above, we needed an RCT in Japan. We have therefore launched a prospective, open-label RCT comparing allogeneic BMT *versus* PBSCT for adult patients with leukaemia. The primary end point of this trial is leukaemia-free survival based on time-to-event analysis. We plan the sample size per one arm to be 160, in order to detect the difference of 1·6 to 1·7 in HR for leukaemia-free survival. If this study can be completed, the impact of stem cell source on survival will be defined more accurately than the previous studies.

In summary, we observed faster engraftment and increased incidence of chronic GvHD in PBSCT compared with BMT for Japanese patients. The incidence of GvHD was lower than the western populations, but there were no differences in relapse, TRM, PFS and OS between PBSCT and BMT. These results suggest that the choice of haematopoietic stem cell source should be considered based on the data for individual ethnic populations. More detailed analysis and future trials may reveal the differential applicability of stem cells from these different sources in each disease category and hence enable us to choose appropriately between BMT and PBSCT based on reliable evidence.

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Appendix A

This study was conducted at the following institutions under the auspices of the following investigators in Japan: M. Sakai (Tokyo Metropolitan Hospital, Tokyo), T. Hamaki (National Cancer Centre, Tokyo), T. Karasuno (Osaka Medical Centre for Cancer and Cardiovascular diseases, Osaka), M. Kasai (Japanese Red Cross Nagoya first Hospital, Aichi), K. Kishi (Tokai University School of Medicine, Kanagawa), S. Okamoto (Keio University School of Medicine, Tokyo), N. Maseki (Saitama Cancer Centre Hospital, Saitama), S. Morishima (Meitetsu Hospital, Aichi), S. Yamasaki (Municipal Kitakyushu Medical Centre, Fukuoka), M. Kasai (Sapporo Hokuyu Hospital, Hokkaido), T. Kamimura (Harasanshin Hospital, Fukuoka), K. Shinagawa (Okayama University Medical School, Okayama), T. Yamane (Osaka City University, Osaka), S. Miyawaki (Saiseikai Maebashi Hospital, Gunma), Y. Miyazaki (Kansai Medical University, Osaka), T. Yamashita (National Medical Defence College, Saitama), N. Uike (National Kyushu Cancer Centre, Fukuoka), A. Maruta (Kanagawa Cancer Centre, Kanagawa), M. Misawa (Hyogo College of Medicine, Hyogo), K. Mitani (Dokkyo University School of Medicine, Tochigi), K. Kamezaki (Kyushu University Graduate School of Medical Sciences, Fukuoka), M. Masuda (Ryukyu University, Okinawa), J. Ishikawa (Osaka University, Osaka), A. Wake (Kokura Memorial Hospital, Fukuoka), A. Kohno (JA Aichi Showa Hospital, Aichi), M. Hara (Ehime Prefectural Central Hospital, Ehime), M. Kuroiwa (Hamanomachi Hospital, Fukuoka), E. Kusumi (Toranomon Hospital, Tokyo), K. Nishiwaki (Jikei University School of Medicine, Tokyo), M. Imamura (Hokkaido University Graduate School of Medicine, Hokkaido), Y. Takemoto (Jiaikai Imamura Hospital, Kagoshima), K. Fujimaki (Yokohama City University School of Medicine, Kanagawa), T. Tamaki (Rinku General Medical Centre, Osaka), Y. Takamatsu (Fukuoka University School of Medicine, Fukuoka), T. Murayama (Hyogo Medical Centre for Adults, Hyogo), M. Hirokawa (Akita University School of Medicine, Akita), T. Kobayashi (Tsuchiura Kyodo General Hospital, Ibaraki), K. Ozawa (Jichi Medical School, Tochigi), T. Ashida (Kinki University School of Medicine, Osaka), S. Imamura (Fukui Medical University, Fukui), Y. Kimura (Tokyo Medical University, Tokyo), K. Hodohara (Shiga Medical University, Shiga), H. Ago (Shimane Prefectural Central Hospital, Shimane), C. Shimazaki (Kyoto Prefectural University of Medicine, Kyoto), H. Teshima (Osaka City General Hospital, Osaka), A. Kubota (National Kyushu Medical Centre, Fukuoka), J. Tsukada (University of Occupational and Enviromental Health, School of Medicine, Fukuoka), C. Hashimoto (Yokohama City University Medical Centre), A. Yokota (Chiba Municipal Hospital, Chiba), H. Tsurumi (Gifu University, Gifu), M. Yamaguchi (Ishikawa Prefectural Central Hospital, Ishikawa), T. Endo (Hokkaido University Graduate School of Medical Sciences, Hokkaido), T. Chujo (Kanazawa University Graduate School of Medical Sciences, Ishikawa), M. Masuda (Tokyo Women's Medical College, Tokyo), S. Murakami (Social Insurance Kyoto Hospital, Kyoto), N. Emi (Nagoya University School of Medicine, Aichi), T. Fujisaki (Matsuyama Red Cross Hospital, Ehime), E. Matsuishi (Saga Prefectural Hospital Koseikan, Saga), F. Sano (St Mariannna University School of Medicine, Yokohama City Seibu Hospital, Kanagawa), Y. Torimoto (Asahikawa Medical College, Hokkaido), K. Yakushiji (Kurume University School of Medicine, Fukuoka), N. Uoshima (Matsushita Memorial Hospital, Osaka), H. Takamatsu (Kurobe City Hospital, Toyama), Y. Kobayashi (Kyoto Prefectural University of Medicine, Kyoto), K. Sunami (National Okayama Medical Centre, Okayama), K. Naito (Hamamatsu University School of Medicine, Shizuoka), H. Taguchi (Kochi Medical School, Kochi), S. Tsuchiya (Institute of Development, Aging and Cancer, Tohoku University, Miyagi), Y. Itoh (National Beppu Hospital, Oita), S. Doi (Kyoto Katsura Hospital, Kyoto), H. Kobayashi (Kyoto Prefectural Hospital, Kyoto), K. Tanimoto (Shin-koga Hospital, Fukuoka), K. Hayashi (Hoshigaoka Koseinenkin Hospital, Osaka), K. Kawachi (Takamatsu Red Cross Hospital, Kagawa), A. Urabe (NTT Kanto Medical Centre, Tokyo), R. Okamoto (Tokyo Metropolitan Komagome Hospital, Tokyo), T. Nishiura (National Kure Medical Centre, Hiroshima), H. Kimura (Kita-Fukushima Medical Centre,

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Graft-versus-tumor effects

Expansion and activation of minor histocompatibility antigen HY-specific T cells associated with graft-versus-leukemia response

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Summary:

The immune system of females is capable of recognizing and reacting against the male-specific minor histocompatibility antigen (mHA), HY. Thus, cytotoxic T-lymphocytes (CTLs) recognizing this antigen may be useful in eradicating leukemic cells of a male patient if they can be generated *in vivo* or *in vitro* from a human leukocyte antigen (HLA)-identical female donor. The HLA-A*0201-restricted HY antigen, FIDSYICQV, is a male-specific mHA. Using HLA-A2/HY peptide tetrameric complexes, we reveal a close association between the emergence of HY peptide-specific CD8$^+$ T cells in peripheral blood and molecular remission of relapsed BCR/ABL$^+$ chronic myelogenous leukemia in lymphoid blast crisis in a patient who underwent female-to-male transplantation. Assessment of intracellular cytokine levels identified T cells that produce interferon-γ in response to the HY peptide during the presence of HY tetramer-positive T cells. These results indicate that transplant with allogeneic HY-specific CTLs has therapeutic potential for relapsed leukemia, and that expansion of such T cells may be involved in the development of a graft-versus-leukemia response against lymphoblastic leukemia cells.

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Minor histocompatibility antigens (mHAs) are immunogenic peptides derived from polymorphic cellular proteins.[1] These peptides bind to human leukocyte antigen (HLA) and are recognized by allogeneic T cells. Following stem cell transplantation (SCT) with HLA-matched donor cells, graft-versus-host disease (GVHD) can arise through disparities in mHAs between the donor and the recipient. Using tetrameric HLA class I–mHA complexes, Mutis *et al*[2] demonstrated that a limited number of mHA-specific T cells expand in peripheral blood (PB) in parallel with increasing GVHD severity. It is believed that donor-derived T cells specific for mHAs play a significant role in the development of graft-versus-leukemia (GVL) effect as well as GVHD after allogeneic SCT. However, there is only limited evidence for the killing of leukemic cells by mHA-specific T cells.

Kern *et al*[3] reported that human cytomegalovirus peptide-specific CD8$^+$ T cells can be detected by flow cytometry in samples whose HLA is known. Kuzushima *et al*[4] showed that the frequency Epstein–Barr virus-specific CD8$^+$ T-cell frequencies are detectable irrespective of HLA typing when PB lymphocytes are incubated with an autologous lymphoblastic cell line (LCL). Both methods are based on multiparameter flow cytometric assays that detect rapid intracellular accumulation of interferon (IFN)γ after *in vitro* antigen stimulation in the presence of an intracellular transport blocker. By assessing the frequency of IFNγ-producing cells, the presence of functional T cells reactive with target antigens and target cells can be detected.

One male-specific mHA is the HLA-A*0201-restricted peptide, FIDSYICQV, from the male-specific antigen HY.[5] Cytotoxic T-lymphocytes (CTLs) recognizing this peptide may be useful in eradicating leukemic cells of a male patient with HLA-A2 if they can be generated *in vivo* or *in vitro* from an HLA-identical female donor. By HLA-A2/HY peptide tetramer staining and intracellular IFNγ assessment, we provide the first evidence that the emergence and activation of transferred HY-specific female CTLs contributes to molecular remission of chronic myelogenous leukemia (CML) in a male patient in lymphoid crisis.

