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Urgent need for a validated tumor response evaluation system for use in immunotherapy

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Hentschke *et al* recently reported a detailed case series on reduced-intensity stem-cell transplantation (RIST) for the treatment of renal cell and colon cancers.[1] While they provided important information on the feasibility of RIST and its possible antitumor effect, we would like to comment on their study design, especially focusing on the feasibility of response evaluation criteria. Although the Response Evaluation Criteria in Solid Tumors (RECIST) system has been used as a gold standard to evaluate the response of solid tumors to treatment,[2] mainly in the field of cancer chemotherapy, it has not been fully validated in the area of allogeneic transplantation for solid tumors, where the immune-mediated destruction of tumor cells is the principle mechanism of tumor destruction (graft-versus-tumor effect, GVT). Compared to hematological malignancies, solid tumors are generally more resistant to the cytotoxic agents used in conditioning regimens administered before transplantation. Consequently, we considered that there may be some important differences in evaluating the response of solid tumors between RIST and conventional chemotherapy.

First, the feasibility of directly applying RECIST, including the optimal timing of response evaluation, should be critically validated before its extensive application in transplantation. Currently available reports on RIST for solid tumors commonly note that tumor regression occurs several months after transplantation.[3] Some responses and GVHD effects, in general, occur during the late period of RIST. Thus, most tumors continue their natural growth until the manifestation of effective alloimmunity to restrain tumor growth. If the original RECIST criteria[2] are applied to patients undergoing RIST for solid tumors, most of the GVT effects would be evaluated as progressive disease (PD), which would preclude subsequent evaluation (Figure 1a). Therefore, RECIST may underestimate the efficacy of RIST. Furthermore, while there is no concept of spontaneous regression in the field of chemotherapy, this is quite common in RIST.

Second, the proper time to measure the tumor size as a baseline for evaluating a subsequent tumor response has not been clearly defined. In contrast to the results with chemotherapy, the tumor often temporarily increases in size following RIST. Some metastases initially progress slowly, while others progress rapidly. Accordingly, when the size at transplantation is used as a baseline, as in chemotherapy, a therapeutic effect following the initial progression could be overlooked or underestimated (Figure 1c). On the other hand, evaluating regression from the largest size after transplant certainly overestimates the effect of treatment (Figure 1b), and gives an unacceptable bias.

Third, the tumor size after RIST often fluctuates in response to a *de novo* GVT effect, post transplant immunotherapy including donor lymphocyte infusion, and adjustment of immunosuppressive agents (Figure 2). In this situation, it is clear that any evaluation of the response duration, such as progression-free survival and the overall response duration, is essentially impossible using the current RECIST criteria.

These limitations in tumor response evaluation are also expected to be present in other areas including tumor vaccination and dendritic cell therapy strategy. Improved overall survival will ultimately be evaluated in phase III trials. To reach this point, a global standard evaluation system that enables the effective screening of a therapeutic effect in an earlier phase II study will need to be established. We hope that this letter will inspire a productive discussion.

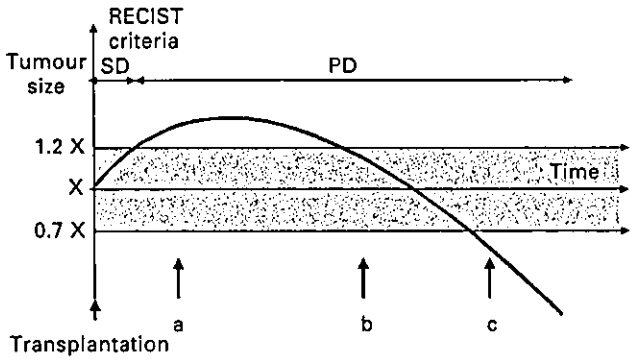

Figure 1 Course of tumor size after transplantation. Primary solid tumors are progressive, despite chemoradiotherapy prior to transplantation. (a) Most tumors continue their natural growth until the development of a GVT effect, which usually occurs several months after transplantation. (b) If the tumor has increased in size compared to that at the time of transplant, regression from the largest size may overestimate the treatment effect. (c) If the tumor size at transplant is defined as a baseline, some treatment effects, observed in patients whose lesions show initial progression followed by regression with the development of GVHD, will be underestimated.

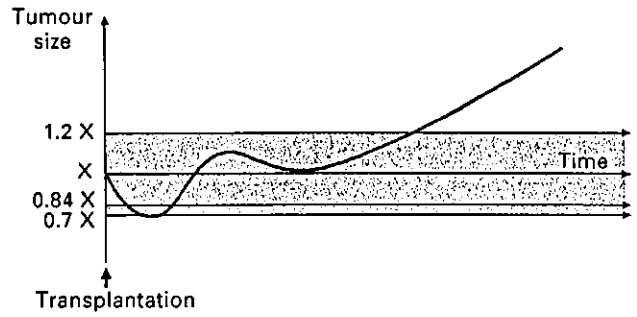

Figure 2 Fluctuation of tumor size after donor lymphocyte infusion or adjustment of immunosuppressive agents. It is difficult to handle patients in whom the tumor size fluctuates in response to post transplant immunotherapy, such as donor lymphocyte infusion and adjustment of immunosuppressive agents. Neither an appropriate timing of response evaluation nor an appropriate time to measure a baseline tumor size has been established in these cases.

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ORIGINAL ARTICLE

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Cryopreservation of mobilized blood stem cells at a higher cell concentration without the use of a programmed freezer

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Abstract Cryopreservation of peripheral blood stem cells (PBSC) mobilized by chemotherapy combined with or without granulocyte colony-stimulating factor (G-CSF) is an essential part of procedure for anti-cancer strategies. We evaluated whether a higher cell concentration (2×10^8/ml) without the use of a programmed freezer was acceptable for the storage of mobilized PBSC in an autologous setting. Mobilized PBSC were enriched to mononuclear cells (MNC) by Percoll separation and then frozen at cell concentrations of $2–5\times10^7$/ml (group I, n=20) or 2×10^8/ml (group II, n=44) without the use of a programmed freezer using 5% DMSO, 6% hydroxy ethyl starch, and 4% autologous serum or human albumin. CD34+ cells purified by ISOLEX300 were frozen at 2×10^7/ml (group III, n=22) using the same method. The median recovery rates of CD34+ cells and CFU-GM were, respectively, n.d. (not determined) and 88% in group I, 103 and 64% in group II, and 98 and 53% in group III. There was a statistical significance between the recovery rate of CFU-GM in group III and that in group I (p=0.02). The median percentage of cell viability after thawing in each group was 89, 87, and 75%, respectively. The median numbers of days after PBSCT to achieve a WBC of $>1.0\times10^9$/l, an absolute neutrophil count of $>0.5\times10^9$/l, and a platelet count of $>50\times10^9$/l were, respectively, 11, 11 and 15 in group I; 12, 12 and 16 in group II; and 12, 12 and 27 in group III. These results suggest that enriched MNC from mobilized PBSC could be frozen at a higher cell concentration (2×10^8/ml) without the use of a programmed freezer, leading to reduction of the toxicities associated with infusion of thawed cells and of costly space required for cell storage.

Keywords Mobilized cells · Cryopreservation · Autologous · Cell concentration

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Introduction

Peripheral blood stem cell transplantation (PBSCT) has replaced autologous marrow transplantation in the treatment of various types of cancers [1].Improvements in mobilization methods with recombinant cytokines enable us to collect larger numbers of PBSC. Harvested peripheral blood stem cells (PBSC) are also expected to be a source of targeting cells for various types of future cell therapies.

Cryopreservation of PBSC is essential in an autologous setting. It has been previously reported that cell concentration (2×10^7/ml) and rate control (−1°C/min) are critical in cell freezing procedure [2]. In case of PBSCT, the traditionally recommended concentration of 2×10^7/ml would result in large product volumes with correspondingly increased amounts of cryoprotectant, which could be toxic for the recipients [3, 4, 5]. On the other hand, rate-controlled freezing with a programmed freezer is expensive as a routine clinical procedure. To make this procedure simpler and more economical, a non-rate-controlled freezing method has been reported by several

investigators [6, 7, 8].It has been shown that rate-controlled cryopreservation with a programmed freezer at a higher cell concentration does not impair the post-thaw recovery of hematopoietic stem/progenitor cells [9].

However, the effect of cryopreservation at a higher cell concentration without the use of a programmed freezer on autologous PBSCT has been tested very little [10]. In this study, we compared the recovery of CFU-GM, CD34+ cells and engraftment kinetics of cryopreserved products, which were frozen without the use of a programmed freezer at low and higher cell concentrations.

Methods and materials

Subjects

Patients who underwent PBSC collection and autologous PBSCT from January 1992 to December 1999 in the Department of Pediatrics, University of Tokushima, were enrolled into this study after obtaining consent. The study subjects consisted of patients with acute lymphoblastic leukemia (ALL, n=26), acute nonlymphocytic leukemia (ANLL, n=11), non-Hodgkin's lymphoma (NHL, n=3), and various solid tumors (n=46). The solid tumors included neuroblastoma (n=13), brain tumor (10), Wilms tumor (6), ovarian cancer (5), breast cancer (4), retinoblastoma (2), rhabdomyosarcoma (2), testicular tumor (2), peripheral neuroectodermal tumor (1), and juvenile rheumatoid arthritis (1). Of 86 patients, 42 were male and 44 were female. Their ages ranged from 1 to 56 years, with a median age of 10 years.

Mobilization and collection of PBSC

PBSC were mobilized by intensive chemotherapy with or without recombinant human granulocyte colony-stimulating factor (G-CSF) as previously reported [11].They were collected in the recovery phase of chemotherapy with a Baxter CS3000 plus continuous-flow blood cell separator (Baxter Healthcare, Deerfield, IL, USA) [12].Collection was performed on the day the patient achieved a WBC of $>3\times10^9$/l and a platelet count of $>100\times10^9$/l in the recovery phase after chemotherapy. In some patients, PBSC were mobilized by G-CSF alone [13].In these cases, patients received 10 μg/kg of G-CSF once a day by subcutaneous injection for 5 days.Apheresis was initiated from days 4 to 6 after G-CSF injection, and 200–300 ml/kg (max. 10 liters) were processed per session.

