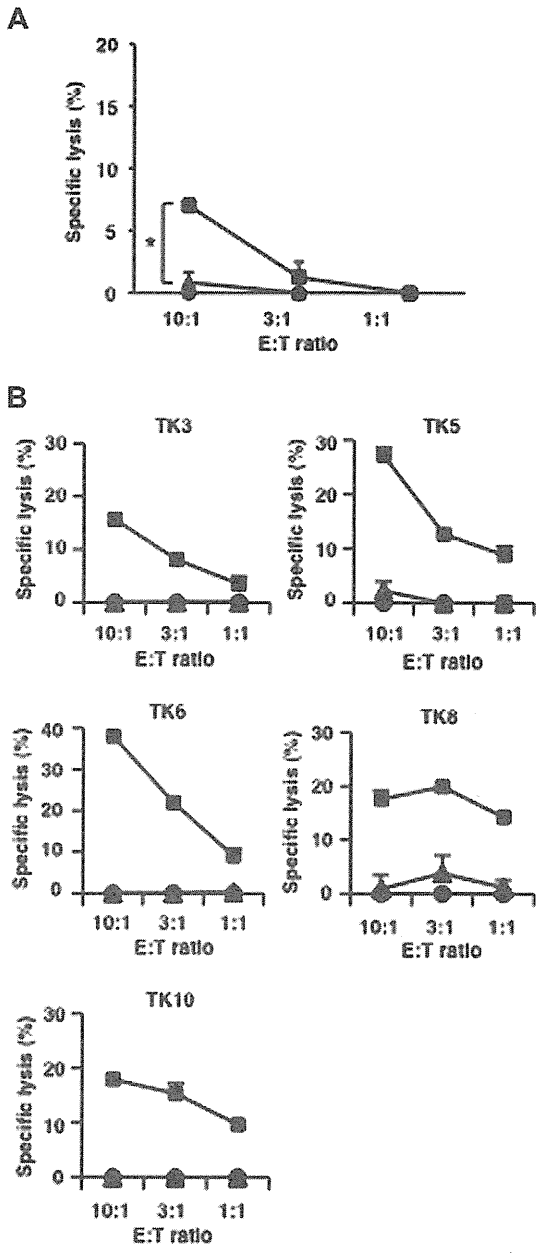

Fig. 5. Cytotoxicities of CTLs against leukemia blasts. (A) Purified pre-transplant leukemia blasts (purity, ~62%) (square), purified post-transplant leukemia blasts (purity, ~99%) (triangle) and donor B-LCL (circle) were used as targets for TK1 CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. *Significant difference ($p = 0.024$: Student's t-test) in the lysis of the pre-transplant leukemia blasts compared with the post-transplant leukemia blasts. Data are representative of three experiments. (B) Purified post-transplant leukemia blasts (purity, ~99%) (triangle), B-LCLs from the patient (square) and donor (circle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Data are representative of three experiments. There was no significant difference in the lysis of the post-transplant leukemia blasts compared with B51-negative donor B-LCL (negative control).

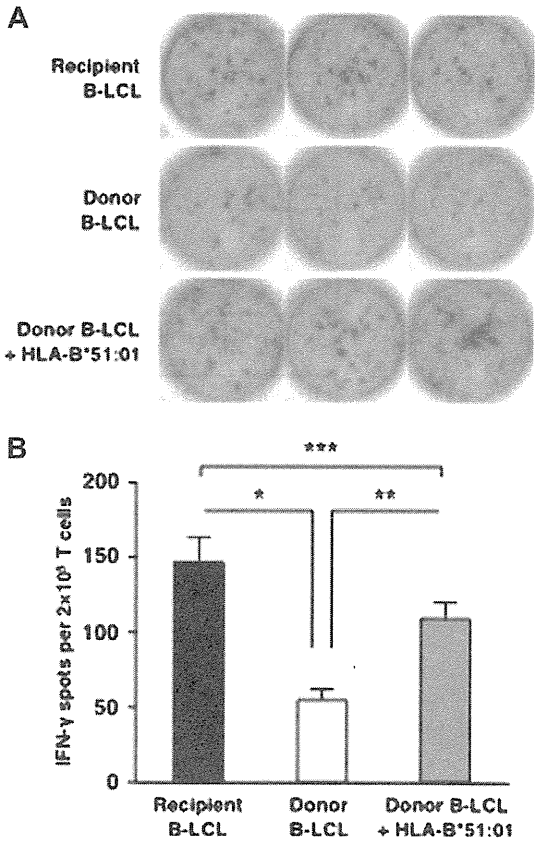

Fig. 6. Detection of HLA-B*51:01-specific CTLs in T lymphocytes obtained from the recipient on day 232 after transplantation. (A) Representative ELISPOT wells show triplicate results of T lymphocytes stimulated by recipient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. Data are representative of three experiments. (B) The frequency of CTLs in T lymphocytes recognizing the HLA-B*51:01 molecule was measured by IFN-γ ELISPOT analysis. The frequency of IFN-γ-producing cells is shown against recipient B-LCL (black), donor B-LCL (white), and *HLA-B*51:01*-transfected donor B-LCL (gray). Data are the means and SD of triplicate determinations. *$p = 0.0057$; **$p = 0.0077$; ***$p = 0.090$ (Student's t-test). Data are representative of three experiments.

non-leukemia peptide complex were generated in the recipient blood during acute GVHD, and these CTLs continued to produce immunological pressure on leukemia blasts for at least 8 months after transplantation, but B*51:01-down-regulated leukemia blasts escaped from the pressure of B*51:01-specific CTLs, and then the leukemia clinically relapsed.