To date, a small number of studies describe a clear dominance in T-cell receptor (TCR) variable (V)-gene segment usage in the recognition of certain HLA class I/peptide complexes in humans after SCT.[6,7] We examined PB CD8$^+$ T cells producing intracellular IFNγ of a male patient with CML in lymphoid crisis for the emergence of clonal T-cell proliferation by analyzing the T-cell repertoire

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as well as *in vitro*-generated HY-specific CTLs. TCR BV spectratyping showed a similar peak with same size in some BV family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific $CD8^+$ T cells. These findings suggest the circulation of a functional T-cell clone capable of eradicating lymphoblastic leukemia cells.

Materials and methods

Case report

A patient was a 15-year-old Japanese male (HLA-A*0201-positive) with chronic-phase CML. He had experienced no GVHD after receiving busulfan- and cyclophosphamide-conditioned bone marrow from his HLA-identical sister. At 40 months after bone marrow transfer, lymphoid blast crisis suddenly developed. After treatment with cyclophosphamide, adriamycin, vincristin and prednisolone, the hematologic relapse persisted. At 2 months after chemotherapy, the patient underwent PB stem cell transplantation (PBSCT) from the same donor following conditioning with cytarabine, cyclophosphamide and total body irradiation. An unmanipulated PB stem cell graft including a total of 5.5×10^6 CD34-positive cells/kg was infused. Cyclosporine, which is used to prevent GVHD, was withdrawn on day 21 to induce GVL effect. At 3 weeks after PBSCT, conversion to full donor chimerism was obtained. The patient developed grade II acute GVHD on day 29 after PBSCT, and the disease progressed to extensive chronic GVHD on day 80. At 20 weeks after PBSCT, no BCR/ABL transcripts were detected, and he remained in molecular remission until a relapse at 13 months after PBSCT. The patient died of veno-occlusive disease shortly after the third transplantation with cytarabine- and idarubicin-conditioned bone marrow from the same donor.

Peptide

An HLA-A201-restricted HY peptide was synthesized using a semiautomatic multiple peptide synthesizer based on the reported sequence.[5] The purity of the peptide was checked by reverse-phase high-pressure liquid chromatography.

Cell preparation

Cells were obtained from the post transplant patient and his stem cell donor. Peripheral blood mononuclear cells (PBMC) were prepared using density-gradient centrifugation. For the establishment of Epstein–Barr virus (EBV)-transformed LCLs, PBMC from the donor were depleted of T cells using the rosette formation method. A total of $2–3 \times 10^6$ non-T cells were incubated in RPMI 1640 (GIBCO, Grand Island, NY, USA) medium containing 10% fetal calf serum (FCS; GIBCO) containing 10% culture medium from an EBV-producing cell line, B95-8, at 37°C for 2 h. The EBV-infected cells were cultured for 3 weeks until transformed LCL cells grew. LCL cells were maintained by changing the medium every 4 to 5 days.

Tetrameric HLA-A2/mHA HY peptide complexes

The generation of HLA-A2/mHA HY tetramers and tetramer labeling of HY-specific T cells was performed as described previously.[8]

Generation of dendritic cells (DCs) from monocytes

PB monocyte-derived DCs were generated as described previously.[9] Briefly, monocytes were isolated by adherence of donor PBMCs to plastic for 2 h. Monocytes were cultured in RPMI 1640 medium supplemented with 10% pooled AB serum, 10 ng/ml recombinant human interleukin-4 (IL-4) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (a gift from Kirin Brewery, Tokyo, Japan). On day 5, 100 U/ml recombinant human tumor necrosis factor (TNF)α was added. On day 8 or 9, the cells were harvested for use as monocyte-derived DCs for antigen presentation. Cultured cells expressed DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class I and class II.

Induction of HY peptide-specific CTLs

DCs were pulsed with an HY peptide for 90 min at 37°C in serum-free RPMI 1640. After washing, 1.0×10^6 peptide-(40 nM) pulsed DCs and 1.0×10^7 donor-derived (autologous) PBMC were cultured together in 24-well culture plates. The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, minimal essential amino acids, sodium pyruvate and ampicillin (all from GIBCO) plus 10% autologous plasma. The cells were kept at 37°C in a humidified, 5% CO_2–air mixture. At days 7, 14 and 21, responder cells were restimulated with peptide-pulsed autologous DCs. From day 21, cultured T cells were suspended in 100 U/ml IL-2- (a gift from Shionogi, Osaka, Japan) containing culture medium and were restimulated weekly with peptide-pulsed autologous monocytes or DCs. T cells were harvested at day 35 and used for the cytotoxicity assay and RNA extraction for T-cell repertoire analysis.

Cytotoxicity (^{51}Cr release) assays

Donor-derived LCL cells and DCs as well as fibroblasts and leukemic cells of the patient were used as target cells in the standard 4-h ^{51}Cr release assay.[10] Specific lysis was calculated using the following formula: 100 × (cpm experimental release–cpm spontaneous release)/(cpm maximal release–cpm spontaneous release).

Preparation of target cells

Patient fibroblasts were isolated from a biopsy specimen and cultured in RPMI 1640 plus 10% FCS for 4 weeks. Single-cell suspensions were prepared by trypsinization. Donor-derived LCL cells and DCs and the patient's fibroblasts were suspended in 100 μl of ^{51}Cr solution containing an HY peptide at a concentration of 4 nM. In some experiments, concentrations of the peptide were changed as noted. Bone marrow mononuclear cells containing 98% BCR/ABL^+ cells were obtained from the patient

just before the second transplantation and were cryopreserved for use as leukemic cells. Thawed leukemic cells were cultured in RPMI 1640 plus 10% pooled AB serum for 24 h before use as a target in the CTL assay.

Blocking of cytotoxicity by monoclonal antibodies (MoAbs)

Polyclonal antibodies (control) or purified MoAbs were added to cultures of HY peptide- (4 nM) pulsed DCs in 96-well plates at a concentration of 10 μg/ml, and CTLs were immediately added to each well. MoAbs were HU-4 (anti-HLA-DR; kindly provided by Dr Akemi Wakisaka, Hokkaido University, Japan) and W6/32 (anti-HLA class I; American Type Culture Collection, Rockville, MD, USA).

Identification and isolation of IFNγ-producing CD8$^+$ T cells by flow cytometry

To detect circulating CD8$^+$ T-lymphocytes that recognize HY peptide, intracellular IFNγ was assessed by flow cytometry as described previously with slight modifications.[3,4] Briefly, donor-derived LCL cells were incubated for an hour with or without HY peptide. PBMCs were taken from the patient 12 weeks after the second transplantation. The CD8$^+$ T-lymphocytes were isolated from PBMCs using magnetic beads coated with an anti-CD8 monoclonal antibody (mAb) according to the manufacturer's instructions (Dynal AS, Oslo, Norway). A total of 10^6 CD8$^+$ cells were mixed with 10^6 autologous LCL cells in a culture tube in RPMI 1640 medium and cultured in a humidified 5% CO_2 incubator at 37°C for 1 h. Brefeldin A (Sigma, St Louis, MO, USA) was added at a final concentration of 10 μg/ml and the cells were cultured for an additional 5 h. After incubation, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with phosphate-buffered saline, cells were permeabilized with IC Perm (Biosourse International, Camarillo, CA, USA) and stained with PE-labeled anti-CD8 (Coulter, Miami, FL, USA) and FITC-labeled anti-human IFNγ (Biosourse International) MoAbs. Stained cells were analyzed and sorted on a FACScan (Becton Dickinson, San Jose, CA, USA).

RNA extraction and cDNA preparation

Total RNA was extracted from PBMCs or CTLs using a technique described elsewhere,[11] and was reverse transcribed into cDNA in a reaction primed with oligo(dT) using SuperScript II reverse transcriptase as recommended by the manufacturer (BRL, Bethesda, MD, USA).

Spectratyping of complementarity-determining region 3 (CDR3)

Conditions for the generation of the CDR3 size spectratyping have been reported previously.[6] Briefly, cDNA was polymerase chain reaction (PCR) amplified through 35 cycles (95°C for 1 min, 55°C for 1 min and 72°C for 1 min) with a fluorescent BC primer and primers specific to 24 different BV subfamilies. Analyses of the pseudogenes BV10 and BV19 were excluded from this study.[12] A measure of 1 μl of each amplified products was mixed with 2.0 l 100% formamide, heated at 90°C for 3 min and electrophoresed on a 6.75% denaturing polyacrylamide gel. The distribution of CDR3 size within the amplified product of each BV subfamily was analyzed with an automatic sequencer (Applied Biosystems Division, Foster City, CA, USA) equipped with a computer program allowing the determination of the fluorescence intensity of each band. The results are given as peaks corresponding to the intensity of the fluorescence. Expansion of a limited number of T cells was judged when a prominent peak appeared in the CDR3 pattern with or without a reduced number of peaks (five peaks). Given the BV-NDN-BJ sequence of the identical CDR3 size between in HY-specific CD8$^+$ cells in PB and the CTLs, a more specific primer covering CDR3 and different BJ subfamilies[13] was designed specifically to amplify cDNA of the BV22$^+$ T-cell clone in both CTLs.

Direct sequencing of PCR products

BV22-BJ PCR products were purified and sequenced as described previously.[14]

Results

Cytolytic activity of in vitro*-generated HY-specific CTLs*

Cultured T female donor cells stimulated with autologous HY peptide-pulsed DCs pulsed were able to lyse HY peptide-pulsed autologous DCs and patient fibroblasts, but could not lyse untreated DCs (Figure 1a). Of note, the CTLs lysed nonpeptide pulsed leukemic cells of the patient more efficiently than his fibroblasts. CTLs showed cytotoxicity to HY peptide-loaded autologous DCs, in a peptide concentration-dependent manner (Figure 1b). Cytotoxicity mediated by the CTLs against HY peptide-pulsed autologous DCs was blocked to a similar degree by the addition of MoAb either against anti-class I or anti-CD3, but was not affected by the addition of anti-HLA-DR (Figure 1c). In addition, the CTLs could effectively lyse LCL cells of unrelated males who shared HLA-A*0201. In contrast, apparent cytotoxic activity was neither observed against allogeneic LCL cells that did not possess HLA-A2 nor against LCL cells of unrelated females (Figure 1d).