Cryopreservation and thawing procedures

Apheresis-collected cells were separated using discontinuous gradients of 40 and 60% Percoll and centrifugation [14].Cells were resuspended in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% autologous serum, as previously reported. The freezing method reported by Makino et al. was introduced with minor modifications from the beginning of this study [7].Briefly, Percoll-separated cells were resuspended in DMEM with 10% autologous serum or 8% human albumin (Albumin Yoshitomi, Yoshitomi Pharmaceutical, Osaka) and mixed slowly with an equal volume of freezing solution containing 12% HES and 10% DMSO to give final concentrations of 5% DMSO and 6% HES. Prior to May 1995, PBSC were cryopreserved at concentrations of $2–5\times10^7$/ml (Group I, n=20). Purified CD34+ cells were frozen at 2×10^7/ml using the same method (Group III, n=22). Subsequently, a concentration of 2×10^8/ml was adopted (Group II, n=44). In all methods, cells were transferred to 5-ml polypropylene cryo-tubes (MS4605 W, Sumitomo Bakelite, Osaka), placed directly into a −80°C electric freezer, and then transferred to −135°C on the following day. The cells were stored in the same freezer until use.

Cells were thawed rapidly in a water bath maintained at 37°C. We divided stored cells into two portions that were infused over 2 days, when the volume of cells suspension was >300 ml [15]. For recovery analysis, an aliquot of cell suspension was quickly transferred to a 50-ml tube and diluted with thawing medium consisting of 10% fetal bovine serum (FBS, Filtron, Brooklyn, Australia) and 50 IU/ml of deoxyribonuclease (Sigma DN-25, Aldrich Japan, Tokyo) in DMEM by the stepwise addition of this medium at room temperature with gentle agitation. The cells were then collected by centrifugation, washed three times with the thawing medium, and resuspended in DMEM supplemented with 10% FBS for further experiments. Trypan blue staining method was used to measure cell viability.

CD34+ cell purification

G-CSF-mobilized PBSC collected by apheresis were enriched for CD34+ cells using an ISOLEX-300 (Baxter Healthcare, Deerfield, IL, USA) according to the manufacturer's suggestions. Briefly, excess platelets were removed by centrifugation for 20 min at 200×G at room temperature. Cells were incubated in phosphate-buffered saline (PBS, Nissui, Tokyo) containing 0.5% human-globulin (Gammagard, Baxter Japan, Tokyo) for 15 min to block Fc-receptors. One vial of anti-CD34 monoclonal antibody (9C5, 2 mg) was added to the cell suspension that contained $<5\times10^{10}$ cells. After 30 min of incubation at room temperature with gentle rotation (4/min), cells were washed three times with PBS containing 1% human serum albumin (Albumin-Yoshitomi, Yoshitomi Pharmaceutical, Osaka). Sensitized cells were incubated with sheep anti-mouse IgG_1-coated paramagnetic microspheres (Dynabeads, 10 ml; Dynal, Oslo). Cells rosetted with beads were captured on permanent magnets, and released by chymopapain or peptide capture included in the kit. These cells were frozen by the same method as described above.

Flow cytometry

CD34+ cells were assayed by Otsuka Assay Institute (Tokyo). Sample cells were shipped by air-cargo and assayed within 24 h. Cells that expressed the surface CD34 antigen were identified by flow cytometry analysis. Briefly, 100 μl of cell suspension were added to a test tube (Falcon 2052, Becton Dickinson, Lincoln Park, NJ, USA) containing isotype control (phycoerythrin-mouse IgG_1) and phycoerythrin-conjugated CD34 monoclonal antibody (Anti-HPCA2 antibody, Becton Dickinson) at a concentration of 1 μg antibody/10^6cells. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson). After function was verified, samples were drawn into the flow cytometer using FSC and SSC, as gating parameters, along with debris subtraction techniques to determine the characteristics of the cells. A total of 20,000 events were counted to identify the mononuclear cell fraction. The flow cytometric data were analyzed using a gated analysis via a set of SSC-FL parameters for CD34+ cells to calculate the percentage of positive cells. When a sample was substantially contaminated with RBC, it was lysed with a solution consisting of 0.826% (w/v) NH_4CL, 0.1% $KHCO_3$, and 0.004% EDTA-4Na.

Hematopoietic progenitor assay

Colony-forming cells were incubated in methylcellulose cultures supplemented with 20% FBS, 450 μg/ml of human transferrin (Sigma T-1147), 2 U/ml of recombinant human erythropoietin (Kirin Brewery, Tokyo), 1% deionized delipidated BSA (Calbiochem 12657, Hoechst Japan, Tokyo), and a combination of recombinant human G-CSF (filgrastim, Kirin), interleukin-3 (Kirin), and stem cell factor (Kirin). These stimulating factors were used at a final concentration of 20 ng/ml, which was the

previously determined optimal concentration in our laboratory. Triplicate or quadruplicate cultures were plated in volumes of 0.4 ml in 24-well tissue culture plates (Corning 258201, New York, NY) that were then placed in an ESPEC N_2-O_2-CO_2 BNP-110 incubator (Tabai ESPEC, Osaka, Japan), which maintained a humid atmosphere of 5% carbon dioxide, 5% oxygen, and 90% nitrogen at 37°C. Plates were incubated for 13–15 days and three types of colonies, including colony-forming unit for granulocyte-macrophage (CFU-GM), were counted using an inverted microscope. The mean number of colonies in four wells was calculated.

Transplant procedures

The transplant procedures in our institute have been previously described in detail [16]. Briefly, frozen cells were thawed rapidly in a water bath maintained at 37°C. The patients were given 5 mg/kg of hydrocortisone and/or antihistamines to prevent allergic reactions before infusion. The recovery speed after autografting was evaluated in terms of the number of days to achieve a WBC of $>1\times10^9$/l, an absolute neutrophil count (ANC) of $>0.5\times10^9$/l, and a platelet count of $>50\times10^9$/l. G-CSF was given to the patients only in group III after autografting.

Statistics

The Mann Whitney U-test was used to analyze the significance of differences. Data were analyzed using StatView (Version 4.5; Abacus Concepts, Berkeley, CA, USA) for a Macintosh computer.

Results

Frozen cells

After Percoll separation (groups II and I) or the purification procedure (group III), the median (range) numbers of frozen cells per kilogram of recipient body weight were determined, as shown in Table 1. MNC in group III (median, 2.6×10^6/kg) was significantly lower than those in groups II (5.9×10^8/kg) and I (13×10^8/kg) ($p<0.001$ each). The number of CD34+ cells in group II (5.7×10^6/kg) was also significantly higher than that in group III (2.2×10^6/kg) ($p=0.002$). The number of CFU-GM in group II (16×10^5/kg) was significantly higher than those in groups III (4.2×10^5/kg) and I (6.1×10^5/kg) ($p<0.001$ each). However, there was no difference in the number of CFU-GM between groups III and I.

Table 1 Numbers of frozen cells

	Group	MNC ($\times10^8$/kg)	CD34+ cell ($\times10^6$/kg)	CFU-GM ($\times10^5$/kg)
I	($2–5\times10^7$/ml) n=20	13 (3.6–67.2)	n.d.	6.1 (1.5–22.0)
II	(2×10^8/ml) n=44	5.9 (0.8–35.5)	5.7 (0.13–83.2)	16 (0.4–73.2)
III	(2×10^7/ml) n=22	2.6×10^6/kg (1.3–19)	2.2 (0.85–15.6)	4.2 (0.75–37.4)

Each value indicates a median value (range). The number of CD34+ cells in group II was significantly higher than that in group III ($p=0.0024$). The number of CFU-GM in group II was significantly higher than in groups I and III ($p=0.0009$ and 0.0003, respectively). *n.d.* not determined

Recovery rate and cell viability

Cell recovery rates after cryopreservation/thawing are shown in Table 2. These numbers ranged widely. Recovery rates of MNC were statistically identical among the three groups, but with different p values (II and I; 0.770, III and I; 0.100, III and I; 0.144). The viability of MNC after thawing in group III [75% (66–89)] was significantly lower than those in groups II and I ($p<0.001$, each). There was no difference in cell viability between groups II and I ($p=0.332$). The recovery rate of CFU-GM in group III [53% (13–202%)] was lower than that in group I ($p=0.023$). However, there were no significant differences between groups II and I ($p=0.091$), or groups III and II ($p=0.271$).

Engraftment kinetics

The median volume of cell suspension infused was 180 ml (range, 85 –700 ml) in group I, 60 ml (23–145 ml) in group II, and 8 ml (3.5–15 ml) in group III. The number of infused CFU-GM in group I [median, 5.6 (range, 1.5–20) $\times10^5$/kg] was statistically identical to that in group II [9.1 (0.3–90) $\times10^5$/kg] ($p=0.086$). The number of infused CD34+ cells in group III [2.1 (0.43 – 7.0) $\times10^6$/kg] was significantly lower than that in group II [5.3 (0.12–75) $\times10^6$/kg] ($p=0.001$), and the number of CFU-GM in group III [2.2 (0.3–12) $\times10^5$/kg] was significantly lower than those in groups II [9.1 (0.3–90) $\times10^5$/kg] and I [5.6 (1.5–20) $\times10^5$/kg] ($p<0.001$ each).

Table 2 Cell recovery rates and viabilities after thawing

	Group	MNC	Viability	CD34+ cell	CFU-GM
I	($2–5\times10^7$/ml) n=20	67 (26–298)	89 (84–95)	n.d.	88 (33–373)
II	(2×10^8/ml) n=44	72 (23–335)	87 (83–91)	103 (27–429)	64 (19–508)
III	(2×10^7/ml) n=22	96 (23–204)	75 (66–89)	98 (33–196)	53* (13–202)

Each value indicates the percentage of a median value (ranges). *n.d.* not determined
*CFU-GM recovery in group III was significantly lower than that in group I with a p value of 0.023

Table 3 Engraftment speed after autografting

	Group	WBC ($>1\times10^9$/l)	ANC ($>5\times10^9$/l)	Platelets ($>50\times10^9$/l)
I	(2×10^7/ml) n=20	11 (8–29)	11 (8–22)	15 (10–88)
II	(2×10^8/ml) n=44	11 (8–18)	11 (8–19)	16 (10–37)
III	(2×10^7/ml) n=22	12 (9–20)	12 (9–19)	27* (11–60)

The data indicate median days (ranges) to achieve each criterion after autografting.
* Platelet recovery speed was significantly slower in group III than in group II and I with p values of 0.002 and 0.022, respectively

The engraftment rates determined by the number of days to achieve a WBC of $>1\times10^9$/l and ANC of 0.5×10^9/l after autografting were identical among the three groups. However, the platelet engraftment rate determined by the number of days to achieve a platelet count of $>50\times10^9$/l in group III was significantly delayed (p=0.022 and 0.002 versus groups II and I, respectively; Table 3).