CTLs recognizing mismatched HLA molecules play an important role in the immune reaction after HLA-mismatched HSCT, including graft rejection [25–27], GVHD [28], and the GVL effect [11]. In this study, the mismatched HLA-B*51:01-specific CTLs could participate both in GVHD and the GVL effect in the recipient. Ten CTL clones were isolated from the recipient's blood just after the onset of grade III acute GVHD involving skin, gut, and liver, and all clones demonstrated HLA-B*51:01-specific cytotoxicity in a non-leukemia peptide-dependent manner (Fig. 3 and 4). The patient was suffering from GVHD until his death on day 279, and in the ELISPOT assay for T lymphocytes obtained from recipient blood on day 232, HLA-B*51:01-reactive T lymphocytes accounted for the majority of alloreactive T lymphocytes (Fig. 6). Meanwhile, weak but clear lysis of pre-transplant leukemia blasts by an HLA-B*51:01-specific CTL clone was confirmed (Fig. 5A), and the primary refractory T lymphoblastic leukemia/lymphoma was in remission until day 261. These data are consistent with participation of the recipient HLA-B locus-specific CTLs both in GVHD and the GVL effect.

Selective HLA down-regulation was seen in this patient's post-transplant leukemia blasts. Mechanisms that alter HLA class I expression have been investigated and summarized as follows [29]: (1) loss of heterozygosity in chromosome 6 and/or 15, in

which class I heavy chain or β_2-microglobulin genes are located [30,31]; (2) mutations in these genes [32]; and (3) down-regulation of the antigen processing machinery, including transporter associated with antigen processing and low-molecular-weight protein genes [33]. Ten *HLA-B* cDNAs were cloned from purified post-transplant leukemia blasts (purity, ~99%) by RT-PCR; 5 (50%) clones were identical to the canonical *B*51:01* cDNA sequence, and the others (50%) were identical to the canonical *B*54:01* cDNA sequence, which was another recipient B allele (data not shown). These data suggest that down-regulation of HLA-B*51:01 expression in the post-transplant leukemia blasts resulted from mechanisms other than loss of heterozygosity of B locus and mutation of the *B*51:01* gene itself, although the entire sequence of *B*51:01* DNA including introns has not been determined. Recently, hypermethylation of the HLA-class I gene promoter regions has been identified as a mechanism for transcriptional inactivation of HLA class I genes in esophageal squamous cell carcinoma lesions [34]. We analyzed *B*51:01* promoter methylation by pyrosequencing of bisulfite-treated DNA from purified post-transplant leukemia blasts and confirmed no hypermethylation of the *B*51:01* gene (data not shown). Other possible mechanisms are down-regulation of translation and post-translational modification of the *B*51:01* gene, although, to the best of our knowledge, these mechanisms have not yet been investigated for HLA-class I genes. Further analysis is required.

The change in expression in HLA-A*11:01 between pre-transplant and post-transplant leukemia blasts was of similar magnitude, but in the opposite direction, to that observed for B*51:01. Because the expression of HLA-A11 on target cells can protect them from lysis by KIR3DL2-positive NK cells [35], the possibility that the post-transplant blasts with high expression of HLA-A11 were resistant to NK cell-mediated cytotoxicity, resulting in leukemia relapse, cannot be ruled out.

A question left unresolved is whether the present observation is unique to this recipient or can be duplicated in additional recipients who receive HLA one locus-mismatched HSCT. However, the present findings can explain, at least in part, the mechanism of how leukemia relapse occurs during persistent GVHD after HSCT. Another question is whether the present observation is unique to T lymphoblastic leukemia/lymphoma, which is a relatively rare subset of acute leukemia in adults. The relevance of this finding to other leukemias, including B lymphoblastic leukemia/lymphoma and myeloid malignancies, should be confirmed. Further efforts to identify the peptides that are presented by HLA-B*51:01 molecules and recognized by isolated CTL clones should help to elucidate the precise mechanisms of leukemia escape.

In conclusion, immune escape of leukemia blasts from CTL pressure toward a mismatched HLA molecule/non-leukemia peptide complex may lead to clinical leukemia relapse.

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総　説

同種造血幹細胞移植後ウイルス感染に対するドナーリンパ球を用いた養子免疫療法

西田徹也

名古屋大学医学部附属病院血液内科

Adoptive Immunotherapy for Virus Infection after Allogeneic Hematopoietic Stem Cell Transplantation

Tetsuya Nishida

Department of Hematology, Nagoya University Hospital

Summary Viral infection has remained one of the primary factors causing morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). The reconstitution of virus-specific T cells is considered to be indispensable to control viral infection after allogeneic HSCT. Although donor lymphocyte infusion (DLI) might improve viral infection, there is a possibility that DLI causes graft-versus-host disease (GVHD) and marrow suppression. Virus-specific T cells have been expected to cure persistent viral infection without GVHD, and clinical trials have shown the safety and efficacy of adoptive transfer of virus-specific T cells for prophylaxis and treatment of viral infections such as cytomegalovirus and Epstein-Barr virus. However, the number of patients who received adoptive immunotherapy has been limited, because time and special institutions are required to generate virus-specific T cells. In Western countries, Cell Banks have been established so that virus-specific T cells are available off the shelf.