Tetramer staining of HY-specific CTLs

The patient in lymphoid crisis with CML relapse developed acute GVHD shortly after the second transplant, which progressed to extensive chronic GVHD. He achieved a molecular remission at 4 months after PBSCT despite the fact that leukemic cells accounted for more than 98% of his bone marrow cells when the conditioning regimen was started. This unusual clinical course appeared to suggest the induction of GVL reactions associated with GVHD.

Other than the HY antigens, there was no disparity in the minor histocompatibility alleles, including HA-1, HA-2,

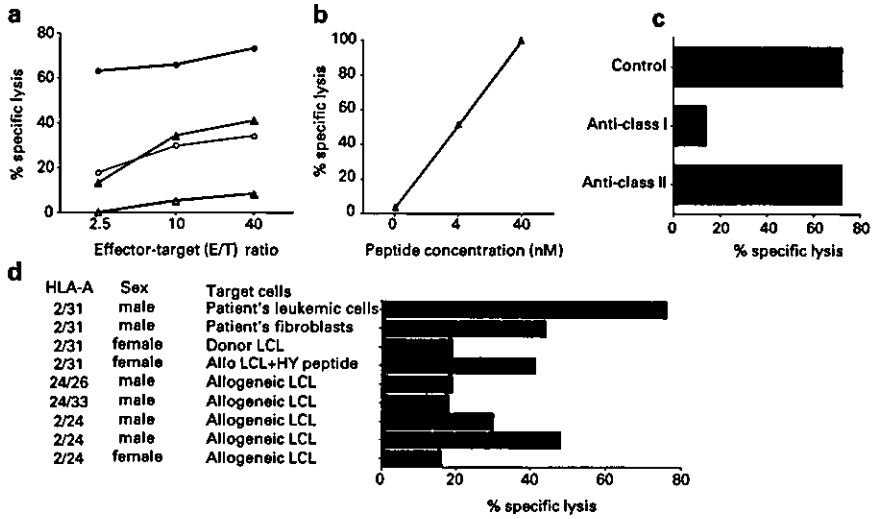

Figure 1 Cytotoxic activity of *in vitro*-generated HY-specific CTLs. (**a**) Cytotoxicity of cultured T cells stimulated by autologous DCs pulsed with HY peptide. The amount of peptide utilized in (a, b and c) was 4 nM. Target cells: autologous DCs without HY peptide (Δ); autologous DCs pulsed with HY peptide (▲); fibroblasts of the patient (○); leukemic cells of the patient (●). In (b, c, and d), cytotoxicity was determined at an E/T ratio of 10:1. (**b**) Effect of concentration of HY peptide on cytotoxicity of CTLs. Cytotoxicity of CTLs to autologous DCs loaded with various concentration of HY peptide was tested. (**c**) Antibody blockade of cytotoxicity against autologous DCs pulsed with HY peptide. Polyclonal antibodies (control), anti-class I MoAb or an anti-class II MoAb were added to cultures for testing blockade of cytotoxicity. (**d**) Cytotoxicity of HY peptide for 6 h. HY-specific CTLs against LCL cells of unrelated males who share HLA-A2 (A*0201).

CD31, CD49b and CD62L,[15,16] between the donor and the recipient. Tetramer staining demonstrated the expansion of HY peptide-specific T cells from undetectable prior to PBSCT to 15.9% of the circulating CD8$^+$ T cells 12 weeks after PBSCT (Figure 2a). Thereafter, frequencies of HY tetramer-positive T cells declined and disappeared from the PB, coinciding with molecular relapse. The *in vitro*-generated HY-specific CTLs were 90% HY tetramer-positive CD8$^+$ T cells (Figure 2b).

Detection of HY-reactive CD8$^+$ T cells by intracellular cytokine assessment

To demonstrate the HY peptide-driven expansion of CD8$^+$ T cells, we assessed intracellular accumulation of IFNγ in PB CD8$^+$ T cells by flow cytometry. At 12 weeks after PBSCT, 6.8% of PB CD8$^+$ T cells produced intracellular IFNγ in response to HY peptide-pulsed autologous LCL cells (Figure 3a), while IFNγ production was negligible in CD8$^+$ T cells in response to autologous LCL cells without the HY peptide (Figure 3b). These findings indicate that *in vivo* expansion and activation of HY peptide-reactive T cells occurred after the second transplantation. At that time, the proportion of circulating CD8$^+$ cells positive for HY-tetramer staining was 15.9% as show in Figure 2a. Although inducibility of IFNγ in HY-tetramer-positive CD8$^+$ T cells was not examined, there could be overlapping between CD8$^+$ T cells producing intracellular IFNγ in response to HY peptide and CD8$^+$ T cells stained with HY tetramer, because HY-tetramer staining must detect functional T cells reactive with HY peptide.

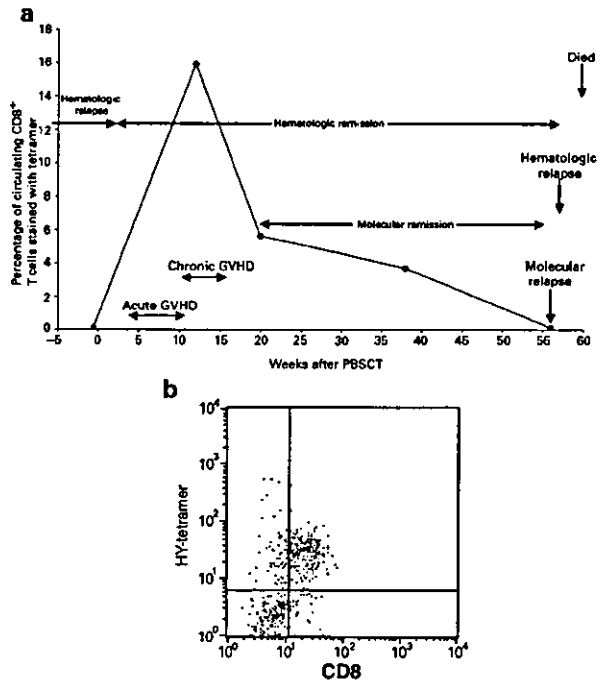

Figure 2 Monitoring and generation of HY-specific CD8$^+$ T cells. (**a**) Correlation between frequency of circulating HY-tetramer-positive CD8$^+$ T cells and clinical events. (**b**) HY-tetramer staining of the *in vitro*-generated HY-specific T cells, showing FITC-conjugated anti-CD8 antibody (*x*-axis) and PE-conjugated HY-tetramers (*y*-axis). Appropriate gates were set on vital lymphocytes according to their typical forward- and side-scattering characteristics.

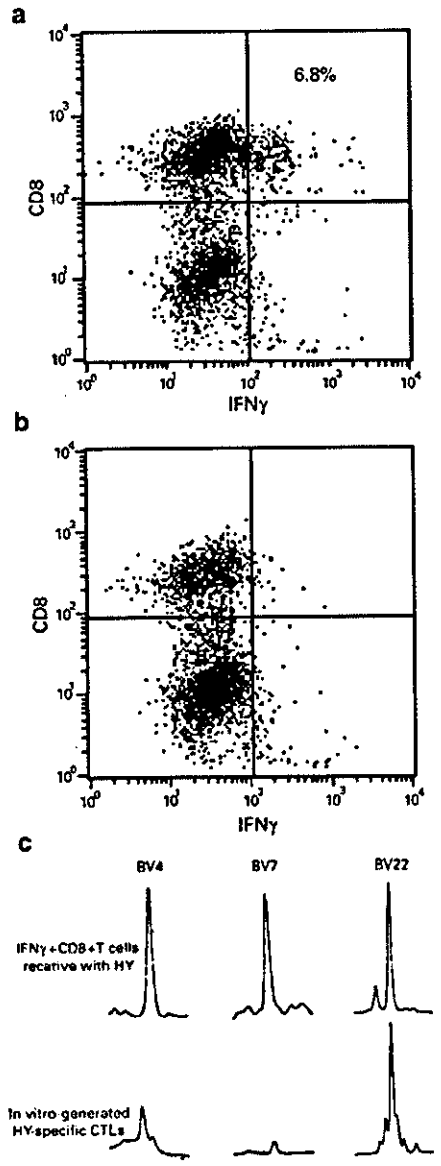

Figure 3 HY-specific IFNγ-producing CD8$^+$ T cells in PB. (**a**) PB CD8$^+$ T cells were incubated with autologous LCL cells pulsed with after fixation and permeabilization, the cells were stained for CD8 and IFNγ. The frequency of CD8$^+$ T cells producing IFNγ in response to HY peptide is shown as a percentage of total CD8$^+$ cells. (**b**) IFNγ production was negligible in CD8$^+$ T cells stimulated by autologous LCL cells not pulsed with the HY peptide. (**c**) CD8$^+$ IFNγ$^+$ T cells detected in Figure 3a were selected by fluorescence-activated cell sorting, and spectratyping was performed. CDR3 sizes of TCR BV subfamilies from CD8$^+$ IFNγ$^+$ T cells are shown together with the data from *in vitro*-generated HY-specific CTLs.

CDR3 size distribution of TCR BV cDNA of HY-specific T cells

Spectratyping of the TCR BV region was performed on *in vitro*-generated HY-specific CTLs and PB CD8$^+$ T cells that were stained with intracellular IFNγ MoAb in response to HY peptide stimulation and sorted as shown in Figure 3a. PB HY-reactive CD8$^+$ cells showed prominent skewing within BV4, BV7, BV22 and BV24, and the *in vitro*-generated HY-specific CTLs showed skewing within BV4, BV7, BV12, BV16 and BV22. The two T-cell populations shared the usage of BV4, BV7 and BV22 (Figure 3c), but only the BV22$^+$ T cells from *in vitro*-generated HY-specific CTLs and HY-responsive PB CD8$^+$ T cells had a similar peak with the same CDR3 size distribution.