Discussion

The rapid hematopoietic recovery after myeloablative therapy has prompted the use of PBSC in preference to BM cells [17]. Apheresis after mobilizing chemotherapy with or without G-CSF enables the collection of a large numbers of PBSC in comparison with marrow aspiration under general anesthesia. However, the collection and cryopreservation of PBSC is associated with intense labor and requires ample space for storage. Optimal conditions for PBSC cryopreservation have not yet been defined. In particular, an increase in the volume of cell suspension which will be frozen results in a concomitant increase in the volume of cryoprotectant, such as DMSO, which may become toxic at cell infusion [3, 5]. Under these circumstances, we applied a gradient centrifugation method with double-layered Percoll to deplete red cells, granulocytes, and platelets for clinical use in pediatric patients [18].

Rowley et al. reported that stem cell survival, as reflected in the post-thaw recovery of MNC, CFU-GM and CD34+ cells, was unaffected even when nucleated cells were frozen at a concentration of $3.7\pm1.9\times10^8$/ml [9].In addition, cryopreservation at different cell concentrations did not predict the time to engraftment or duration of aplasia [9]. Another study showed a reduced recovery of CFU-GM when a higher cell concentration of PBSC was compared with a lower cell concentration of bone marrow at the time of freezing. However, this did not translate into delayed hematopoietic recovery in clinical transplantation [19].Based upon these studies, we have initiated to cryopreserve PBSC at higher cell concentrations without the use of a programmed freezer. Benefit and efficacy of the procedure was evaluated by comparing hematopoietic recovery after autologous PBSCT.

In this study, we did not observe a significant difference in the freeze/thaw recovery rates of CD34+ cells or CFU-GM between groups II and I. However, recovery rate of CFU-GM and the number of reinfused cells in group III were significantly smaller than in the other two groups. The speeds of engraftment were not significantly different between groups II and I, although patients in group III showed a slower recovery of platelets. The neutrophil recovery might be enhanced by the administration of G-CSF in group III. On the other hand, there is another possibility that purification procedures with ISOLEX300 selectively affect on platelet-lineage progenitor cells or make them vulnerable to freeze/thaw procedure. Further investigations will be required to solve this problem.

Balint et al. reported that presence of 10% DMSO is an essential part of the cryopreservation procedure of very primitive murine stem cells [20]. On the other hand, DMSO is the primary factor related to toxicities at graft infusion and reduction of amount of DMSO by reducing the total volume of grafts should merit patients, particularly pediatric population. Thus, there is no suspicion for the superiority of using 5% DMSO when recovery rates of frozen cells and engraftment kinetics are identical.

In conclusion, the present results indicate that MNC in apheresis products be cryopreserved at 2×10^8/ml without the use of a programmed freezer, without jeopardizing their engraftment potential. Definition of the upper limit for the cryopreservation cell concentration will require further studies.

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Graft-*versus*-Tax Response in Adult T-Cell Leukemia Patients after Hematopoietic Stem Cell Transplantation

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ABSTRACT

Adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) is characterized by poor prognosis after chemotherapy. Recent clinical trials have indicated, however, that allogeneic but not autologous hematopoietic stem cell transplantation (HSCT) for ATL can yield better clinical outcomes. In the present study, we investigated cellular immune responses of ATL patients who obtained complete remission after nonmyeloablative allogeneic peripheral blood HSCT from HLA-identical sibling donors. In the culture of peripheral blood mononuclear cells (PBMCs) from a post-HSCT but not pre-HSCT ATL patient, $CD8^+$ CTLs proliferated vigorously in response to stimulation with autologous HTLV-I-infected T cells that had been established before HSCT *in vitro*. These CTLs contained a large number of monospecific CTL population directed to a HLA-A2-restricted HTLV-I Tax 11-19 epitope. The frequency of Tax 11-19-specific CD8+ CTLs in this patient markedly increased also *in vivo* after HSCT, as determined by staining with HLA-A2/Tax 11-19 tetramers. Similar clonal expansion of HTLV-I Tax-specific CTLs exclusively directed to a HLA-A24-restricted Tax 301-309 epitope was observed in the PBMCs from another ATL patient after HSCT from a HTLV-I-negative donor. Among four post-HSCT ATL patients tested, HTLV-I-specific CTLs were induced in the PBMC culture from three patients but not from the remaining one who had later recurrence of ATL. These observations suggested that reconstituted immunity against antigen presentation in ATL patients after HSCT resulted in strong and selective graft-*versus*-HTLV-I response, which might contribute to graft-*versus*-leukemia effects.

INTRODUCTION

Adult T-cell leukemia (ATL) is a T-cell malignancy that develops in ~5% of human T-cell leukemia virus type I (HTLV-I)-infected individuals and is characterized by mostly $CD4^+$ and $CD25^+$ mature T-lymphocyte phenotypes, onset at middle age or later, immune suppression, and poor prognosis (1–3). Clinical use of combination chemotherapy for ATL brought the 4-year overall survival rate up to 8 to 12%, which is still lower than those of other types of leukemia (4, 5). Recently, hematopoietic stem cell transplantation (HSCT) has been applied to a limited number of ATL patients. Initial studies of autologous HSCT revealed frequent recurrence of ATL (6). However, more recent studies have revealed that allogeneic HSCT could produce better results, although there was also a risk of graft-*versus*-host-disease (GVHD; Ref. 7). This strongly suggests that the cellular immune responses of donor against recipient, *i.e.*, graft-*versus*-leukemia (GVL) effects, contribute to eradicating ATL cells, as observed in other types of leukemia.

It has been demonstrated that allogeneic HSCT from HLA-identical siblings can cause GVHD to some degree, and the minor histocompatibility antigen (mHA) in the recipient has been referred to as the target antigen of GVHD (8). Several mHA, including the male-specific H-Y transplantation antigen (9), HA-1 antigen (10), CD31 molecule (11, 12), and human platelet antigens (12, 13), have been suggested to be involved in GVHD. It is known that the probability of recurrence of leukemia after allogeneic HSCT increases when the graft has been depleted of T cells or the donor is a genetically identical twin, indicating that GVL effects are important in preventing the recurrence of leukemia (14). Therefore, an augmentation of the donor T-cell response specific for mHA expressed in the recipient's hematopoietic cells but not in the nonhematopoietic cells has been proposed as one strategy for inducing GVL effects without causing GVHD (15). Tumor antigens such as bcr/abl fusion protein and WT-1, which are specific for or overexpressed in tumor cells, are also candidates for the target antigens of GVL effects (16, 17).

Host cellular immune responses against HTLV-I, especially outgrowth of cytotoxic T cells, are frequently found in peripheral blood mononuclear cell (PBMC) culture of asymptomatic HTLV-I carriers and HTLV-I-associated myelopathy/Tropical spastic paraparesis patients but infrequently in ATL patients (18, 19). Of the HTLV-I antigens such as *env*, *gag*, *pol*, and *pX* gene products, it has been shown that HTLV-I Tax is a dominant target antigen of HTLV-I-specific CTL (20, 21). Tax is also known to play a critical role in HTLV-I leukemogenesis by accelerating cell growth and inhibiting apoptosis (22, 23). These findings suggest that Tax-specific CTL may play a role in immune surveillance for HTLV-I leukemogenesis.

In a recently established animal model for HTLV-I-infected T-cell tumors, we demonstrated an antitumor effect of Tax-specific CTL *in vivo* (24, 25). In this model, otherwise fatal T-cell lymphomas in nude rats inoculated with syngeneic HTLV-I-infected cells could be eradicated by transferring fresh T cells from syngeneic immunocompetent rats vaccinated with either Tax-encoded DNA or peptides corresponding to a CTL epitope (26, 27). However, it is unclear whether such observations in experimental models apply to humans because HTLV-I expression is extremely low in human ATL cells in the periphery (28–30).

In the present study, we investigated the cellular immune responses of ATL patients after HSCT against spontaneously HTLV-I-infected T cells derived from the same patient before HSCT. These HTLV-I-infected cells were thought to possess antigens originating from the recipients, including targets for GVL effects. We found that in response to the recipient-origin cells, the PBMCs from post-HSCT patients exhibited vigorous HTLV-I-specific CTL responses that were directed to a limited number of Tax epitopes. Furthermore, such oligoclonal expansion of HTLV-I-specific CTL in post-HSCT PBMCs was observed also *in vivo*. These observations indicated that

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a strong graft-*versus*-HTLV-I response occurred in ATL patients after HSCT.

MATERIALS AND METHODS

Recipient/Donor Pairs and Blood Samples. Four acute type ATL patients, #37 (case 1), R07 (case 2), R11 (case 3), and #97 (case 4) and their corresponding HLA-identical sibling donors, #36, D07, D11, and #98, respectively, donated peripheral blood samples under written informed consent. The patients were participants in the clinical trial protocol for allogeneic HSCT for ATL with a reduced-intensity conditioning regimen that was supported by the Ministry of Health, Welfare, and Labor of Japan. After cyclophosphamide, doxorubicin, vincristine, prednisolone therapy, patient #37 at the beginning of recurrence, patients R07 and #97 in partial remission, and patient R11 in complete remission received conditioning treatment consisting of fludarabine (30 mg/m^2 i.v. days −8 to −3), busulfan (4 mg/kg p.o. days −6 and −5), and ATG (2.5 mg/kg days −2 and −1) before the infusion of granulocyte-colony stimulating factor-mobilized peripheral blood stem cells from the donors. Prophylaxis for GVHD was cyclosporine A alone starting from day −1. Although patients #37, R07, and #97 obtained complete remission within 2 months after HSCT, R11 had recurrence of ATL lymphoma in the neck 6 months after HSCT. Donor #36 was a HTLV-I carrier, but the other donors were not. The HLA and other clinical characteristics of the patients and donors are summarized in Table 1.