Key words: adoptive immunotherapy, cytotoxic T lymphocyte, allogeneic hematopoietic stem cell transplantation, viral infection

1. はじめに

同種造血幹細胞移植は，白血病などの難治性造血器悪性腫瘍に対して，治癒が期待できる治療法として確立されてきた．長年，腫瘍細胞の根絶のためには強力な移植前治療は必須のものと考えられ，高齢者や臓器障害を有する患者は同種移植の適応外であった．しかしながら，1997年に前治療の強度を弱め，生着のための免疫抑制を強化した骨髄非破壊的同種移植の有用性が報告されて以来[1]，骨髄破壊的前治療が困難な高齢者や合併症を有する患者に対しても移植適応が拡大され，移植数は飛躍的に増加してきている．

移植片対宿主病（graft-versus-host disease：GVHD）予防のためにT細胞除去をした同種移植では移植後白血病再発が多く，また，GVHDを合併した患者では再発が少ないことから，移植したドナー由来のリンパ球が患者の白血病細胞を認識して攻撃する移植片対白血病（graft-versus-leukemia：GVL）効果が示唆され[2]，1990年にはKolbらから移植後再発に対するドナーリンパ球輸注（donor lymphocyte infusion：DLI）の有効性が報告された[3]．ドナー由来リンパ球は，同種移植において腫瘍根絶に重要な役割を果たしているだけでなく，GVHDや感染症などの移植後の予後を左右する合併症に関与している．免疫抑制剤を用いて免疫担当細胞を抑制することでGVHDは制御されるが，感染症や再発が増加してくる．逆に，感染症や再発に対して，免疫抑制を解除するとGVHDが惹起される可能性がある．また，DLIは移植後再発やウイルス感染に対して有効なケースがあるが，GVHDや骨髄抑制などの副作用もあり，その適応は慎重に検討される必要がある．そこで，GVHDを誘導することなく，感染や腫瘍をコントロールしうる治療法として，ドナー末梢血単核球からウイルスや腫瘍細胞を標的とした抗原特異的T細胞を体外で誘導・増殖させ，患者に輸注する養子免疫療法の効果が期待され，臨床応用が試みられている（図1）．本稿においては，同種造血幹細胞移植後ウイルス感染に対するドナーリンパ球を用いた養子免疫療法の現状と今後の展望について