Deduced amino-acid sequence of CDR3 of BV22 cDNA

To determine if HY-specific CTLs circulate in the patient, we subcloned the amplified cDNA of the *in vitro*-generated BV22$^+$ CTLs and BV22$^+$ T cells that produced intracellular IFNγ in response to HY peptide stimulation as shown in Figure 3a and determined the CDR3 sequence (Table 1). One of three N-D-N sequences of HY-specific CD8$^+$ cells from PB was identical to one of those of the CTLs. These findings indicate that the same cells isolated from the donor are expanding *in vivo*, and suggest that HY-specific CD8$^+$ T cells in PB of the patient have cytotoxic activity against leukemic cells.

Discussion

In mHAs HA-1- and HA-2-matched stem cell transfer between a female donor and a male recipient, we have observed the emergence of HY peptide-specific CTLs that result in the durable remission of relapsed leukemia. The present study utilized tetramer staining to show that HLA-A*0201-restricted HY peptide-specific CD8$^+$ T cells were present in the PB after the development of GVHD and led to the eradication of BCR/ABL transcript-positive leukemic cells. Further, these cells disappeared upon molecular relapse of disease. Assaying for the frequency IFNγ-producing cells by intracellular cytokine staining, we demonstrated the emergence of functional T cells in PB that were reactive with the HY peptide during the presence of HY tetramer$^+$ T cells. The data imply that HY-specific CTLs may have therapeutic potential as adoptive immunotherapy for relapsed leukemia after allogeneic SCT.

In this patient, leukemic relapse had occurred as full-blown disease of CML in lymphoid blast crisis. Although a beneficial effect of donor immunity was expected in the control of leukemic relapse, it would take months to start to work. Thus, we had chosen multiple transplants instead of donor leukocyte infusion (DLI) to reduce leukemic cells sufficiently.

Recently in mHAs HA-1 and/or HA-2 incompatible donor–recipient pairs an association between the emergence of HA-1 or HA-2 tetramer-positive CTLs and the complete disappearance of BCR/ABL$^+$ cells or of myeloma cells was reported.[17] Of the three reported patients, one who underwent female-to-male transplantation experienced an increase in HLA-B7-restricted HY-specific T cells as well as an increase in HA-2-specific T cells, but not that of

Table 1 Junctional amino-acid sequence of TCR BV22 of HY-specific T cells

V	*N-D-N*	*J*	*BJ family*
(a) BV22⁺ T cells from in vitro-generated HY-specific CTLs			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	REGGRS	GYTFCSGTKLTV	1.2
CASS	KQKGNPPPI	SPLHFGNGTRLTVT	1.6
(b) Functional BV22⁺ T cells from HY-reactive CTLs circulating in PB			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	RQSQGS	GYTFCSGTKLTV	1.2
CASS	RQGRGVSEF	SPLHFGNGTRLTVT	1.6

HLA-A2-restricted HY-specific T cells. However, the cell population(s) contributing to the GVL effect were unable to be identified. It is worth noting that this reported case with lymphoid blast crisis of BCR/ABL transcript-positive CML obtained molecular remission after the development of GVHD and the emergence of HY-specific CTLs as seen in the present case.

The *in vitro*-generated HY peptide-specific CTLs efficiently lysed leukemic cells of the patient, and were also to a lesser extent cytotoxic to the nonhematopoietic cells such as fibroblasts of the patient. Gratwohl *et al*[18] reported that male recipients with CML of female blood or marrow stem cell grafts are at a high risk of GVHD, but benefit from reduced incidence of disease recurrence. These findings provide evidence that HY-specific CTLs may be commonly induced in male patients given a stem cell graft from a female donor, leading to the development of GVL reactions and GVHD. This implies that the availability of a female blood or marrow graft may be beneficial to a leukemic male recipient at high risk of relapse.

In contrast to the ubiquitous expression of HY, HA-1 and HA-2 are exclusively expressed on hematopoietic cells.[1] *In vitro*-generated HA-1- and HA-2-specific CTLs specifically lyse leukemic cells, but not nonhematopoietic cells in a ^{51}Cr release assay.[1,19] Thus, upon HA-1- or HA-2-mismatched SCT and adoptive immunotherapy such as DLI, a low risk of GVHD would be expected. However, HA-1 disparity between a patient and a donor has been associated with the development of GVHD without reducing a rate of relapse.[15,20] Marijt *et al*[17] demonstrated the emergence of HA-1- and HA-2-specific CD8⁺ T cells in PB of three patients after DLI preceding complete remission of relapsed leukemia. Relapse was associated with the development of GVHD in all three patients. A recent report showed that GVHD does not require alloantigen expression on host epithelium, and its development is primarily mediated by inflammatory cytokines such as TNFα and IL-1.[21] This may account for discrepancies between *in vitro* behavior of HA-1- and HA-2-specific CTLs and clinical observations. Based on these findings, we believe that in mHA-oriented allogeneic immunotherapy the ability of mHAs to induce powerful immune reactions is more important than restriction of mHAs to hematopoietic tissue, and so far it appears that GVHD is an inevitable consequence. In the future, selective blockade of cytokines mediating GVHD[21] may be a strategy to preserve GVL, while reducing toxicity of GVHD after mHA-oriented immunotherapy.

TCR BV spectratyping showed a similar peak with same size in a BV22⁺ family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific CD8⁺ T cells, and one shared N-D-N sequence. These findings suggest the expansion of a functional T-cell clone that participates in eradicating lymphoblastic leukemia cells positive for BCR/ABL transcripts, although we were not able to provide direct evidence demonstrating antileukemic activity of HY-specific CD8⁺ T cells taken from PB of the patient. It would have been beneficial to sort the HY-tetramer-positive cells detectable in PB of the patient, to expand these cells using HY peptide-pulsed LCL cells, and to test their cytotoxicity against the leukemic targets. Restricted TCR BV usage for HA-1-specific CTLs has also been described.[7] Spectratyping could be beneficial in monitoring HY-specific CTLs *in vivo*, because spectratype analysis is more sensitive than tetramer analyses, and can be performed using as little as 500 cells.[6]

Compared with tetramer staining, a flow cytometric assay assessing intracellular IFNγ levels can be used to screen a large number of allogeneic peptides with a relatively little effort. In allogeneic SCT, this approach should be useful for initial screening of candidates for mHAs derived from polymorphic cellular proteins. Moreover, intracellular IFNγ assessment between CD8⁺ T cells during the GVL effect or GVHD as a responder and hematopoietic cells or nonhematopoietic cells of a host as a stimulator may enable the detection of undefined mHAs-specific CTLs. As the IFNγ capture assay enables isolation of live T cells stained for surface-associated IFNγ,[22] further studies with regard to the function of responding effector T cells could elucidate their putative target antigens.

Another advantage of the flow cytometric cytokine production assay is that it is possible to assess the production of multiple cytokines on an antigen-specific, single-cell basis. It has already been demonstrated by Nazaruk *et al*[23] that a subset of EBV-specific CD8⁺ T-cell lines produce IL-4 or IL-13 in addition to IFNγ upon stimulation with phorbol myristate acetate and ionomycin. Such a technique could be utilized in determining the cytokine production capabilities of mHA-specific CTLs in PBSCT.

In conclusion, the present data provide evidence that the emergence and activation of HY-specific CD8⁺ T cells may participate in eradicating lymphoblastic leukemic cells. This implies that *in vitro*-generated HY-specific CTLs may have therapeutic potential for relapsed leukemia after allogeneic SCT.

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Unrelated donor transplants

Allogeneic peripheral blood stem cell transplantation from two- or three-loci-mismatched related donors in adult Japanese patients with high-risk hematologic malignancies

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Summary:

With the increasing frequency of haploidentical transplantation, it is becoming more important to establish the degree of HLA mismatch that can be accepted. We retrospectively analyzed clinical data of 50 adult Japanese patients with high-risk hematologic malignancies who underwent allogeneic peripheral blood stem cell transplantation (PBSCT) from two- or three-loci-mismatched related donors with HLA class I and II gene disparities in the graft-versus-host direction. They were treated at 20 transplant centers between 1996 and 2002. In all, 18 patients received unmanipulated PBSC, while 32 received purified CD34+ blood cells. Conventional ($n=31$) or reduced-intensity ($n=19$) conditioning regimens were used. Of the 39 patients (78%) who survived for $\geqslant 28$ days after transplant, 37 (95%) achieved neutrophil engraftment, while graft failure and rejection occurred in two of 39 (5%) and three of 37 (8%) patients, respectively. Stepwise Cox regression analysis revealed a significantly lower incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells (hazard ratio 0.32; 95% CI 0.12–0.84; $P=0.022$). By 1 year post transplant, 28 patients (56%) had died of transplant-related problems, including infectious complications (30%). Although the number of patients is small, our data suggest that transplant-related problems, particularly infectious complications, are major obstacles to the success of this therapy.

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Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapeutic approach for a number of life-threatening hematologic malignancies.[1] Unfortunately, only 30–40% of patients have a matched related donor (MRD) or a one-locus-mismatched related donor (MMRD), mismatched for either HLA-A, -B or -DR antigens, availabe.[2] Therefore, most transplant centers perform allogeneic HSCT from a matched unrelated donor (MUD) as a second option for those who do not have an MRD. Even though a large number of volunteers are willing to donate bone marrow, there are candidate patients for whom an MUD is not available. Moreover, the aggressive nature of their diseases often precludes a lengthy search for an MUD. Recently, unrelated cord blood (CB) has been used as a source of allogeneic HSCT.[3] However, in some cases, it is difficult to collect a sufficient number of stem cells from CB to engraft adult patients, which has been reported as an important factor of improved event-free survival.[4] Therefore, two- or three-loci-MMRD, which are readily available, have been proposed as a potential stem cell source.