Cell Lines. PBMCs from the donors and recipients isolated on a Ficoll-Hypaque PLUS (Amersham Biosciences, Piscataway, NJ) gradient were partially stored in liquid nitrogen until use and partially used to obtain HTLV-I-infected IL-2-dependent T-cell (ILT) lines and EBV-transformed lymphoblastoid B-cell lines LCL. ILT-#37, ILT-R07, ILT-R11, and ILT-#97were spontaneously HTLV-I-infected T-cell lines originating from pre-HSCT recipients #37, R07, R11, and #97, respectively. To establish these ILT lines, PBMCs were stimulated with 1 μg/ml phytohemagglutinin (PHA)-P (Sigma, St. Louis, MO) after depletion of CD8$^+$ cells using a Dynabeads M450-CD8 (Dynal, Oslo, Norway) and then maintained in RPMI 1640 (Invitrogen-Life Technologies, Inc., Grand Island, NY) containing 10% FCS (Sigma), 10 units/ml recombinant human interleukin (IL)-2 (Shionogi, Osaka, Japan), or 10 ng/ml recombinant human IL-15 (Sigma) at 37°C with 5% CO_2 for over 2 months. An EBV-transformed B-cell line, LCL-#36, was established by maintaining positively separated CD19$^+$ PBMCs from donor #36 in RPMI 1640 with 10% FCS after infection with an EBV-containing culture supernatant of the B95-8 cell line (31). TCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9; Ref. 32), ILT-As-2 (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), ILT-Myj-3 (HLA-A2/A24, B54/B60, Cw1/Cw3, and DR4/DR5; Ref. 21), ILT-Nkz-2 (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and ILT-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are HTLV-I-infected T-cell lines, and LCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9), LCL-As (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), TOK (HLA-A24/-, B52/-, and DR2/-; Ref. 33), LCL-Nkz (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and LCL-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are EBV-transformed B-cell lines. An erythroblastoid cell line, K562 (34), was also used.

Flow Cytometry for Phenotyping and HTLV-I Expression. Cell surface phenotypes were determined using directly FITC-conjugated murine antihuman monoclonal antibodies (mAbs) followed by analysis on a FACSCalibur (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest software (Becton Dickinson). The mAbs used were anti-CD4 (clone: RPA-T4; BD PharMingen), anti-CD8 (clone: RPA-T8; BD PharMingen), anti-CD19 (clone: HIB19; BD PharMingen), and for isotype controls, antimouse IgG1. For detection of intracellular HTLV-I proteins, cells were stained with anti-Tax mAb (Lt-4; Ref. 35) and anti-Gag mAb (GIN-7; Ref. 36) after cell membrane permeabilization. These mAbs were kindly provided by Dr. Yuetsu Tanaka (University of the Ryukyus, Okinawa, Japan).

Induction of HTLV-I-Specific CTL. One million whole PBMCs from post-HSCT patient #37 were stimulated with 1 μg/ml PHA-P and then mixed with the same number of ILT-#37 cells, derived from pre-HSCT patient #37, and pretreated with 1% formaldehyde/PBS. These T cells were maintained in AIM-V medium (Invitrogen-Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 0.5 mg/ml streptomycin, 10% heat-inactivated FCS, and 100 units/ml recombinant human IL-2 with periodic stimulation with formaldehyde-fixed respective ILT cells at 10–14-day intervals. PBMCs from donor #36 and pre-HSCT patient #37 were similarly stimulated with PHA and subsequently with formaldehyde-fixed ILT-#37 in cultures for CTL induction. CTL induced from CD8$^+$ cell-enriched PBMCs of donor #36 were also used in some experiments. In the other ATL cases tested (patients R07, R11, and #97), PHA-stimulated CD8$^+$ cell-enriched PBMCs from each post-HSCT

Table 1 *Summary of clinical status and T-cell immune response of the participants in hematopoietic stem cell transplantation (HSCT)*

Patient's ID	HSCT case	Donor/ recipient	Age (yrs)	Sex	Status	HTLV-I[a] infection	HLA	*In vitro* immune analysis of PBMC[b]			HTLV-I proviral DNA (copies/1000 PBMC)[d]		Clinical outcome after HSCT
								Sampling date (days after HSCT)	Stimulated with	Induction of CTL[c]	Pre-HSCT	Post-HSCT	
#36	Case 1	Donor	57	M	Healthy	+	A2/−, B46/−, Cw1/−, DR8/−	0	ILT-#37	+	Undetectable	N.T.	
#37	Case 1	Recipient	63	M	Acute ATL	+	A2/−, B46/−, Cw1/−, DR8/−	+183	ILT-#37	+	1150.3	Undetectable	Complete remission for more than 24 months
D07	Case 2	Donor	48	F	Healthy	−	A24/A32, B35/B60, DR4/−	N.T.	N.T.	N.T.	N.T.	N.T.	
R07	Case 2	Recipient	51	M	Acute ATL	+	A24/A32, B35/B60, DR4/−	+255	ILT-R07	+	26.7	Undetectable	Complete remission for more than 23 months
D11	Case 3	Donor	52	F	Healthy	−	A2/A26, B35/B61, DR4/6	N.T.	N.T.	N.T.	N.T.	N.T.	
R11	Case 3	Recipient	54	M	Acute ATL	+	A2/A26, B35/B61, DR4/6	+153	ILT-R11	−	440.8	Undetectable	Relapse of lymphoma at 6 months after HSCT
#98	Case 4	Donor	61	M	Healthy	−	A2/A26, B51/, DR4/5	N.T.	N.T.	N.T.	N.T.	N.T.	
#97	Case 4	Recipient	66	F	Acute ATL	+	A2/A26, B51/, DR4/5	+104	ILT-#97	+	3297.2	Undetectable	Died of GVHD at 9 months after HSCT

[a] HTLV-I, human T-cell leukemia virus type I; ATL, adult T-cell leukemia; PBMC, peripheral blood mononuclear cell; N.T., not tested; GVHD, graft-*versus*-host disease; ILT, IL-2-dependent T-cell line.

[b] PBMCs isolated from patients #37, R07, R11, and #97 at the indicated days after HSCT were repeatedly stimulated in culture with formalin-fixed autologous ILT cells established before HSCT, as described in the "Materials and Methods."

[c] Culture in which CTL specific for autologous ILT cells grew is indicated as (+) and that without CTL induction is indicated as (−).

[d] HTLV-I proviruses in the peripheral blood were measured just before and after HSCT at similar dates when *in vitro* immune responses were analysed. A level less than 0.5 copies/1000 cells was undetectable.

patient were stimulated with formaldehyde-fixed autologous ILT cells established before HSCT and otherwise similarly maintained.

Synthetic Peptides. We prepared a total of 38 peptides (9 to 24-mer) to cover the entire sequence of the HTLV-I Tax protein. Some of the peptides were synthesized as described previously (27, 33). All 9-mer peptides were purchased from Hokudo Co. (Hokkaido, Japan). To identify potential HLA-A2- or A24-binding peptides within HTLV-I Tax, a computer-based program, BioInformatics and Molecular Analysis Section (BIMAS),[6] was used as described previously (37–39).

CTL Assay. Cytotoxic activities were measured by 6-h ^{51}Cr-release assay at various E:T cell ratios as described previously (27, 40). Specific cytotoxicity was calculated as [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100%. IFN-γ production by the effector cells was also measured in response to specific antigens. Briefly, effector cells were added to microtiter wells containing some target cells in RPMI 1640 containing 10% FCS at various E:T ratios. After incubation for 18 h at 37°C, 50 μl of supernatant were collected to measure IFN-γ by ELISA (human IFN-γ ELISA kit; Endogen, Woburn, MA) in duplicate assay. Absorbances were detected at 450 nm using a microplate reader (Bio-Rad, Hercules, CA), and data were analyzed with Microplate Manager III software. The limit of detection of the IFN-γ production ELISA assay was 3 pg/ml.

Mapping of CTL Epitopes. HLA-matched EBV-transformed B-cell lines were pulsed with a series of peptides at a final concentration of 10 μM for 1 h at 37°C and then CTLs were added to each well. CTL activity was measured by 6-h ^{51}Cr-release assay or ELISA assay for IFN-γ production in the supernatants after additional incubation for 18 h.

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses WR-p27^{X} (41) containing HTLV-I pX genes and WR-HA without the HTLV-I gene were kindly provided by Dr. Hisatoshi Shida (Hokkaido University, Sapporo, Japan). The recombinant vaccinia viruses-infected cells were used as targets for CTL assay after 16 h infection at a multiplicity of infection of 50 as described previously (21, 27).

Enzyme-Linked Immunospot (ELISPOT) Assays. IFN-γ-producing antigen-specific T cells were counted using IFN-γ-specific ELISPOT assays as described previously (42, 43). A 96-well polyvinylidene difluoride plate (MAIPS4510; Millipore, Bedford, MA) was coated overnight at 4°C with 100 μl of 15 μg/ml anti-IFN-γ mAb, 1-D1K (Mabtech, Nacka, Sweden) in 0.1 M carbonate-bicarbonate buffer (pH 9.6; Sigma). After six washes with PBS, PBMCs were added in triplicate at 5 × 10^4 cells/well in the absence or presence of the same number of stimulator cells or 10 μg/ml peptides in RPMI 1640 containing 10% FCS and incubated overnight at 37°C in 5% CO_2. The next day, cells were removed by washing with PBS/0.05% Tween 20 and biotinylated anti-IFN-γ mAb, and 7-B6-1 biotin (Mabtech) was added 100 μl of 1 μg/ml and left for 2 h at room temperature, followed by incubation with streptavidin-alkaline phosphatase (Mabtech) for an additional 1 h. Individual cytokine-producing cells were detected as dark purple spots after 10-min reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate (Sigma). After washing in tap water to stop color development, colored spots on the dried membranes were counted using a KS-ELISPOT microscopy system (Carl Zeiss, Jena, Germany).

Tetramer Staining. Phycoerythrin-conjugated HLA-A*0201/Tax11-19 (LLFGYPVYV) tetramer was provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University Vaccine Center at Yerkes (Atlanta, GA). Lymphocytes (1 × 10^6) were stained for 30 min at 4°C with Cy-Chrome-conjugated mouse antihuman CD8 mAb (BD PharMingen) and then for an additional 60 min with tetramer (1:100 diluted) at 4°C. After washing in PBS, the cells were fixed in 1% formaldehyde/PBS, and the samples were subjected to two-color analysis on a FACSCalibur using CellQuest software (Becton Dickinson; Ref. 44).

Quantification of HTLV-I Provirus. HTLV-I proviral load in peripheral blood was quantified by real-time PCR on a LightCycler PCR Instrument (Roche Diagnostics, Mannheim, Germany) using primers specific for HTLV-I pX region and β-globin as described elsewhere (7).

[6] Internet address: http://bimas.dcrt.nih.gov/molbio/hla_bind/.

RESULTS

Establishment of HTLV-I-Infected T-Cell Lines from Pre-HSCT ATL Patients. To examine the immune responses of the post-HSCT recipients to hematopoietic cells of pre-HSCT-recipients origin, we established T-cell lines from PHA-stimulated PBMCs from patients #37, R07, R11, and #97 before HSCT by maintaining them for longer than 2 months in the presence of IL-2 or IL-15. The phenotypes of the resulting IL-15-dependent T-cell lines, ILT-#37, ILT-R07, ILT-R11, and ILT-#97, are shown in Fig. 1. ILT-#37, ILT-R07, and ILT-#97 were positive for surface CD4, whereas ILT-R11 was positive for CD8. All of these ILT lines expressed intracellular HTLV-I antigens such as HTLV-I Tax and p19 (Fig. 1). Thus, spontaneously HTLV-I-infected T-cell lines of the pre-HSCT ATL patients were obtained.