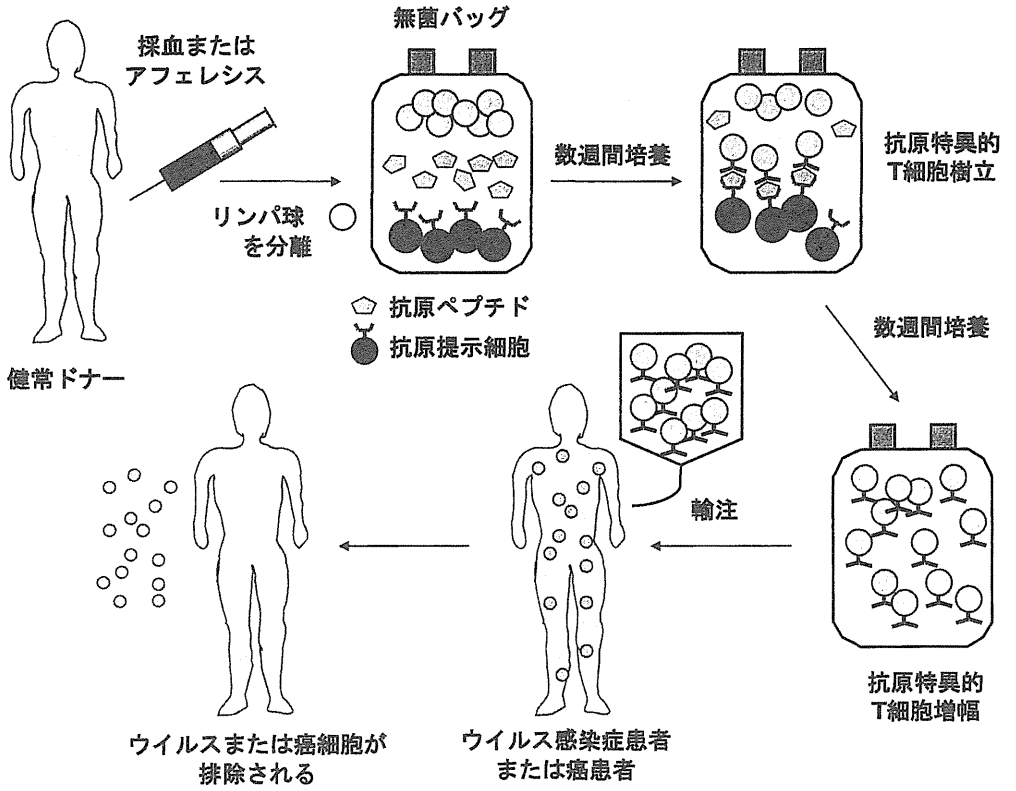

図1　抗原特異的 CTL による養子免疫療法

紹介する．

2. ウイルス感染に対する養子免疫療法

サイトメガロウイルス（cytomegalovirus：CMV）やエプスタインバールウイルス（Epstein-Barr virus：EBV）などのヘルペス属ウイルスは，ほとんどの成人において既感染であるものの，健常人では自己の免疫力によってウイルスの活性化が制御され，感染症を起こすことはほとんどない．しかし，免疫力が低下した場合，ウイルスの再活性化を抑えることができず，特に，同種造血幹細胞移植後などの高度な免疫不全状態では致死的な転帰をとることがある．これらのウイルスの排除に対しては，ウイルス特異的 T 細胞が重要な役割を担っている．

2.1 C M V

同種造血幹細胞移植後に最も高頻度に合併する CMV 感染は，肺炎・胃腸炎・網膜炎・肝炎など多彩な臨床症状を呈する．近年，CMV アンチゲネミアなどによる感染モニタリングや抗ウイルス剤であるガンシクロビルの早期投与により，致死的な CMV 感染症は減少してきている．しかし，薬剤耐性ウイルスなどの問題があり，CMV 感染症は現在も移植後の克服すべき課題である．ウイルス排除には宿主の免疫能が重要であり，1992 年に Riddell らから骨髄移植後の 3 名の患者に対して CMV 特異的細胞傷害性 T 細胞（cytotoxic T lymphocyte：CTL）クローンを用いた養子免疫療法の有用性が示されて以来[4]，欧米において，移植後 CMV 感染症治療および予防に，CMV 特異的 CTL 輸注療法が行われ，その有用性が報告されている（表 1）．

2.2 E B V

EBV は，B 細胞に親和性が高いが，上皮細胞，T/NK 細胞などにも感染し，バーキットリンパ腫，上咽頭癌，ホジキンリンパ腫，T/NK 細胞リンパ腫などの悪性腫瘍の発症と関連している．これらの悪性腫瘍に対して，自家リンパ球から誘導した EBV 特異的 CTL を用いた養子免疫療法の有効性が報告されている[11~13]．同種造血幹細胞移植において T 細胞除去や抗胸腺細胞グロブリンを使用するなど高度な免疫抑制状態では，EBV 関連リンパ増殖症（lymphoproliferative disorder：LPD）を発症する．ドナー由来の B 細胞から生じることが多い．移植後 EBV-LPD に対しては，DLI が有効であり，日本骨髄バンクでの DLI 申請時の A 適応（DLI の効果の期待度が高い）

表1　造血幹細胞移植後 CMV 感染に対する細胞療法

対象	投与細胞	症例数	有効例	有害事象	GVHD	文献
予防	$CD8^+$ CMV CTL clone	14	14	0	3 (G I-II)	5
抗ウイルス剤抵抗性	CMV CTL line	8	6	0	0	6
感染	CMV CTL line	16	8	0	3 (G I)	7
感染	CMV CTL line	9	8	0	3 (G I-II)	8
予防	CMV CTL line	9	6	0	3 (G II-III)	9
予防	CMV CTL line	12	8	0	4 (G II-III)	10

となっているが，DLI による GVHD 誘因の危険性もある．EBV 特異的 CTL による EBV-LPD の治療効果は高く，GVHD などの合併症がほとんどないことから，EBV-LPD 発症のリスクが高い症例に対しては予防的に用いられ有用性が報告されているが[14,15]，時間や施設などの点から EBV 特異的 CTL の調整は容易ではない．近年，抗 CD20 モノクローナル抗体である rituximab の EBV-LPD に対する有効性が報告され[16]，DLI や EBV 特異的 CTL による細胞療法は第一選択ではなくなっている．

2.3　その他のウイルス感染

同種造血幹細胞移植後の出血性膀胱炎などの原因となるアデノウイルス（Adenovirus：ADV）に対しては，抗ウイルス薬 cidofovir の有効性が報告されているが，腎毒性などの副作用の他に，我が国では保険承認されていないという問題があり，ADV に対する有効な治療法の開発が必要である．ADV 特異的 CTL による養子免疫療法の有用性が海外から報告されており[17,18]，現在，我々は，日本人に対する ADV 特異的 CTL を用いた養子免疫療法の可能性について研究を進めている．

ポリオーマウイルス科に属する BK ウイルスは，アデノウイルス同様に同種造血幹細胞移植後の出血性膀胱炎の原因ウイルスであり[19]，BK ウイルス特異的 T 細胞の反応が出血性膀胱炎発症と関連していることが報告されている[20]．BK ウイルス特異的 T 細胞による養子免疫療法の開発が進んでおり[21]，今後の臨床応用が期待される．

3. 複数ウイルスに対する CTL

CMV pp65 蛋白を組み込んだアデノウイルスベクターを遺伝子導入した EBV 形質変換リンパ芽球細胞株（EBV-transformed B-lymphoblastoid cell line：EBV-LCL）を抗原提示細胞（Ad5f35pp65-EBV-LCL）として T 細胞を刺激することで，1 回の培養にて CMV，ADV，EBV に対するウイルス特異的 CTL を同時に誘導して，これらのウイルス感染に対する治療に用いられている[22]．