The degree of HLA disparity between patients and donors has been reported to have a major impact on the outcome of allogeneic HSCT, particularly on engraftment and acute GVHD.[5,6] Although the incidence of graft failure

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in BMT from a three-loci-MMRD or a haploidentical related donor has been reported to be higher than in BMT from a one-locus- or two-loci-MMRD,[7] allogeneic HSCT from a two- or three-loci-MMRD was demonstrated to be feasible for the achievement of stable engraftment.[8] Allogeneic HSCT from a two-loci-MMRD was reported to be associated with a similar incidence and severity of acute GVHD as conventional allogeneic HSCT from a one-locus-MMRD,[9] especially in patients receiving purified CD34+ blood cells.[10] It is becoming more important to establish the degree of HLA mismatch that can be accepted.

The Japanese population is believed to be relatively homogeneous both ethnically and genetically, with less HLA genetic diversity than Caucasians.[11,12] This could make unmanipulated allogeneic HSCT between two- or three-loci-mismatched pairs more feasible in this population because there may be lower incidence of graft failure and GVHD. Allogeneic peripheral blood stem cell transplantation (PBSCT) is increasingly undertaken in Japan since it became eligible for reimbursement from health insurance in the year 2000. We conducted a nationwide retrospective survey to investigate the time course of engraftment, incidence of acute and chronic GVHD, transplant-related problems and survival in patients undergoing allogeneic PBSCT from two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the graft-versus-host (GVH) direction.

Patients and methods

Patient characteristics

This study included 50 Japanese patients over age 16 who underwent allogeneic PBSCT from two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the GVH direction for a variety of high-risk hematologic malignancies. They were transplanted between January 1996 and March 2002 at 20 different transplant centers in Japan. The patients consisted of 17 (34%) with AML, six (12%) with ALL, six (12%) with myelodysplastic syndrome (MDS), eight (16%) with CML, 11 (22%) with non-Hodgkin's lymphoma (NHL) and two (4%) with multiple myeloma (MM). All of them were considered to be at high risk for relapse or to be refractory to intensive chemotherapy: 18 patients (36%) were transplanted at primary refractory status, 17 (34%) at chemorefractory status, nine (18%) in relapse after prior autologous PBSCT, three (6%) in relapse after prior allogeneic BMT from an MUD (MUD-BMT), one (2%) in relapse after prior unrelated CB transplantation and MUD-BMT, and two (4%) after graft rejection following MUD-BMT for MDS. A total of 33 patients (66%) underwent primary allogeneic PBSCT and 17 (34%) underwent allogeneic PBSCT after prior autologous PBSCT ($n=10$, 20%) or prior allogeneic HSCT ($n=7$, 14%). The median time interval between prior autologous PBSCT or allogeneic HSCT and this therapy was 10.8 (range 3.1–27.3) or 5.3 months (1.7–29.2 months), respectively. Patient characteristics are summarized in Table 1. Treatment protocols were approved by local institutional review boards and all patients provided informed consent.

Stem cell collection and graft manipulation

G-CSF (Filgrastim, Kirin Brewery/Sankyo Co., Tokyo, Japan) was administered subcutaneously to donors at a dose of 400 $\mu g/m^2$ per day for 4 or 5 days as previously reported.[13] Leukaphereses were performed using a continuous-flow blood cell separator (Cobe Spectra, Cobe Laboratories, Lakewood, CO, USA) for 1–3 days beginning on day 4 of G-CSF administration until 3.0×10^6 CD34 cells/kg (patient body weight) had been collected. The percentages of CD34+ and CD3+ cells in the graft were determined by flow cytometry. Median doses of nucleated, CD34+ and CD3+ cells infused are shown in Table 1. Immunomagnetic selection was performed using a CliniMACS device ($n=17$; Kirin Brewery, Tokyo, Japan)[14] or an Isolex system ($n=15$; Baxter, Munich, Germany)[15] according to the manufacturer's recommendations. The graft was cryopreserved until infusion as previously reported.[16]

Conditioning regimen and GVHD prophylaxis

A conventional conditioning regimen was administered to 31 patients (62%). In all, 26 (52%) received 8–13.2 Gy TBI, and eight (16%) were given antithymocyte globulin (ATG) as an additional immunosuppressive. All patients treated with a conventional conditioning regimen received GVHD prophylaxis (CYA-based, $n=18$; FK506-based, $n=13$), as summarized in Table 2.

A total of 19 patients (38%) were treated with a reduced-intensity conditioning regimen: eight (16%) received 2–6 Gy TBI and 11 (22%) were given ATG. All but one of these patients treated with a reduced-intensity conditioning regimen received GVHD prophylaxis (CYA-based, $n=8$; FK506-based, $n=9$; prednisolone alone, $n=1$).

Definition of outcome

Neutrophil engraftment was defined as an absolute neutrophil count (ANC) exceeding $0.5 \times 10^9/l$ for 3 consecutive days after transplant. The day of neutrophil engraftment was determined to be the first of these 3 consecutive days. Platelet engraftment was defined as a platelet count exceeding $20 \times 10^9/l$ without platelet support. In all 42 patients (84%) received G-CSF until neutrophil engraftment after transplant. If the ANC never exceeded $0.5 \times 10^9/l$ or if it was not maintained above $0.5 \times 10^9/l$ for at least 3 consecutive days by day 28 post transplant, the patient was considered to have 'graft failure'. If ANC of greater than $0.5 \times 10^9/l$ could not be maintained after initial engraftment, the patient was considered to have 'graft rejection'.

The severity of acute GVHD was graded according to the consensus criteria[17] among 43 evaluated patients (86%) who developed acute GVHD within 28 days or who survived ≥ 28 days after transplant. Chronic GVHD was assessed and graded according to the standard criteria[18]

Table 1 Patient, donor and graft characteristics

	Manipulation		
	No. (n = 18)	*CD34+ cell selection (n = 32)*	
		CliniMACS (n = 17)	*Isolex (n = 15)*
Median age (range) (years)			
Patients	32 (20–55)	35 (18–69)	29 (17–48)
Donors	42 (16–60)	42 (18–59)	44 (21–67)
Gender match, donor/patient			
Male/male	4[a]	8	8
Female/male	5	5	3
Female/female	8	2	2
Male/female	1	2	2
Relationship of donor to patient			
Father/mother	1/7	2/2	3/2
Sibling	5	9	9
Son/daughter	0/5	3/1	0/0
Aunt	0	0	1
Genotypic disparities			
GVH direction			
HLA-A/-B and -DRB1	1/9	1/7	1/3
HLA-A, -B and -DRB1	8	9	11
HVG direction			
HLA-A/-B/-DRB1	0/0/0	0/1/2	1/0/0
HLA-A and -B	0	1	1
HLA-A/-B and -DRB1	1/10	1/5	1/3
HLA-A, -B and -DRB1	7	7	9
Disease at transplant			
AML	2	9	6
ALL	1	2	3
MDS	4	0	2
CML	4	3	1
NHL	6	2	3
MM	1	1	0
Prior autologous PBSCT/allogeneic HSCT	3/4	2/3	5/0
Median time interval[b] (range) (months)	10.5 (0.2–24.2)	4.0 (0.4–96.8)	1.0 (0.1–38.1)
ECOG PS 0/1/2/3	3/8/5/2	5/8/3/1	6/7/1/1
Median graft size (range)[c]			
Nucleated cell dose ($\times 10^7$/kg)	78.2 (11.3–324.0)	0.71 (0.36–1.4)	0.38 (0.19–0.56)
CD34+ cell dose ($\times 10^6$/kg)	4.2 (1.5–9.5)	6.8 (2.9–13.5)	4.4 (0.67–9.8)
CD3+ cell dose (/kg)	$2.7\ (1.3\text{-}5.4) \times 10^8$	$2.8\ (0.30\text{-}5.0) \times 10^8$	$4.0\ (1.7\text{-}24.7) \times 10^8$
Median follow-up (range) (months)	2.5 (0.10–15.4)	3.8 (0.20–16.8)	2.7 (0.30–35.5)

[a]Number of patients unless indicated otherwise.
[b]Time from diagnosis to transplant.
[c]The dose of CD34+ cells was significantly higher in patients receiving CD34+ blood cells purified by a CliniMACS device than in those receiving unmanipulated PBSC ($P = 0.0027$) or CD34+ blood cells purified by an Isolex system ($P = 0.014$). The dose of CD3+ cells was significantly higher in patients receiving unmanipulated PBSC than in those receiving CD34+ blood cells purified by a CliniMACS device ($P = 0.0004$) or an Isolex system ($P = 0.0009$).GVH, graft-versus-host; HVG, host-versus-graft; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; ECOG PS, Eastern Cooperative Oncology Group performance score.

among 25 patients with sustained engraftment for $\geqslant 100$ days after transplant.