Induction of CTL from a Post-HSCT Recipient Reacting with Pre-HSCT HTLV-I-Infected Cells. The T-cell response in the PBMCs of post-HSCT patient #37 to ILT-#37 cells was examined at +183 days after HSCT. The hematopoietic cells of this patient had been completely replaced by those of donor origin in the first 2 months after HSCT as determined by short tandem repeat polymorphism in the genome. Because donor #36 was a HTLV-I carrier, we also examined the T-cell response of donor #36 to ILT-#37. The PBMCs from post-HSCT patient #37 and donor #36 stimulated *in vitro* with 1% formaldehyde/PBS-treated ILT-#37 twice with a 10-day interval in the presence of IL-2 were examined for IFN-γ-producing ability against ILT-#37 and K562 cells at 19 days after initiation of culture. As shown in Fig. 2*A*, significant levels of IFN-γ were produced from post-HSCT #37 in the culture against ILT-#37 but not against K562 cells after an overnight incubation. The PBMCs from

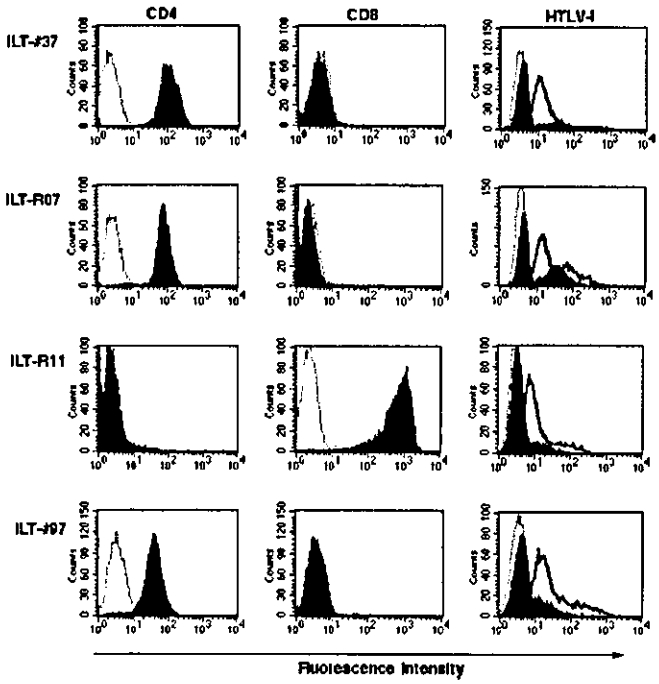

Fig. 1. Surface phenotype and human T-cell leukemia virus type I (HTLV-I) expression in T-cell lines spontaneously established from prehematopoietic stem cell transplantation adult T-cell leukemia (ATL) patients. ILT-#37, ILT-R07, ILT-R11, and ILT-#97 cells that were cultured for >2 months in the presence of 10 ng/ml recombinant human interleukin 15 were stained with FITC-conjugated antibodies to CD4 and CD8 (closed histogram) on the cell surface or intracellularly stained with monoclonal antibodies to HTLV-I Tax (closed histogram), Gag p19 (*solid line*), or control antibody (*broken line*) followed by FITC-conjugated second antibodies and analyzed by flow cytometry. Histograms represent the log of fluorescence (*x* axis) *versus* relative cell number (*y* axis).

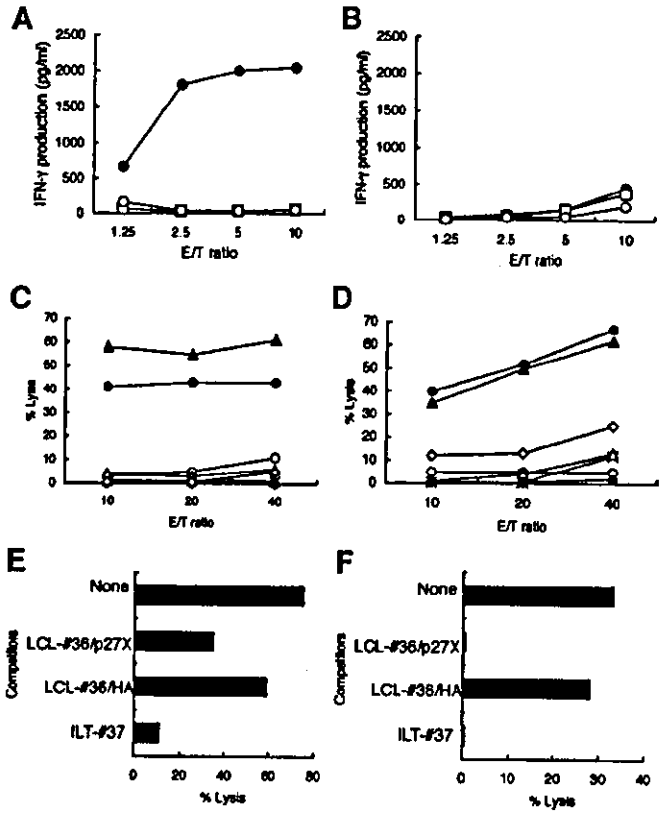

Fig. 2. Induction of and human T-cell leukemia virus type I (HTLV-I)-specific CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37 and donor #36 in response to ILT-#37 cells. Peripheral blood mononuclear cells (PBMCs) from patient #37 (+183 days post-HSCT) (*A*, *C*, and *E*) and donor #36 (*B*, *D*, and *F*) were cultured with periodical stimulations with formalin-fixed ILT-#37 cells, and their IFN-γ-producing ability at 19 days (*A* and *B*) and cytotoxicity at 53 days (*C* and *D*) after initiation of culture were evaluated against various targets cells by ELISA and 6-h ^{51}Cr-release assay, respectively. The target cells used were formalin-fixed ILT-#37 (●), K562 (□), or none (○) in *A* and *B*, HLA-identical ILT-#37 (●), LCL-#36 (○), and PHA-activated PBMCs of pre-HSCT patient #37 (×), HLA-A2- and B46-matched TCL-Kan (▲) and LCL-Kan (△), and HLA-mismatched ILT-As-2 (♦) and LCL-As (◇) in *C* and *D*. *Closed symbols* represent HTLV-I-infected while *open symbols* represent HTLV-I-negative cells. Values represent the mean of IFN-γ concentrations of duplicate assays (*A* and *B*) and percentage of specific lysis of triplicate assays (*C* and *D*). Specificity to HTLV-I Tax of the PBMCs from post-HSCT patient #37 (*E*) and donor #36 (*F*), which had been stimulated five times with formalin-fixed ILT-#37 cells in culture for 90 days, was examined by ^{51}Cr-release assay against radiolabeled ILT-#37 in the presence of unlabelled LCL-#36 cells infected with vaccinia recombinants expressing HTLV-I pX gene products (LCL-#36/p27X) or control vaccinia vector (LCL-#36/HA) or ILT-#37 cells. Both the E:T and competitor-to-target ratios were 30 to 1.

donor #36 cultured for the same period grew more slowly than those from patient #37 and did not significantly produce IFN-γ against stimulation with ILT-#37 (Fig. 2*B*), although the activities increased at later time points of culture (Fig. 2*D*).

HTLV-I Specificity of CTL Induced from a Post-HSCT Recipient and Donor. We then assessed cytotoxicity and specificity of the responder cells expanding in response to stimulation with ILT-#37 cells in the PBMCs from post-HSCT patient #37 and donor #36 at 53 days after initiation of culture. The responder PBMCs from both patient #37 (Fig. 2*C*) and donor #36 (Fig. 2*D*) showed significant levels of cytotoxicity against ILT-#37 but not against PHA-stimulated PBMCs of pre-HSCT #37 patient. This indicated that the main target antigens of these CTL were those preferably expressed on ILT-#37 but not on PHA-stimulated PBMCs, although both of these target cells originated from pre-HSCT #37 patient. Furthermore, these CTLs efficiently killed allogeneic HTLV-I-infected TCL-Kan cells sharing HLA-A2 and B46 but not HLA-mismatched HTLV-I-infected ILT-As-2, EBV-infected LCL-#36 derived from HLA-identical donor #36, LCL-Kan nor LCL-As cells. These results strongly indicated that the CTL line established from post-HSCT #37 patient (CTL-post-HSCT-#37) and donor #36 (CTL-donor-#36) in response to ILT-#37 was specific for HTLV-I antigens.

Recognition of HTLV-I Tax by CTL-Post-HSCT-#37. The target antigens of CTL-post-HSCT-#37 were then analyzed. Because it is known that HTLV-I Tax is a major target antigen for HTLV-I-specific CTLs in HTLV-I-infected individuals, we examined whether HTLV-I Tax was recognized by CTL-post-HSCT-#37. A CTL line similarly induced from donor #36 (CTL-#36) with stimulation by ILT-#37 cells was also examined. The results are shown in Fig. 2, *E* and *F*. Unlabelled LCL-#36 infected with vaccinia recombinants expressing HTLV-I *pX* gene products including Tax (LCL-#36/p27X) significantly inhibited the cytotoxicity of CTL-post-HSCT-#37 against ILT-#37, compared with unlabelled LCL-#36/HA that was infected with control vaccinia vector. Unlabelled ILT-#37 cells more efficiently competed with the cytotoxicity of CTL-post-HSCT-#37 for radiolabeled ILT-#37 (Fig. 2*E*). The cytotoxicity of CTL-#36 for ILT-#37 was almost completely inhibited by unlabeled LCL-#36/p27X as well as ILT-#37 cells (Fig. 2*F*). These findings suggest that the majority of CTL-#36 and a substantial part of CTL-post-HSCT-#37 consisted of HTLV-I Tax-specific CD8$^+$ CTL capable of lysing ILT-#37 cells.