4. 臍帯血を用いた養子免疫療法

臍帯血に含まれている T 細胞は骨髄や末梢血中の T 細胞と異なり，ほぼすべての T 細胞がナイーブ T 細胞であり，同種抗原に対する反応性が低い．そのため，HLA 不一致でも臍帯血移植は可能であり，骨髄移植や末梢血幹細胞移植より GVHD のリスクは低いが，造血の回復が遅く，感染症の合併に注意が必要である．臍帯血は細胞数が少ないことに加え，ナイーブ T 細胞から抗原特異的 CTL を誘導することは困難であり，移植臍帯血を用いた養子免疫療法の実現のためにはこれらの課題を克服する必要がある．

Park KD らが，臍帯血単核球から樹立した樹状細胞（dendritic cells：DC）を用いて，臍帯血 T 細胞より CMV 特異的 T 細胞を誘導できることを報告した[23]．その後，Hanley PJ らは，CMV pp65 蛋白を組み込んだアデノウイルスベクターを遺伝子導入した臍帯血由来 DC（Ad5f35pp65-DC）で臍帯血ナイーブ T 細胞を IL-7，IL-12，IL-15 存在下で刺激し，その後，Ad5f35pp65-EBV-LCL を抗原提示細胞として用いることで，臍帯血中のナイーブ T 細胞から CMV，ADV，EBV 特異的 CTL を誘導，増殖させることが可能であることを示し[24]，今後の臨床応用が期待される．

5. 当院における移植後 CMV 感染に対する養子免疫療法

名古屋大学医学部附属病院血液内科では，現在，小児科と共同で「同種造血幹細胞移植後において生じる難治性 CMV 感染症に対する CMV 抗原特異的 CTL を用いた治療の安全性に関する臨床第 I 相試験」を実施している．

CMV 抗原特異的 CTL は，ドナー末梢血 50 ml から分離した末梢血単核球に CMV pp65 エピトープペプチドと IL-2 を加えて誘導し，14 日間培養後に CMV

pp65 エピトープペプチドを添加した抗原提示細胞と共培養して樹立する（図 2）．抗原提示細胞としては，抗原提示能に優れた DC が使用されることが多いが，DC を大量に調製するためにはドナーからアフェレシスにより十分量の単核球を採取する必要があり，ドナーへの負担が大きくなる．また，抗原提示能が高い成熟 DC に分化誘導するには，GM-CSF，IL-4，TNF-α など数種類のサイトカインを異なったタイミングで添加しなくてはならないため調製に手間と費用がかかる．そこで，OKT-3 と IL-2 で容易に増やすことができる活性化 T 細胞を抗原提示細胞として用いることを検討した．CTL 活性化・増幅には，T 細胞レセプターを介した抗原特異的なシグナルに加えて，CTL 細胞上の CD28 と抗原提示細胞上の CD80/CD86 との結合などによる共刺激シグナルが必要であるが，T 細胞を OKT-3 と IL-2 で活性化することで細胞表面上の