Regimen-related toxicity (RRT) of organ systems before day 100 post transplant was graded according to the criteria proposed by Bearman *et al.*[19] A clinical diagnosis of veno-occlusive disease (VOD) was made based on the presence of jaundice (bilirubin $\geqslant 2$ mg/dl), hepatomegaly and/or right upper quadrant pain, and $\geqslant 5\%$ weight gain from admission, with or without ascites.[20] The diagnosis of thrombotic microangiopathy (TMA) was made if the patient had thrombocytopenia (defined as a platelet count $< 100 \times 10^9$/l), microangiopathic hemolytic anemia as indicated by red blood cell fragmentation present in a peripheral blood smear and elevated lactate dehydrogenase (LDH), without an identifiable cause for the thrombocytopenia or microangiopathic hemolytic anemia (eg, sepsis, disseminated intravascular coagulation, carcinoma, eclampsia).[21]

Relapse was defined either by morphologic evidence of the disease in the peripheral blood, marrow or extramedullary sites, or by recurrence and sustained presence of pretransplant chromosomal abnormalities in cytogenetic analysis of the marrow cells. Patients with CML, in whom the sole evidence of the disease was positivity for the bcr/abl RNA transcript by polymerase chain reaction, were not classified as having relapse. Both relapse and progression were defined as disease progression with transplant-related deaths being censored. Treatment-related mortality (TRM)

Table 2 Treatment characteristics

	Manipulation		
	No. (n = 18)	CD34+ cell selection (n = 32)	
		CliniMACS (n = 17)	Isolex (n = 15)
Conventional conditioning regimen	7 (39%)[a]	9 (53%)	15 (100%)
TBI + CY + others[b]/TBI + melphalan	3/0	8/1	14/0
BU + CY + others[c]	4	0	1
ATG-containing	1 (6%)	0	7 (47%)
GVHD prophylaxis			
CYA + MTX/CYA + prednisolone/CYA	1/0/0	5/0/1	7/2/2
FK506 + MTX/FK506	6/0	3/0	0/4
Reduced-intensity conditioning regimen	11 (61%)	8 (47%)	0
TBI + CY/TBI + Flu + BU/TBI + Flu + ATG + others[d]/TBI + BU + ATG	1/0/0/0	0/2/4/1	0/0/0/0
Flu + others[e]	10	1	0
ATG-containing	6 (33%)	5 (29%)	0
GVHD prophylaxis			
CYA + MTX/CYA + prednisolone/CYA + MMF/CYA	1/1/0/1	0/0/3/2	0/0/0/0
FK506 + MTX/FK506 + prednisolone + MMF/FK506 + prednisolone/FK506	6/1/1/0	0/0/0/1	0/0/0/0
Prednisolone/none	0/0	1/1	0/0
G-CSF after transplant	12 (67%)	16 (94%)	14 (93%)

[a]Number of patients (%) unless indicated otherwise.
[b]Others = ATG, BU, Ara-C, thiotepa or VP-16.
[c]Others = ATG, Ara-C, Flu or melphalan.
[d]Others = BU, CY or thiotepa.
[e]Others = BU, CY, Ara-C, idarubicin or melphalan.
ATG, antithymocyte globulin; Flu, fludarabine; MMF, mycophenolate mofetil.

was defined as death from any cause other than relapse or disease progression. Progression-free survival (PFS) was defined as the time interval from transplant to the first event including relapse, disease progression and death. Overall survival (OS) was defined as the time interval from transplantation to death.

Data collection

Questionnaires were returned by 216 of 432 (50%) transplant centers in Japan.

A total of 91 adult patients underwent allogeneic HSCT from a genotyped related donor mismatched at two or three loci in the GVH direction for the treatment of hematologic malignancies in a total of 32 centers (listed in the Appendix).[22] These data were collected from medical records. Histocompatibility was determined by serology for HLA-A, -B and -DR antigens and/or by DNA typing for HLA-A, -B and -DRB1. All of these HLA data were reviewed, and inquiries concerning patient and donor HLA typings were verified by the central committee. Of these 91 patients, 24 were excluded because they received BM, five because they were thought not to be at high risk for relapse or refractory to intensive chemotherapy, and six because their donors were not anticipated to be serological two- or three-loci-MMRD. As in allogeneic HSCT from related as well as unrelated donors, both HLA class I and II gene disparities contribute to increased incidence of graft failure or acute GVHD prophylaxis,[23,24] identification of the fact that both HLA class I and II genes were disparate would be helpful. Thus, six patients were excluded because they received allogeneic PBSC from a two- or three-loci-MMRD with only HLA class I or II gene disparity in the GVH direction. Finally, we confirmed that 50 evaluable patients underwent allogeneic PBSCT from a two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the GVH direction for high-risk hematologic malignancies in 20 centers.

Statistical analysis

Comparisons of variables were performed using the two-tailed Fisher exact test or the χ^2 test. Continuous variables were compared by the Mann–Whitney U-test or the Kruskal–Wallis test. Competing risks for grades II–IV acute GVHD included death without grades II–IV acute GVHD, relapse, and graft failure and rejection. Relapse was a competing risk for TRM at day 100 post transplant, and TRM was a competing risk for relapse. The association of variables with the outcome was evaluated in multivariable analyses, with the use of stepwise Cox regression to adjust for differences in potentially confounding variables.[25–27] The variables considered were age and gender of patients or donors, number of transplantations, performance status (PS, which was evaluated before the start of the conditioning regimen and graded according to the Eastern Cooperative Oncology Group (ECOG) performance score), CD34+ cell doses, degree of HLA genotypic disparity in the GVH direction, conditioning regimens including reduced regimens, TBI and ATG used, GVHD

prophylaxis and G-CSF used after transplant. Each of these factors was checked for the assumption of proportional hazards by using a time-dependent covariate. The interactions with outcomes, such as the incidence of neutrophil and platelet engraftment, complications including infection before day 100 post transplant, grades II–IV acute GVHD and TRM at day 100 post transplant, were assessed in a model that included these outcomes and the variables under consideration. Only factors significantly associated with the outcomes ($P<0.05$) were retained in the final models. End points were calculated on the day of the last patient contact. The outcomes PFS and OS following transplant were estimated by the Kaplan–Meier method and significance assessed by the log-rank test ($P<0.05$).

Results

Graft characteristics

The dose of CD34+ cells given was significantly higher in patients who received CD34+ blood cells purified by a CliniMACS device (CliniMACS-purified CD34+ cells, $n=17$) than in those who received unmanipulated PBSC ($n=18$; $P=0.0027$) or CD34+ blood cells purified by an Isolex system (Isolex-purified CD34+ cells, $n=15$; $P=0.014$; Table 1). The target dose of CD34+ cells (3.0×10^6 CD34 cells/kg) was achieved in 36 donors (72%) by performing two or more leukaphereses. In all, 14 patients (28%) received $<3.0\times10^6$ CD34 cells/kg in unmanipulated PBSC ($n=7$), or CliniMACS-purified CD34+ cells ($n=1$) or Isolex-purified CD34+ cells ($n=6$). The dose of CD3+ cells was also significantly higher in those receiving unmanipulated PBSC than in those receiving CliniMACS- or Isolex-purified CD34+ cells ($P=0.0004$ or 0.0009, respectively). As the Isolex systems have changed over the years, variable CD34+ cell doses from the Isolex system may be related to the use of earlier or later version of this equipment.

Engraftment

Among 39 patients (78%) who survived for $\geqslant$28 days after transplant, 37 (95%) and 25 (64%) achieved neutrophil and platelet engraftment, respectively. There was no significant difference in the number of days required for neutrophil engraftment between patients receiving unmanipulated PBSC (median 14 days, range 10–27 days), CliniMACS- and Isolex-purified CD34+ cells (14, 9–20 and 12, 9–20, respectively). There was also no significant difference in the number of days required for platelet engraftment between patients receiving unmanipulated PBSC (18.5, 0–46 days), and CliniMACS- and Isolex-purified CD34+ cells (14, 9–23 and 16, 12–37, respectively).

A total of 11 patients (22%) died before 28 days (median 12 days range 3–27 days) post transplant without evidence of engraftment. Of these patients, two receiving $<3.0\times10^6$ CD34 cells/kg of unmanipulated PBSC or Isolex-purified CD34+ cells died of RRT.

Graft failure and rejection

Graft failure occurred in two patients (5%). One of them had received CliniMACS-purified CD34+ cells from a three-loci-MMRD, mismatched for HLA-A, -B and -DR antigens in the HVG direction, and was given a reduced-intensity conditioning regimen containing ATG and GVHD prophylaxis with prednisolone. This patient developed grade IV pulmonary toxicity and died of pulmonary hemorrhage. The other patient with graft failure received Isolex-purified CD34+ cells from a two-loci-MMRD, mismatched for HLA-B and -DR antigens in the HVG direction, and was given a conventional conditioning regimen containing ATG and GVHD prophylaxis with CYA plus prednisolone. This patient developed grade III stomatitis and gastrointenstinal toxicity and died of TMA after secondary allogeneic BMT. Additionally, they received purified CD34+ cells mismatched for HLA-C antigen in the HVG direction.

Three patients (8%) developed graft rejection. All of them had received CliniMACS-purified CD34+ cells and a conventional conditioning regimen including 12 Gy TBI without ATG. One patient received purified CD34+ cells of $<3.0\times10^6$ CD34 cells/kg. Their donors were all mismatched for HLA-B and -DR antigens in the HVG direction, of whom two were also mismatched for HLA-C + -A antigens in the HVG direction. They received GVHD prophylaxis with CYA plus methotrexate ($n=2$) or CYA alone ($n=1$).

GVHD

Grades II–IV acute GVHD developed in nine (64%) of 14 evaluable patients receiving unmanipulated PBSC, in three (20%) of 15 receiving CliniMACS-purified CD34+ cells and five (36%) of 14 receiving Isolex-purified CD34+ cells. By stepwise Cox regression analysis, the incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells (28%) was significantly lower than in those receiving unmanipulated PBSC (64%) (hazard ratio (HR) 0.32; 95% confidence interval (CI) 0.12-0.84; $P=0.022$). While no patients receiving CliniMACS- ($n=4$) or Isolex- ($n=7$) purified CD34+ blood cells with conditioning regimens containing ATG developed grade III or IV acute GVHD, four of 18 patients (22%) receiving CliniMACS- ($n=11$) or Isolex- ($n=7$) purified CD34+ cells without ATG developed grade III or IV acute GVHD. Chronic GVHD developed in three of the 25 evaluable patients (12%; Table 3).