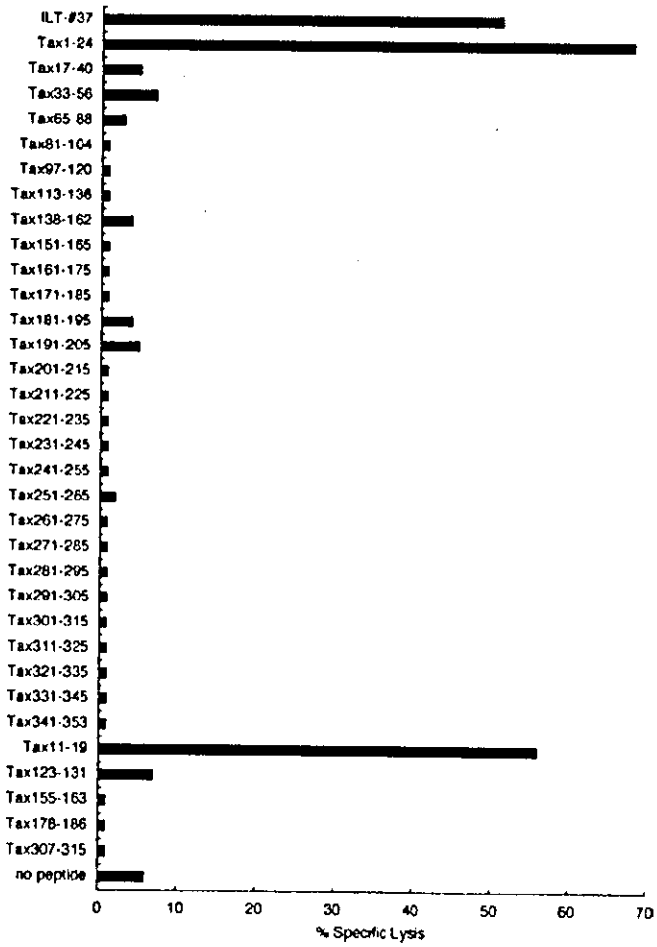

Fig. 3. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37. LCL-#36 cells were pulsed with 10 nM of 33 kinds of 9–24 mer synthetic oligopeptides corresponding to the Tax amino acid sequence, and their susceptibility to CTLs of post-HSCT patient #37 was measured by ^{51}Cr-release assays at an E:T ratio of 10. Values represent the mean of percentage specific lysis of triplicate assays.

Mapping of HTLV-I Tax-Specific CTL Epitope. We additionally examined the epitopes in HTLV-I Tax recognized by CTL-post-HSCT-#37 with a panel of 15–24-mer oligopeptides corresponding to the Tax amino acid sequence, and five 9-mer peptides that were the most probable HLA-A2-restricted Tax epitopes as predicted by a computer program based on the anchor motifs. As shown in Fig. 3, LCL-#36 cells pulsed with the oligopeptides Tax 1-24 (MAHFPGFGQSLLFGYPVYVFGDCV) and Tax 11-19 (LLFGYPVYV) were selectively killed by CTL-post-HSCT-#37, indicating that the major population of HTLV-I Tax-specific CTL in the CTL-post-HSCT-#37 culture was directed to a HLA-A2-restricted Tax 11-19 epitope.

Different HTLV-I-Specific Responses among Pre-HSCT Patient, Post-HSCT Patient, and Donor. We next investigated whether there are any qualitative or quantitative differences in HTLV-I-specific CTL responses among pre-HSCT #37, post-HSCT #37, and donor #36. Unlike the PBMCs of post-HSCT #37, pre-HSCT PBMCs failed to multiply in culture when stimulated with ILT-#37 cells in the presence of IL-2 and could not be maintained for >7 weeks. The cytotoxic ability of this cell line at 40 days after initiation of culture was examined and compared with similarly cultured PBMCs from post-HSCT #37 and donor #36 at 41 days of culture. As shown in Fig. 4*A*, the PBMC cultures from post-HSCT #37 and donor #36, but not from pre-HSCT #37 patient, exhibited significant levels of HTLV-I-specific cytotoxicity.

We also stained these cultured PBMCs with phycoerythrin-conjugated HLA-A*0201/Tax11-19 tetramer and Cy-chrome-conjugated mAb to CD8. As shown in Fig. 4*B*, the PBMC culture from pre-HSCT #37 patient mostly consisted of $CD8^-$, $tetramer^-$ cells, consistent with the results of cytotoxicity assay shown above. In contrast, in the PBMC culture from post-HSCT #37 patient, 63% of the cells were $CD8^+$, HLA-A*0201/Tax11-19^+ (Fig. 4*B*), indicating oligoclonal expansion of CTLs directed to the single epitope in this culture. In the PBMCs from donor #36, which were cultured for the same period, the proportion of HLA-A*0201/Tax11-19^+ cells was 1%. These observations indicated that HTLV-I-specific CTL response in the patient #37 was strongly activated after HSCT in an extremely selective manner distinct from that in the HTLV-I-carrying donor #36.

Induction of HTLV-I-Specific CTL after HSCT from HTLV-I-Negative Donors. T-cell immune responses of the other three ATL patients, R07, R11, and #97, after HSCT from HTLV-I-negative HLA-identical sibling donors were similarly investigated *in vitro* against 1% formaldehyde/PBS-treated autologous ILT cells established before HSCT. Although all of these post-HSCT patients were in complete remission when tested, patient R11 had recurrence of ATL 6 months after HSCT (Table 1). Patient #97 had chronic GVHD. In response to stimulation with autologous ILT cells, PBMCs isolated from patients R07 and #97 at +255 and +104 days after HSCT, respectively, proliferated well in culture as was similarly observed in PBMCs of post-HSCT patient #37. However, PBMCs isolated from patient R11 at +153 days after HSCT neither grew *in vitro* nor exhibited cytotoxicity against stimulation with autologous ILT-R11 cells. Because ILT-R11 cells expressed HTLV-I antigens (Fig. 1) and were susceptible to HLA-A2-restricted Tax-specific CTL (data not shown), the unresponsiveness of the PBMCs from post-HSCT patient R11 could not be due to the absence of specific antigens.

The specificities of the responding $CD8^+$ PBMC from post-HSCT patients R07, R11, and #97, in cultures that were stimulated with ILT-R07, ILT-R11, or ILT-#97, respectively, at 14-day intervals, are shown in Table 2. At 21–24 days after initiation of culture, $CD8^+$ PBMCs from patient R07 produced significant levels of IFN-γ in response to overnight incubation with autologous ILT-R07 cells, allogeneic HTLV-I-infected ILT-Myj-3 cells that shared HLA-A24, B60, and DR4, ILT-As-2 that shared HLA-A24 but not to EBV-infected TOK cells that shared HLA-A24. Cytotoxicity assays at later time points (63 days of culture) confirmed that this culture contained HLA-A24-restricted HTLV-I-specific CTL mainly recognizing HTLV-I *pX* gene products as they killed EBV-transformed TOK cells infected with vaccinia recombinants expressing HTLV-I *pX* gene products (TOK/p27X) more significantly than the ones expressing the HTLV-I envelope (TOK/pEnv) or HTLV-I core (TOK/pGag). $CD8^+$ PBMC culture from post-HSCT patient #97 stimulated with autologous ILT-#97 cells showed HTLV-I-specific IFN-γ production and cytolysis against ILT-#97 and allogeneic HTLV-I-infected ILT-Har cells sharing HLA-A2 and B51 but not with EBV-transformed LCL-Har cells from the same donor (Table 2). This HTLV-I-specific cell population present in the PBMC culture from post-HSCT patient #97 was, however, lost during a longer period of culture.

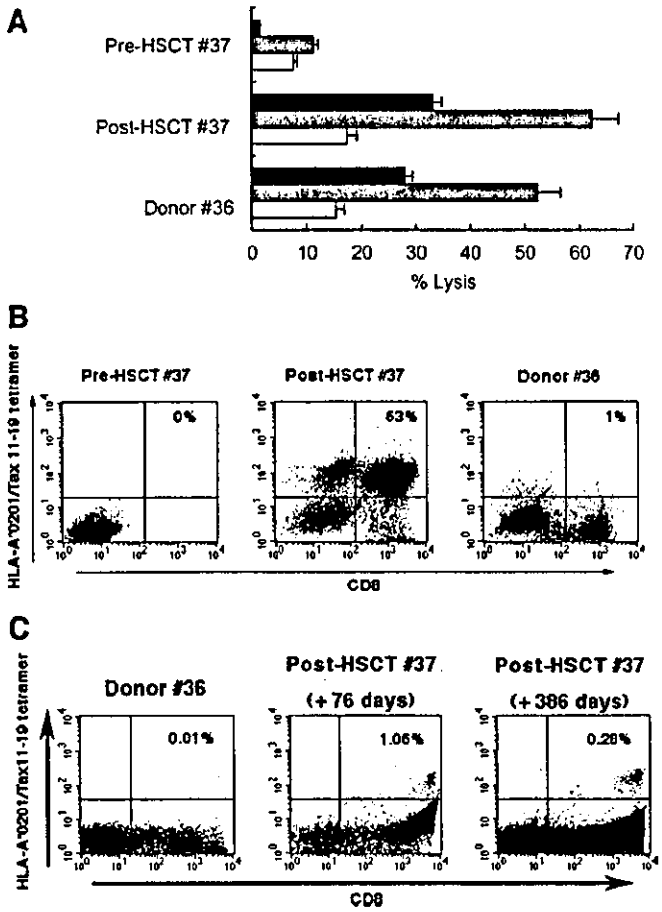

Fig. 4. Selective expansion of Tax11-19-specific CTL in peripheral blood mononuclear cell (PBMC) cultures from patient #37 after hematopoietic stem cell transplantation (HSCT). *A*, PBMCs from pre-HSCT patient #37 at 40 days of culture which were stimulated with formalin-fixed ILT-#37 cells two times, and those from post-HSCT patient #37 and donor #36 at day 41 after three stimulations were examined for cytotoxicity against ILT-#37 (■), TCL-Kan (▩), and LCL-Kan (□) cells at an E:T ratio of 30. Values represent the mean of triplicate assays. *B*, flow cytometric analysis of HLA-A*0201/Tax11-19 tetramer-binding $CD8^+$ T cells in the indicated PBMC cultures stimulated with ILT-#37. The PBMC cultures from post-HSCT patient #37 (*middle*) and donor #36 (*right*) were used at day 46 of culture, whereas those from pre-HSCT patient #37 (*left*) were used at day 36 because they failed to grow long term. Tetramer specificity was confirmed by staining a human T-cell leukemia virus type I Tax11-19-specific cell line, Tc-Myj (Ref. 21; data not shown). Numbers in *top right corners* indicate percentages of PBMCs bound to the tetramer. A total of 100,000 events was collected in each case. *C*, kinetics of human T-cell leukemia virus type I Tax 11-19-specific $CD8^+$ cells in uncultured PBMCs in post-HSCT patient #37. Uncultured cryopreserved PBMCs isolated from donor #36 (*left*) or from post-HSCT patient #37 at +76 days (*middle*) and +386 days (*right*) after HSCT were stained with phycoerythrin-conjugated HLA-A*0201/Tax 11-19 tetramer. The percentages of $CD8^+$ and HLA-A*0201/Tax 1--19 $tetramer^+$ cells in the PBMCs are indicated in the *top right quadrants*.