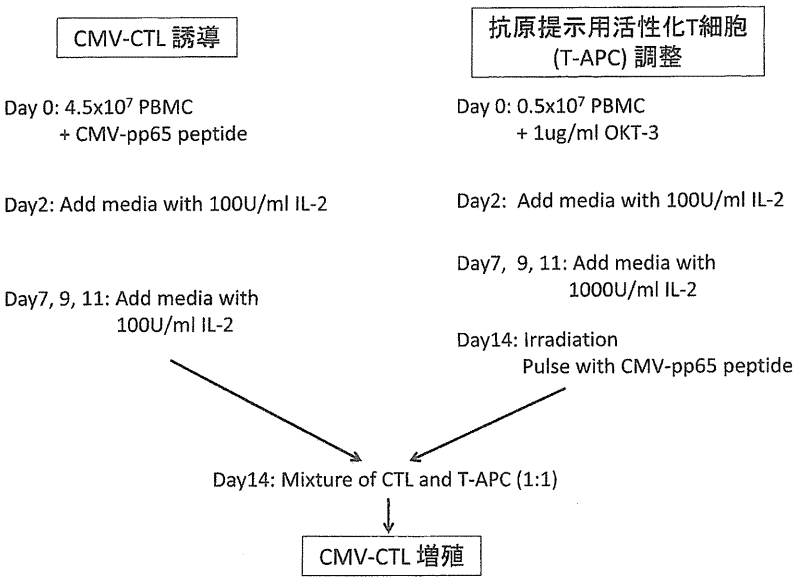

図 2　CMV 抗原特異的 CTL の樹立方法

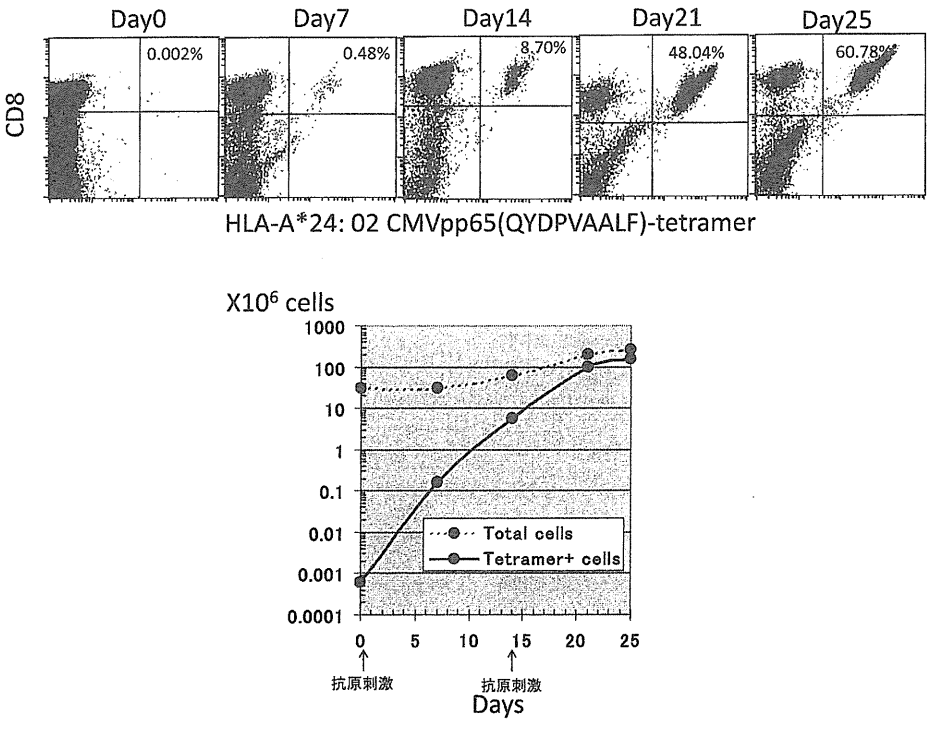

図 3　HLA-A*24：02 拘束性 CMV-pp65（QYDPVAALF）特異的 CTL の調製例

CD80/CD86の発現が高くなり，抗原提示細胞としての能力を有するようになる．実際に，活性化T細胞を抗原提示細胞としてCMV抗原特異的CTLの樹立を試みたところ，10万倍以上に増殖可能であることが示された（図3）．この方法により，末梢血50 mlというドナーへの負担が非常に少ない採血量で，効率よくCMV抗原特異的CTLの誘導・増殖が可能となり，現在，名古屋大学医学部附属病院内のGMP準拠細胞加工センター（ISO 9001，ISO 13485取得）にて調製した細胞を用いて臨床研究を行っている．

6. 今後の課題と展望

6.1 骨髄バンクドナーのリンパ球を用いた養子免疫療法

慢性骨髄性白血病・骨随異形成症候群・急性白血病・悪性リンパ腫・多発性骨髄腫の血液学的再発およびEBV-LPDに対する骨髄バンクドナーからのDLIは，ドナーの適格性が認められれば実施可能である．前述の当院で行っているCMV特異的CTLを用いた養子免疫療法のために骨髄バンクドナーからの採血を申請した際には，臨床研究に伴うDLI申請の前例がなく，骨髄バンクと採取施設と協議などを重ね，申請から採血まで約2カ月要した．その後，骨髄移植推進財団より，養子免疫療法をはじめとして臨床研究を伴うDLI用採血のために以下の申請条件が定められた．

① 施設内倫理委員会に提出し，承認を得られた申請書，研究計画書を提出すること
② 同様の症例があれば文献報告例を提出すること
③ 患者がDLIに同意していること
④ ドナーへの説明書が準備されていること
⑤ ドナーへの説明と同意の確認を採取施設が代行することを採取施設が了承すること
⑥ ドナーの同意があること
⑦ ドナーに対する対応の手続きを，具体的に提案すること
⑧ 採取施設内の倫理委員会でも承認を得ること

これらの条件が定められた後に，2例目の申請を行った際には，申請から採血まで1カ月半と約半月短縮されたものの，CMV特異的CTL培養中に病状が悪化し，輸注のための適格条件を満たさず輸注を実施することができなかった．養子免疫療法のためには抗原特異的CTLを樹立・増殖させるために時間を要することから，通常のDLI同様に申請から採血までの時間をさらに短縮させる必要がある．そのため，骨髄バンクドナーのリンパ球を用いた養子免疫療法が必要な患者に早期の治療が可能となるよう，申請された臨床研究について予め骨髄移植推進財団にて検討し，承認が得られた臨床研究のDLI用採血には，採取施設内での倫理委員会承認は不要とする等の条件変更を検討している．

6.2 細胞調製の改善

養子免疫療法を行うためには，抗原特異的T細胞の誘導と増殖のための時間が必要であり，その時間を短縮するための様々な研究が行われている．Schmitt Aらは，アフェレシスにて末梢血単核球を採取し，生理活性を維持した抗原特異的T細胞を単離できるstreptamer[25]を用いてCMV抗原特異的T細胞を分離精製した．CMV抗原特異的T細胞（2.2×10^5/kg）を培養せずに同種末梢血幹細胞移植後難治性CMV感染患者に輸注してCMVを排除できたことを報告した[26]．この方法を用いることで，抗原特異的CTLを誘導・増殖する必要がないため，CTLによる免疫療法が容易にできるようになる．