Regimen-related toxicity

Six patients (33%) of those receiving unmanipulated PBSC and 13 (41%) of those receiving purified CD34+ cells developed RRT including grades II–IV organ toxicity before day 100 post transplant according to Bearman's criteria. Additionally, two patients (11%) and five (28%) of those receiving unmanipulated PBSC, and one (3%) and three (9%) of those receiving purified CD34+ cells developed VOD or TMA, respectively (Table 3). Seven of

Table 3 Engraftment, GVHD and regimen-related toxicity

	Manipulation		
	No. (n = 18)	*CD34+ cell selection (n = 32)*	
		CliniMACS (n = 17)	*Isolex (n = 15)*
Median time of engraftment (range) (days)			
Neutrophil	14 (10–27)	14 (9–20)	12 (9–20)
Platelet	18.5 (0–46)	14 (9–23)	16 (12–37)
Graft failure/rejection	0*/0	1/3	1/0
Acute GVHD[b]			
0/I	3/2	9/3	5/4
II/III/IV	1/6/2	2/0/1	2/2/1
Median onset (range) (days) of ⩾II acute GVHD	14 (6–77)	26.5 (3–50)	12.5 (5–32)
Chronic GVHD[c] *(onset, days)*			
None/limited/extensive	7/1 (105)/0	9/0/1 (112)	6/1 (101)/0
RRT[d] II/III/IV	2/2/2	1/1/2	2/6/1
VOD/TMA	2/5	1/1	0/2

*Number of patients unless indicated otherwise.
[b]A total of 43 patients who developed acute GVHD within 28 days or who survived ⩾28 days after transplant were evaluated for acute GVHD.
[c]A total of 25 patients who engrafted and survived ⩾100 days after transplant were evaluated for chronic GVHD.
[d]Maximum early RRT was graded according to the criteria documented by Bearman *et al*.RRT, regimen-related toxicity; VOD, veno-occlusive disease; TMA, thrombotic microangiopathy; ⩾II acute GVHD, grades II–IV acute GVHD.

11 (64%) patients receiving unmanipulated PBSC and eight of 14 (57%) receiving purified CD34+ cells died of RRT.

Infectious complications

By day 100 post transplant, the most serious treatment-related problem was some form of severe infectious complication, which was seen in 11 patients (61%) receiving unmanipulated PBSC, as follows: bacterial sepsis ($n=8$, 44%), bacterial pneumonia ($n=3$, 17%), fungal infection ($n=2$, 11%) including Candida ($n=1$) and others ($n=1$), and adenoviral disease ($n=2$, 11%). In 20 patients (63%) receiving purified CD34+ cells, these figures were as follows: bacterial sepsis ($n=12$, 39%), bacterial pneumonia ($n=2$, 6%), fungal infection ($n=10$, 31%) including Aspergillus ($n=2$), Candida ($n=1$) and others ($n=7$), and viral disease ($n=10$, 31%) including CMV ($n=5$) and adenoviral ($n=3$) disease, and VZV reactivation ($n=2$).

Five of these 11 patients (45%) receiving unmanipulated PBSC and eight of these 20 (40%) receiving purified CD34+ cells died of infectious complications before day 100 post transplant. Four of these five patients (80%) receiving unmanipulated PBSC and two of these eight (29%) receiving purified CD34+ cells suffered from infectious complications before achieving neutrophil engraftment. Two patients, who developed grade IV acute GVHD and died of acute GVHD, suffered the complication of Candida infection or CMV disease, respectively, before day 100 post transplant (Table 4).

Eight patients (16%) had carried over active infection to transplant. By day 100 post transplant, three patients who had bacterial infections (methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* or *Stentrophomonas maltophilia*) (infectious sites unknown) before the start of the conditioning regimen suffered sepsis, of whom one died of *P. aeruginosa* sepsis and one of adenoviral pneumonia after recovery from MRSA sepsis. One patient who had pneumonia of unknown origin died of bacterial sepsis, also of unknown origin. Two patients who had Candida liver abscesses or infections of unknown origin and site had *Staphylococcus hemolyticus* or bacterial sepsis of unknown origin, respectively (but recovered). One patient who had active chronic hepatitis C infection died of VOD. One patient who had *Staphylococcus epidermidis* tonsillitis had no infectious complications after transplantation.

Cause of death, relapse, disease progression and survival

In all 11 patients are surviving at a median of 11.7 months (range 4.4–35.5 months) and 39 patients died at a median time of 67 days (range 3–254 days) after transplant. The primary causes of death up to 1 year post transplant in 39 patients (78%) are listed in Table 5. The most common cause of death was treatment-related problem, which was seen in 28 patients (56%). Of the 39 patients who survived ⩾28 day after transplant with confirmed evidence of engraftment, two of 13 (15%) receiving unmanipulated PBSC and two of 26 (8%) receiving purified CD34+ cells relapsed before day 100 post transplant.

Six patients were treated for relapse or disease progression with donor lymphocyte infusion (DLI), the median dose of CD3+ cells being 9.6×10^6/kg (range 1.5×10^5–6.6×10^7/kg), and one underwent a third allogeneic PBSCT. Although grades II acute GVHD developed in two patients who received DLI, and grade III in one, there was no noticeable effect of DLI on disease progression.

Within 168 days after transplant, two of seven patients (29%) undergoing allogeneic PBSCT after prior allogeneic HSCT relapsed and received DLI. One of these two died of

Table 4 Infectious complications before day 100 post-transplant

	Manipulation							
	No. (n = 18)				*CD34+ cell selection (n = 32)*			
	Total	*Neut*	*aGVHD*	*Both*	*Total*	*Neut*	*aGVHD*	*Both*
Bacterial								
Sepsis	8 (2)[a]	6 (2)	1	0	12 (3)	6 (1)	1	0
Pneumonia	3 (1)	3 (1)	0	0	2 (2)	0	0	0
Fungal								
Aspergillus	0	0	0	0	2	1	0	0
Candida	1 (1)	0	1 (1)	0	1	0	0	0
Others[b]	1 (1)	1 (1)	0	0	7 (1)	3 (1)	2	1
Viral								
CMV disease	0	0	0	0	5 (2)	0	1 (1)	0
Adenoviral disease	2	0	1	0	3	0	0	0
VZV reactivation	0	0	0	0	2	1	0	0
CMV antigenemia	6	1	0	3	15	7	0	3

[a]Number of patients (number of deaths) is shown.
[b]Others = *Rhodotorula rubra*, *Pneumocystis carinii* or origin unknown.
Neut, number of patients with incidence of infectious complications before achieving neutrophil engraftment; aGVHD, number of patients with incidence of infectious complications after treating grades II–IV acute GVHD with prednisolone; both, number of patients with incidence of infectious complications before achieving neutrophil engraftment (Neut) and after treating grades II–IV acute GVHD with prednisolone (aGVHD); CMV antigenemia, positive for CMV antigen without diseases caused by CMV infection.

Table 5 Causes of death before 1 year post transplant

	Manipulation	
	No. (n = 18)	*CD34+ cell selection (n = 32)*
Relapse/progressive disease	2 (11%)	9 (28%)
Treatment-related problem	11 (61%)	17 (53%)
Infectious complication	5 (28%)	10 (31%)
Organ toxicity[a]	5 (28%)	5 (16%)
Acute GVHD + infectious complication	1 (5%)	2 (6%)

[a]Organ toxicity = pulmonary hemorrhage ($n = 2$), VOD ($n = 2$), TMA (n = 2), interstitial pneumonia ($n = 2$), intracerebral hemorrhage ($n = 1$) and asphyxia due to oral hematoma ($n = 1$).

relapse and the other died of interstitial pneumonia. However, five of the seven (71%) undergoing allogeneic PBSCT after prior allogeneic HSCT died of infectious complications. The probability of PFS at 1 year in patients undergoing primary allogeneic PBSCT ($n = 33$) or allogeneic PBSCT after prior autologous PBSCT ($n = 10$) was 25.4 and 20.0%, respectively. The probability of OS at 1 year in patients undergoing primary allogeneic PBSCT or allogeneic PBSCT after prior autologous PBSCT was 26.0 and 20.0%, respectively. None of the patients undergoing allogeneic PBSCT after prior allogeneic HSCT survived $\geqslant 196$ days after transplant (Figure 1).

There was no significant difference in the incidence of TRM at day 100 post transplant between patients receiving unmanipulated PBSC ($n = 18$, 56%) and purified CD34+ cells ($n = 32$, 44%). The probability of PFS at 1 year in patients receiving unmanipulated PBSC or purified CD34+ cells was 22.2 and 17.6%, respectively ($P = 0.63$). The probability of OS at 1 year in patients receiving unmanipulated PBSC or purified CD34+ cells was 26.7 and 17.9%, respectively ($P = 0.93$; Figure 2).

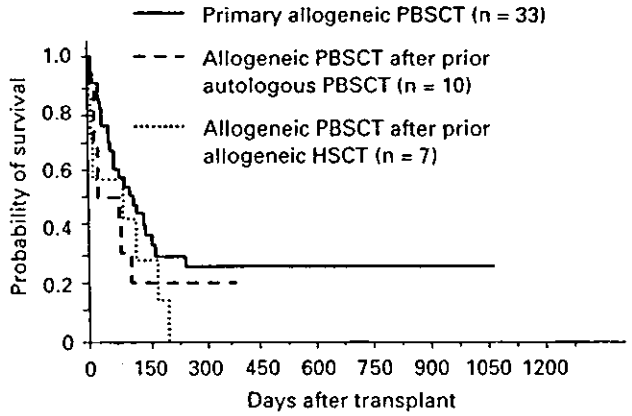

Figure 1 Probability of OS for patients undergoing primary allogeneic PBSCT, and allogeneic PBSCT after prior autologous PBSCT and allogeneic HSCT. The probability of OS at 1 year in patients undergoing primary allogeneic PBSCT or allogeneic PBSCT ($n = 33$, solid line) after prior autologous PBSCT ($n = 10$, dashed line) was 26.0 and 20.0%, respectively. None of the patients undergoing allogeneic PBSCT after prior allogeneic HSCT ($n = 7$, dotted line) survived $\geqslant 196$ days after transplant.