Table 2 *Human T-cell leukemia virus (HTLV-I)-specific CTL responses of peripheral blood mononuclear cell cultures from post-hematopoietic stem cell transplantation (HSCT) patients R07, R11, and 97*[a]

Patient ID (days after HSCT)	PBMC culture period	Stimulator	Target cells for immunoassays			IFN-γ (pg/ml)[b]	Cytolysis (%)[c]
			Cell lines	HTLV-I antigen	Shared HLA alleles with patient		
R07 (+255)	Day 21	ILT-R07	IRT-R07	Positive	Identical	1090 ± 67	N.T.[d]
			ILT-As-2	Positive	A24	866 ± 117	N.T.
			TOK	Negative	A24	160 ± 7	N.T.
			None			36 ± 12	N.T.
	Day 24	ILT-R07	ILT-R07	Positive	Identical	778 ± 55	N.T.
			ILT-Myj-3	Positive	A24, B60, DR4	355 ± 0	N.T.
			TOK	Negative	A24	62 ± 9	N.T.
			None			25 ± 19	N.T.
	Day 63	ILT-R07	ILT-R07	Positive	Identical	N.T.	39 ± 9
			ILT-As-2	Positive	A24	N.T.	48 ± 5
			TOK	Negative	A24	N.T.	5 ± 6
			TOK/p27X[e]	pX products	A24	N.T.	27 ± 11
			TOK/pEnv[e]	Envelope	A24	N.T.	11 ± 4
			TOK/pGag[e]	Core	A24	N.T.	6 ± 10
R11 (+153)	Day 25	ILT-R11	ILT-R11[f]	Positive	Identical	11 ± 1	N.T.
			ILT-Nkz-2	Positive	A2, A26	12 ± 2	N.T.
			LCL-Nkz	Negative	A2, A26	19 ± 6	N.T.
			None	Negative		14 ± 0	N.T.
#97 (+104)	Day 21	ILT-#97	ILT-#97	Positive	Identical	477 ± 34	N.T.
			ILT-Har	Positive	A2, B51, DR4	415 ± 2	N.T.
			LCL-Har	Negative	A2, B51, DR4	104 ± 15	N.T.
			None			63 ± 4	N.T.
			None			63 ± 4	N.T.
	Day 50	ILT-#97	ILT-#97	Positive	Identical	128 ± 36	29 ± 2
			ILT-Har	Positive	A2, B51, DR4	111 ± 10	23 ± 3
			LCL-Har	Negative	A2, B51, DR4	10 ± 1	16 ± 3
			None			1 ± 0	N.T.

[a] PBMCs isolated from patients R07, R11, and #97 were cultured for the indicated periods with stimulations with formalin-fixed autologous ILT-R07, ILT-R11, or ILT-#97 cells, respectively; then IFN-γ production or cytotoxicity of these PBMCs were examined against the various target cells indicated.
[b] IFN-γ producing responses after overnight incubation with various target cells indicated at an E:T ratio of 5 was shown as the mean ± SD of duplicate determinations by ELISA.
[c] Specific cytolysis at an E:T ratio of 40 measured by 6-h ^{51}Cr-release assays was shown as the mean ± SD of triplicate determinations.
[d] N.T., not tested.
[e] TOK cells infected with recombinant vaccinia viruses expressing HTLV-I pX products (TOK/p27X), envelope (TOK/pEnv), and core (TOK/pGag) antigens were used as targets.
[f] ILT-R11 target cells used were confirmed for their susceptibility to HLA-A2-restricted Tax-specific CTL induced from post-HSCT patient #37 by 6-h ^{51}Cr-release assay (53% lysis at an E:T ratio of 20).

Recognition of a Single HLA-A24-Restricted Tax Epitope by CTL Induced from Post-HSCT Patient R07. Subsequently, epitope mapping of the CTL from post-HSCT R07 was performed. The result is shown in Fig. 5. Of the panel of 15–24-mer oligopeptides of Tax and five 9-mer oligopeptides, the most probable HLA-A24-restricted epitopes as predicted by a computer program, Tax 301-315 (SFHSLHLLFEEYTNI) and Tax 301-309 (SFHSLHLLF), were selectively reacted with the responder cells. These observations indicated that HTLV-I-specific CTL response to selective Tax epitopes was induced from patient R07 after HSCT as similarly observed in the case of post-HSCT patient #37.

***Ex Vivo* Analysis on Tax-Specific CTL Expansion in Post-HSCT Patients.** The almost exclusive expansions of Tax 11-19-specific CTL and Tax 301-309-specific CTL in PBMCs from post-HSCT patients 337 and R07, respectively, were observed in *in vitro* culture stimulated with pre-HSCT autologous ILT cells as shown above. Finally, we examined whether Tax-specific CTL response was also augmented *in vivo* by using uncultured PBMCs from these patients.

For patient #37, fluorescence-activated cell sorting analysis on frozen stored uncultured PBMCs was performed after staining with the HLA-A*0201/Tax11-19$^+$ tetramer. Because the hematopoietic chimerism in patient #37 had shifted completely to the donor type by 2 months after HCST, we compared the number of Tax11-19-specific CTL in uncultured PBMCs taken from patient #37 at +76 days after HSCT to that from donor #36. As shown in Fig. 4*C*, >1% of CD8$^+$ cells in uncultured PBMCs from patient #37 (+76 days after HSCT) were stained with the HLA-A*0201/Tax11-19 tetramer, whereas only 0.01% of CD8$^+$ PBMCs from donor #36 bound the tetramer. The proportion of peripheral Tax11-19-specific CTL in the PBMCs of post-HCST patient #37 decreased to 0.28% at a later time point (+386 days after HCST) but was still much higher than that in donor #36. This indicated that the Tax11-19-specific CTL population of donor-origin was activated and expanded to a high level in the recipient after HSCT and then gradually decreased.

For patient R07, uncultured PBMCs before and after HSCT (+255 days) that had been stored frozen were subjected to ELISPOT assay for IFN-γ production after overnight stimulation with ILT-R07 or Tax 301-309 peptide (Table 3). The number of IFN-γ-producing cells was significantly higher in post-HSCT PBMCs than in pre-HSCT PBMCs from patient R07. The number of IFN-γ-producing cells responding to Tax 301-309 peptide was also elevated in the post-HSCT PBMCs in a lesser degree than ILT-R07-responding cells. These PBMCs did not react with control peptide Tax 11-19. Although PBMC samples from patient R07 at any earlier date after HSCT or the donor were not available, these results suggested that CTLs reacting with ILT-R07 and Tax 301-309 in patient R07 were activated *in vivo* after HSCT and were still detectable at +255 days after HSCT.

DISCUSSION

In the present study, the cellular immune responses in ATL patients after nonmyeloablative HSCT from HLA-identical siblings against pre-HSCT T-cell lines spontaneously infected with HTLV-I were investigated. We demonstrated that HTLV-I-specific CTLs were induced in the PBMCs from three patients with complete remission and that CTLs induced from two of these patients showed strong activity directed against a limited number of Tax epitopes. The donor in the first case of HSCT was an HTLV-I carrier, but the other donors were negative for HTLV-I. Therefore, the HTLV-I-specific CTL response

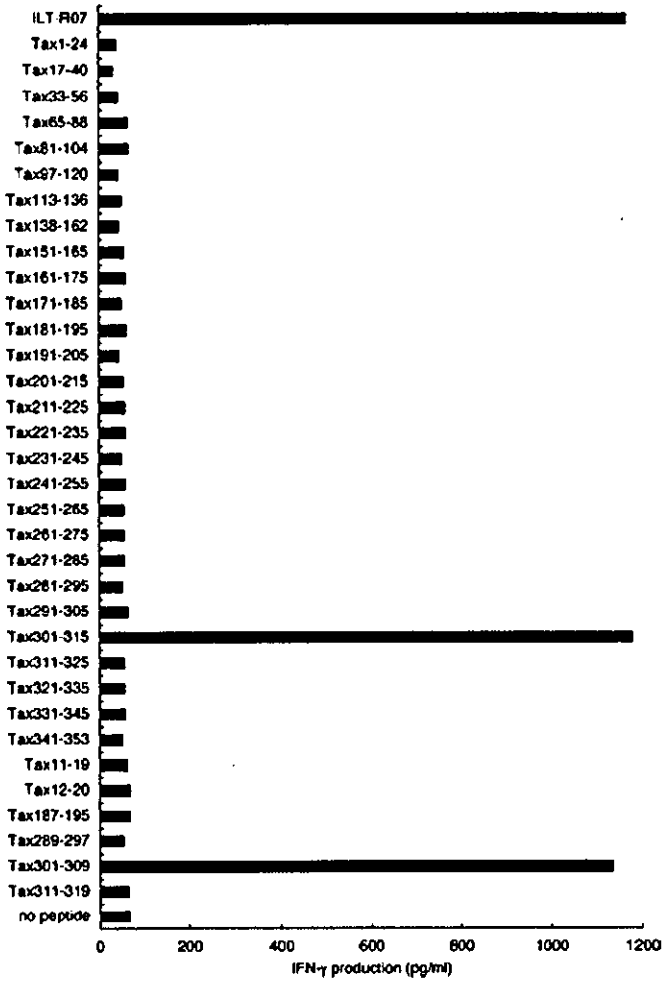

Fig. 5. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by the CTLs induced from post-hematopoietic stem cell transplantation patient R07. $CD8^+$ cell-enriched peripheral blood mononuclear cell that had been cultured for 32 days with three stimulations with formalin-fixed ILT-R07 cells were mixed with HLA-$A24^+$ EBV-transformed B-cell line, TOK, pulsed with a series of 33 synthetic oligo-peptides for Tax at an E:T ratio of 8, and IFN-γ in the supernatants after 18 h of incubation was measured by ELISA assay. Values represent the mean of duplicate assays.

in post-HSCT ATL patients cannot be explained by the transfer of memory CTLs from the donor but must be a result of the new immune response by reconstituted donor-derived T cells after HSCT against HTLV-I antigen-presenting cells present in the ATL patients.