さらに，個々の患者に対して抗原特異的CTLを準備する必要性を回避するために，HLAの一部が一致した第三者から樹立したCTLを用いた養子免疫療法も行われている．Haque Tらは，臓器移植または造血幹細胞移植後EBV-LPDを合併した患者33人に対して，HLA-A，B，DR 2/6から5/6抗原一致第三者から樹立したEBV特異的CTL（2×10^6/kg）を週1回投与（1回3人，2回3人，3回1人，4回23人，6回1人，8回2人）した．5週間後と6カ月後にそれぞれ64%，52%に反応が見られ，GVHDの発症はないという非常に有望な結果が示された[27]．さらに，米国ではCMV，EBV，ADV特異的CTLバンクが作られており，臨床試験として既に治療に用いられている．そして，同種造血幹細胞移植後ウイルス感染症を合併した29人（EBV-LPD 6，CMV 13，ADV 9，CMV＋ADV 1）に対して，HLAが1〜3抗原一致したCTLが輸注され，輸注直後の有害事象はなく，GVHD発症率は低く（29人中4人），42日目までに84.6%に反応（CR＋PR）が認められたと報告されている[28]．現在，我が国においてもCTLバンク設立に向けて準備が開始されており，このCTLバンクが設立されれば，施設内でのCTL培養が不要となり，養子免疫療法が必要な患者にいつでもどこでも治療できるようになる．

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Original Article

Outpatient peripheral blood stem cell collection in Japan: Donor follow-up survey by the Japan Society for Hematopoietic Cell Transplantation

Minako Iida[1], Ritsuro Suzuki[2], Takashi Yoshida[3], Ken Ishiyama[3], Hiroyasu Kaya[3], Jun Taguchi[4], Atsushi Kikuta[5], Kaoru Kahata[6], Eiji Ohno[7], Koichi Miyamura[8], Yoshihisa Kodera[1]

[1]Aichi Medical University School of Medicine, Department of Promotion for Blood and Marrow Transplantation [2]Nagoya University Graduate School of Medicine, Department of HSCT Data Management/Biostatistics, [3]Toyama Prefectural Central Hospital, Department of Internal Medicine, [4]Nagasaki University, Department of Hematology [5]Fukushima Medical University, Department of Pediatric Oncology [6]Hokkaido University, Third Department of Internal Medicine [7]Oita Prefectural Hospital, Second Department of Internal Medicine [8]Japanese Red Cross Nagoya First Hospital, Department of Hematology; Donor Committee, Japan Society for Hematopoietic Cell Transplantation (JSHCT)

It has been the general consensus in Japan that allogeneic peripheral blood stem cell (PBSC) donors should be hospitalized from the beginning of G-CSF administration to the end of harvest for their safety. Consequently, the admission period (about 1 week) is a heavy burden compared to bone marrow donors (3 to 4 days). Since PBSC mobilization and harvest has been successfully implemented in outpatient settings in most other countries, we think it is necessary to establish a system to deal with PBSC donors safely without admission. In Japan, since it came into service, JSHCT obliges all institutes to register all related PBSC donors on the Donor Registry Center preliminarily and it collects all the information about the donors. According to this data, 72 donors in the 25 institutes experienced G-CSF injection and PBSC harvest in outpatient settings. We sent additional questionnaires to these institutes to know the details. As a result, it was made clear that there were no severe adverse health effects, but at the same time many institutes adopted cautious stances toward the outpatient harvest system. It is important to gather these opinions and use the transplant centers' past experiences to make outpatient PBSC harvest safer for donors. (Journal of Hematopoietic Cell Transplantation Vol. 1 No. 2; 52-58, 2012)

Introduction

According to the Japan Society for Hematopoietic Cell Transplantation (JSHCT) guidelines, donors for allogeneic peripheral blood stem cell transplantation (PBSCT) in Japan have been hospitalized for approximately one week from the start of granulocyte-colony stimulating factor (G-CSF) to the end of apheresis to ensure their safety. As a result, the donors' hospitalization is prolonged and it increases their burden. In contrast, outpatient administration of G-CSF and apheresis has been common in other countries.[1,2] Since starting unrelated PBSC in Japan in 2010, it has become essential to establish a safety system for PBSC harvest in outpatient clinics. Taking these trends into consideration, we conducted a survey in combination with the allogeneic PBSC Donor Follow-up System by JSHCT.

Materials and Methods

Outpatient PBSC harvest

A total number of 3,264 donors from 237 institutes were registered in the Donor Registry Center from April 1, 2000 to March 31, 2005. Among them, 2,882 donors' day 0 reports of past donations were collected by September 30, 2006. The

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Address correspondence to: Minako Iida, MD, PhD; Department of Promotion for Blood and Marrow Transplantation, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi, 480-1195, Japan; TEL: +81-561-62-3311 (Ext: 2375), FAX: +81-561-61-3180; Email miida@aichi-med-u.ac.jp.

duration of hospitalization was 0 in 72 donors (2.2% of all registered donors) from 25 institutes (11% of all registered institutes), which were subjected to further study (Fig. 1A: The institutes of outpatient harvest). Additional questionnaires were sent to these institutes to collect more information (Suppl. 1). The questions included 1) reasons for outpatient harvest, 2) dosage and administration of G-CSF, 3) adverse events (AEs) of G-CSF, 4) treatments against the AEs, 5) days and locations of the PBSC harvest and 6) opinions from donors and doctors about outpatient harvest.