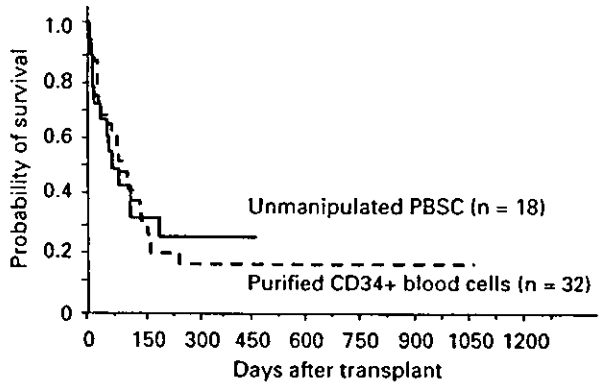

Figure 2 Probability of OS of patients receiving unmanipulated PBSC and purified CD34+ blood cells. The probability of OS at 1 year in patients receiving unmanipulated PBSC ($n = 18$, solid line) or purified CD34+ blood cells ($n = 32$, dashed line) was 26.7 and 17.9%, respectively ($P = 0.93$).

There was no significant difference in the incidence of TRM at day 100 post transplant between patients receiving a conventional ($n = 31$, 48%) and a reduced-intensity conditioning regimen ($n = 19$, 47%). The probability of PFS at 1 year in patients receiving a conventional or a reduced-intensity conditioning regimen was 26.3 and 16.2%, respectively ($P = 0.86$). The probability of OS at 1 year in patients receiving a conventional or a reduced-intensity conditioning regimen was 26.3 and 17.0%, respectively ($P = 0.77$).

Discussion

Our data support the notion that the present approach may be a possible option for a realistic alternative rescue for patients who lack an HLA-matched or a one-locus-mismatched related or unrelated donor, because we found that a significant proportion of patients with high-risk hematologic malignancies survived this procedure. As expected, we found that graft manipulation reduced the incidence of grades II–IV acute GVHD. However, there was no significant difference between the probabilities of PFS and OS at 1 year in patients receiving unmanipulated PBSC and purified CD34+ cells. We confirmed that transplant-related problems, particularly infectious complications, before day 100 post transplant were still major obstacles to the success of this therapy.

In the present analysis, we found that there were no significant differences in the time interval to neutrophil engraftment between patients receiving unmanipulated PBSC and purified CD34+ cells. On the other hand, we found that all graft failures (5%, 2/39) and rejections (8%, 3/37) were observed in those receiving purified CD34+ cells. We previously reported that in 13 Japanese children receiving purified CD34+ cells from a partially MMRD,[28] the observed rates of graft failure and rejection were 31% (4/13) and 22% (2/9), respectively. Kato *et al*[29] analyzed the clinical course in 135 young Japanese patients who underwent allogeneic HSCT with purified CD34+ cells from a haploidentical related donor, and found that graft failure occurred in 13% (13/103) of the patients with malignant diseases. Hence, the incidence of graft failure in the current study appears to be less than those in the previous Japanese reports. However, Aversa *et al*[30] reported that graft failure and rejection was 5% (2/43) and 0% (0/41), respectively. Recently, Redei *et al*[31] showed that all evaluable patients, who received $\geqslant 5 \times 10^6$ CD34+ blood cells purified by an Isolex system without cryopreservation, and were given a conditioning regimen including high-dose TBI, thiotepa, fludarabine and ATG without post transplant immunosuppressive treatment, established successful neutrophil engraftment. As we felt we were able to employ less immunosuppressive conditioning regimens to reduce transplant-related problems, we assumed that graft failure and rejection occurred at higher rates in our study than these two published studies. Additionally, in an attempt to reduce the risk of graft failure, various methods, including purification of blood cells to acquire very large numbers of CD34+ cells, follow-up DLI or T-cell add-back, and various types of anti-lymphocyte or thymocyte antibodies have been investigated,[27,32,33] but we could not find a benefit of employment of these well-established methods in our data. The explanation may again be that most patients were heavily pretreated prior to allogeneic PBSCT from two- or three-loci-MMRD . On the other hand, because all patients with graft failure and two of three of those with graft rejection had received purified CD34+ cells mismatched for HLA-C antigen in the HVG direction, matching for this locus may be an important factor to prevent graft failure and rejection when using purified CD34+ cells. The results of our study again highlight the notion that this approach with purified CD34+ cells should be conducted in a well-designed larger study, focusing on the prevention of graft failure and rejection.

As expected, in this study, the incidence of grades II–IV acute GVHD was significantly lower in patients receiving purified CD34+ cells (28%) compared with unmanipulated PBSC (64%). Similarly, several other studies reported that graft manipulation reduced the incidence of grades II–IV acute GVHD (range 0–21%).[29–31] As our patients received higher doses of CD3+ cells contained in the Isolex-purified CD34+ cells, or less immunosuppressive conditioning regimens with or without various kinds of GVHD prophylaxis, the incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells may be higher in our study than in other studies. Nonetheless, overall, PFS and OS did not differ significantly between patients receiving unmanipulated PBSC and purified CD34+ cells. This may have been due to the many transplant-related problems before day 100 post transplant observed with this approach. Ruggeri *et al*[34] showed that donor-versus-recipient natural killer (NK)-cell alloreactivity could protect patients against GVHD in allogeneic haploidentical HSCT. Procedures for purifying CD34+ cells have recently been introduced for allogeneic PBSCT from two- or three-loci-MMRD with the development of the CliniMACS and Isolex systems. Although further studies are needed to determine whether cell separation approaches can be applied to this approach, our data suggest that technological advances in cell separation of

PBSC allografts may influence the incidence of acute GVHD.

We could not find risk factors of RRT, VOD and TMA in pretransplant clinical status, such as PS and prior HSCT, in pretransplant treatment, such as conditioning regimens, or in post transplant treatment, such as GVHD prophylaxis and G-CSF use. Recently, new methods of transplantation, that is, nonmyeloablative pretransplant conditioning with or without ATG,[35] and improvements in supportive care in allogeneic HSCT, such as prevention and treatment of GVHD and infectious complications, have been introduced. However, none of the patients who underwent prior allogeneic HSCT survived ⩾196 days after transplantation. Five of the seven (71%) undergoing allogeneic PBSCT after prior allogeneic HSCT died of infectious complications. These results emphasize that this approach after prior allogeneic HSCT should be conducted in a well-designed study, focusing on the prevention of infectious complications, regardless of the application of conventional or reduced-intensity conditioning regimens.

We found that the major cause of death was infectious complications before 1 year post transplant (30%, 15/50), as has also been reported in several other studies (range 33–83%).[7,28–31] In our study, 20 patients (40%) suffered from bacterial sepsis before day 100 post transplant and 12 (24%) suffered from bacterial sepsis before achieving neutrophil engraftment. Thus, the effective prevention and treatment of bacterial infection occurring early after transplantation, especially before achieving neutrophil engraftment, may be of primary importance for the success of this approach. Although Volpi *et al*[36] have shown that G-CSF promotes T-helper (Th)-2 immune deviation, which, unlike Th1 responses, does not protect against fungi, G-CSF after transplant may be useful to reduce the incidence of infectious complications, particularly bacterial ones, because of achieving early neutrophil engraftment. On the other hand, delayed reconstitution of T cells may cause a high incidence of infectious complications, especially fungal and viral infections. Handgretinger *et al*[37] suggested that the transplantation of higher doses of purified CD34+ cells may hasten immune reconstitution. Thus, the number of transplanted CD34+ cells may be important to prevent fungal and viral infections. Although post transplant therapy, such as DLI or T-cell add-back, and reduced GVHD prophylaxis or reduced *in vivo* T-cell depletion may increase the incidence of GVHD, they may reduce the incidence of fungal and viral infections. They should be further investigated in future prospective trials. The incidence of infectious complications was high (75%) in our patients with a carry-over infection before the start of the conditioning regimen. Thus, we should therefore consider selection of patients very carefully and may exclude those with a carry-over infection, particularly MRSA and multidrug-resistant *P. aeruginosa*, from eligibility in any future study.

Our study was limited by the inevitable bias associated with a small population of patients treated at 20 different centers. Nevertheless, we found that a small but significant proportion of patients with high-risk hematologic malignancies survived this approach. In Japan, Kawano *et al*[28] previously reported that the probability of PFS and OS at 1 year was 30 and 40%, respectively, and Kato *et al*[29] reported that the probability of PFS at 67 months was 5.7%. Aversa *et al*[30] reported that the probability of OS at 18 months was 28% (including seven (16%) of 43 standard-risk patients). The probability of OS at 2 years in a study reported by Redei *et al*[31] was 25%. Although this approach is not significantly better in Japan than elsewhere in contrast to allogeneic HSCT from MUD, our data also support the notion that this modality may be a possible option as a realistic alternative rescue for patients who lack an HLA-matched or a one-locus-mismatched related or unrelated donor, regardless of whether they received unmanipulated PBSC or purified CD34+ cells, or the application of conventional or reduced-intensity conditioning regimens.

In conclusion, because only a small number of patients survived this procedure, future studies of allogeneic PBSCT with purified CD34+ cells should be conducted on the prevention of graft failure and rejection, and the observed high risk of treatment-related problems requires eligibility criteria for the selection of patients and the effective prevention and treatment of infectious complications occurring early after transplant. Our data suggested that patients undergoing prior allogeneic HSCT and those with a carry-over infection before the start of the conditioning regimen should also be excluded from this approach because their mortality from infectious complications was extremely high, regardless of whether they received unmanipulated PBSC or purified CD34+ cells, or the application of conventional or reduced-intensity conditioning regimens with or without various kinds of GVHD prophylaxis. The strategy for graft manipulation, and the application of conditioning regimens and GVHD prophylaxis should be further investigated in future prospective trials.

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