We received 62 replies (response rate: 86%) from 23 institutes.

Control data

To compare the results, we sent controlled questionnaires (Suppl. 2) to institutes in which all the procedures were performed on an inpatient basis. In order to distribute roughly evenly to the outpatient institutes, we divided Japan into 8 regions and selected 4 to 5 institutes from each by order of the number of harvests. Out of 38, 31 institutions which experienced 1,443 PBSC harvests replied to the questionnaire (collection rate: 82%) (Fig. 1B: The institutes of inpatient harvest). The questions differed between the two groups. As mentioned above, the questionnaire to the institutes performing outpatient harvest included questions about each donor, while to those performing inpatient harvests, involved questions to obtain opinions about outpatient harvest. This questionnaire included the actual status of PBSC apheresis in the hospital, especially the administration of G-CSF such as duration, dosage and AEs. It also asked how to deal with harvesting if outpatient harvest becomes common in Japan.

Results

The results of the questionnaire for the outpatient cases are listed in Table 1. The median age of the 62 donors whose PBSCs were harvested in outpatient settings was 35 years old, and there were 27 male and 35 female donors. There were many reasons for outpatient harvest. The most common reason for avoiding hospitalization was the donors' preference, which was mainly due to working schedules and schoolwork. The next most common was inconvenience for the institutes. In some pediatric cases, the patient's parent became the PBSC donor. He/she stayed in the hospital the entire day to take care of his/her child; however, the donor in this case was recognized to be different from an inpatient. G-CSF was administered to outpatient donors by their personal physicians and none was administered to another neighboring clinic. Although the duration of G-CSF administration ranged from 4 to 6 days, and was the same between outpatient and inpatient cases, the number of G-CSF administrations in a day was 1 in almost all outpatient donors. On the other hand, half of the inpatient donors were administered G-CSF twice a day. Sixteen donors experienced 21 adverse events (AEs) of G-CSF, 12 of which were bone pain (lumbago, back pain), 4 were general fatigue, 3 were fever, 1 was sleeplessness and 1 was splenomegaly, but all were treated by their personal physicians, not at other neighboring medical facilities. All donors except one had their PBSC harvested in their doctors' outpatient clinic. It took one or two days for most donors (88%) to harvest but it was reported that only one donor needed five days to finish the harvest. Ten AEs associated with PBSC harvest were reported: 5 numbness of the limbs, 2 thrombocytopenia, 2 nausea and 1 citrate intoxication. None were serious enough to be hospitalized. More than half of the donors and doctors reported that outpatient procedures did not have any problems (54%, 66%, respectively). These doctors made positive remarks such as "PBSC harvest is possible in outpatient clinics," "PBSC harvest is sufficient in outpatient settings," or "In case of donors who had experienced a previous uncomplicated harvest, outpatient harvest was permitted." Others also expressed favorable opinions on outpatient harvest like "The first three days for G-CSF injection is available in outpatient clinic" and "Doctors should take account of the donors' request." Only 2% (one case) of both donor and doctor said "Should have admitted because of the donor's sensitive character."

From the results of the questionnaire for the inpatient institutes (Table 2), 20% of the institutes in which all the procedures were supposedly performed on an inpatient basis had experienced outpatient G-CSF administrations and some asked a neighboring medical agency to inject G-CSF. Some hospitalized donors continued to stay at home overnight during the period of G-CSF administration. Nevertheless, all of the PBSC harvest was performed in an inpatient setting and at their own institutes. Seven donors or their family members wanted their harvest series performed in an outpatient setting, but their doctors convinced them to stay in the hospital during the harvest. Fortunately, no donor declined to donate because of hospitalization and 90% of the

(A) Outpatient harvest

(B) Inpatient harvest

Figure 1. The distribution of the surveyed institutes

Table 1. Results of the questionnaire: Outpatient donors (N=62)

Items	Results
Age	10–66 (median 35)
Sex Male	27
Female	35
Reason for being an outpatient	
Donor's preference	49%
Institute's convenience	29%
Donor family's wishes	4%
Others	18%
Institutes administering G-CSF	
Their own hospitals	100%
Schedule for G-CSF administration (duration)	
4 days	13%
5 days	71%
6 days	16%
Schedule for G-CSF administration (frequency)	
Once a day	98%
Twice a day	2%
Adverse events during G-CSF administration (some overlapped)	
Bone pain	12
General Fatigue	4
Fever	3
Sleeplessness	1
Splenomegaly	1
Institutes harvesting PBSC	
Their own hospitals	97%
Other institutes	3%
Schedule for PBSC harvest	
1 day	54%
2 days	34%
3 days	10%
More	2%
Adverse events during PBSC harvest (some overlapped)	
Numbness	5
Thrombocytopenia	2
Nausea	2
Citrate intoxication	1
Donors' opinion about the outpatient PBSC harvest	
Outpatient was good	54%
Should have admitted	2%
No opinion	44%
Doctors' opinion about the outpatient PBSC harvest	
Outpatient was good	66%
Should have admitted	2%
No opinion	32%