It is a striking phenomenon that >60% of CTL induced from post-HSCT patient #37 were stained with the HLA-A*0201/Tax11-19 tetramer. Such an almost exclusive expansion of Tax11-19-specific CTL was not merely caused by *in vitro* selection because a similarly cultured CTL line from HTLV-I-carrying HLA-identical donor #36 contained a much smaller number of Tax11-19-specific cells (Fig. 4*B*). Although CTLs induced from donor #36 also recognize Tax as shown in Fig. 2*F*, they were directed to multiple heterologous Tax epitopes, including Tax 11-19 (data not shown). It is intriguing that oligoclonal expansion of the HTLV-I Tax11-19-specific CTL response has been observed in HTLV-I-associated myelopathy/Tropical spastic paraparesis patients whose viral load is generally high (33, 45, 46), suggesting that the pattern of HTLV-I-specific response observed in the post-HSCT ATL patients in the present study might be due to abundant antigen presentation *in vivo*. CTLs induced from patient R07 who received HSCT from an uninfected donor also exhibited preference for a limited epitope, Tax 301-309 epitope restricted by HLA-A24, supporting the notion that the selective CTL responses are more likely to be influenced by the *in vivo* conditions of the recipient rather than those of the donor.

Ex vivo analyses revealed that the number of cells stained with the HLA-A*0201/Tax11-19 tetramer in uncultured PBMCs was 100 times greater in post-HSCT patient #37 at +76 days after HSCT than in donor #36 (Fig. 4*C*). The number of tetramer-bound cells in the PBMCs of this patient then gradually decreased, presumably as the viral load decreased. Similarly, in uncultured PBMCs from patient R07, elevation of the number of IFN-γ-producing cells against ILT-R07 or Tax 301-309 peptide was shown by ELISPOT assay, although the only sample available from this patient was taken a long time after HSCT (+255 days; Table 3). These findings indicated that the Tax-specific CTLs of these ATL patients selectively activated not only *in vitro* but also *in vivo* after HSCT.

Proviral loads before HSCT varied among patients and decreased down to undetectable levels after HSCT in all patients tested, including R11 who did not show a CTL response. This suggests that the spread of HTLV-I *in vivo* in the reconstituted immune system after HSCT may not be as effective as primary HTLV-I-infection, even in the absence of CTL. The reason for the unresponsiveness of patient R11 against ILT-R11 remains to be clarified.

It has been reported that most ATL cases who obtained complete remission after HSCT were associated with GVHD (7), indicating that graft-*versus*-host (GVH) responses contribute to GVL effects for ATL. In patient #37 in the present study, ATL cells increased at +4 weeks after HSCT but decreased again on withdrawing cyclosporine A, although some episodes of grade 2 GVHD occurred. In patient R07, acute GVHD of the stomach (grade 2) and no chronic GVHD were observed after transplantation, but the clinical course was essentially uneventful without recurrence of ATL. Acute and chronic GVHD in patients #37 and R07 were eventually controlled. In patient R11, who was in complete remission before HSCT and later had recurrence of ATL 6 months after HSCT, a transient acute GVHD of the stomach but no chronic GVHD was observed. In patient #97, no acute but some chronic GVHD in the skin, liver, and lungs have been observed. Complete remission has been sustained for >18 months for patients #37 and R07 and 7 months for patient #97. Successful induction of HTLV-I-specific CTL only from the patients who sustained complete remission implies that HTLV-I-specific CTL as well as effector cells for GVHD might contribute to the GVL effects. Previous findings such as cytotoxicity of HTLV-I Tax-specific CTL against ATL cells *in vitro* (19, 29) and antitumor effects demonstrated in animal models of ATL (27, 40) support this notion. The contribution of Tax-specific T-cell responses to the anti-ATL effects remains to be clarified, most likely by a clinical trial of Tax-directed immunotherapy for ATL in the future.

Several mHA that have been suggested to be involved in GVHD

Table 3 *Ex vivo IFN-γ-producing response of peripheral blood mononuclear cells (PBMCs) from patient R07 before and after hematopoietic stem cell transplantation (HSCT) in response to ILT-R07 or Tax peptides*[a]

Stimulator	IFN-γ-producing SFC/5 × 10^4 PBMC[b]	
	Pre-HSCT R07	Post-HSCT R07
ILT-R07	5 ± 6	44 ± 16
Tax301-309 (SFHSLHLLF)	1 ± 1	5 ± 1
Tax11-19 (LLFGYPVYV)	1 ± 1	0 ± 1
Medium	0 ± 0	0 ± 0

[a] Uncultured PBMCs directly thawed from frozen stocks from patient R07 before and after (+255 days) HSCT were subjected to IFN-γ-enzyme-linked immunospot assay after overnight incubation with formalin-fixed ILT-R07, synthetic oligopeptides Tax 301-309, and Tax 11-19, or control medium at a concentration of 5 × 10^4/well as described in "Materials and Methods." Values represent the mean ± SD of triplicate assays.

[b] Results of IFN-γ-enzyme-linked immunospot assay are expressed as spot-forming cells (SFCs)/5 × 10^4 PBMCs.

(8–10, 12, 13) are candidates for GVL targets. In the present study, the T-cell lines used as stimulators (ILT-#37, ILT-R07, ILT-R11, and ILT-#97) originating from the ATL patients before HSCT possessed antigens of recipient-origin as well as HTLV-I antigens. Therefore, it is likely that these cells express GVH target antigens to induce GVH-effector cells *in vitro* from the PBMCs of post-HSCT patients when used as a stimulator. In fact, the cytotoxicity of post-HSCT-#37 CTL against ILT-#37 was not completely competed by Tax-expressing cells (Fig. 2*E*), indicating the presence of CTL populations recognizing other antigens such as mHA. In the PBMC culture from post-HSCT patient #97 with chronic GVHD, HTLV-I-specific CTLs were induced at an early culture period (Table 2), but additional culture resulted in expansion of another CTL population capable of killing ILT-#97 but not directed against HTLV-I Tax. The target antigens of these CTL are currently under investigation. These observations suggest that HTLV-I and some other antigens associated with GVH responses could act as strong target antigens for post-HSCT CTL responses.

We and others (33, 47) previously demonstrated that Tax 11-19 is one of the major target epitopes of HLA-A2-restricted HTLV-I-specific CTLs in HTLV-I-infected individuals. In the present study, Tax 11-19 was also the major CTL epitope, especially in post-HSCT patient #37. In the second HSCT case, the CTL line of post-HSCT patient R07, induced by only two stimulations with ILT-R07 cells, almost exclusively recognized the Tax 301-309 epitope restricted by HLA-A24, which is one of the most common HLA alleles in Japanese individuals. We assume that Tax 301-309 is one of the major epitopes for HLA-A24. These major CTL epitopes could be candidate antigens for potential immunotherapy for ATL.

In conclusion, a new balance between host immunity and HTLV-I-infected cells was established after allogeneic HSCT into ATL patients from HLA-identical donors. The phenomenon of *in vitro* induction and proliferation of CTL specific for selected Tax epitopes observed in these recipients is very similar to that in HTLV-I-associated myelopathy/Tropical spastic paraparesis patients. In this sense, allogeneic HSCT converted HTLV-I-specific T-cell immunity in the recipients from one extreme to the other in which host immunity could control the malignant expansion of HTLV-I-infected cells *in vivo*.

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Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation

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Summary:

To evaluate the clinical significance of a cytomegalovirus (CMV) antigenemia assay in the prediction and diagnosis of CMV gastrointestinal (CMV-GI) disease after hematopoietic stem cell transplantation (HSCT), 19 allogeneic HSCT recipients developing CMV-GI disease were retrospectively reviewed. All patients were monitored by a CMV antigenemia assay, at least once weekly after engraftment. The median onset of CMV-GI disease occurred 31 days post transplant (range: 19–62). Only four of 19 patients (21%) developed a positive CMV antigenemia test before developing CMV-GI diseases. Although all 19 patients subsequently developed positive CMV antigenemia tests during their clinical courses, the values remained at a low-level in nine (47%) patients. Among the 14 patients in whom results of real-time polymerase chain reaction (PCR) were available, seven (50%) yielded positive results of real-time PCR before developing CMV-GI disease. In contrast to the values of CMV antigenemia, all 14 patients exclusively yielded high viral loads (median: 2.8×10^4 copies/ml plasma). We conclude that CMV antigenemia testing has limited value in prediction or early diagnosis of CMV-GI disease, and that real-time PCR could have a more diagnostic significance.

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Cytomegalovirus (CMV) disease remains a serious infectious complication that causes morbidity and mortality in recipients of allogeneic hematopoietic stem cell transplantation (HSCT).[1] Although the incidence of CMV disease has decreased over time, prophylactic therapy (the administration of antiviral agents to all HSCT recipients), largely due to its toxicities, resulted in no survival benefit.[2–4] Subsequently, many investigators focused on pre-emptive therapy, treating only patients with proven CMV infection or reactivation.[4–8] Rapid, sensitive, quantitative, and reliable methods, which can detect CMV reactivation before CMV disease develops, are essential for the refinement of pre-emptive therapy. The CMV antigenemia assay is one of the most widely used methods to detect CMV reactivation in a variety of clinical settings,[9,10] and CMV antigenemia-based pre-emptive therapy has been shown to effectively prevent the occurrence of CMV pneumonitis.[4,7,8] However, several reports, including ours, have suggested that pre-emptive therapy based on CMV antigenemia allowed the development of CMV diseases other than pneumonitis, including gastrointestinal (GI) disease, retinitis, and hepatitis, in a small number of patients.[4,7,8] In the present study, we focused on CMV-GI disease in allogeneic HSCT recipients, and evaluated the predictive and diagnostic values of the CMV antigenemia assay in CMV-GI disease in comparison with the efficacy of quantitative real-time polymerase chain reaction (PCR).

Patients and methods

Patients and transplant procedures

Among the recipients of allogeneic bone marrow or peripheral blood stem cells at five transplant centers, those who developed histologically diagnosed CMV-GI disease were enrolled into this study. Clinical data from each patient were collected from the medical records and reviewed retrospectively.

CMV antigenemia assay

All patients enrolled into this study were monitored at least once a week for CMV reactivation with a CMV antigenemia assay after engraftment. CMV antigenemia assay using the monoclonal antibodies C10/C11 (Biotest, Dreieich, Germany) or HRP-C7 (Teijin, Tokyo, Japan) was performed as previously reported.[7–10] High-level CMV antigenemia was defined as 10 or more positive cells per applied 150 000 granulocytes for C10/11, and 10 or more positive cells per 50 000 granulocytes for HRP-C7.

Real-time PCR

Real-time PCR was performed basically as previously reported.[11,12] In brief, DNA extracted from 100 μl of plasma, using a QIAamp Blood Mini Kit (QIAGEN, Valencia, CA, USA), was subjected to PCR. The sequences of PCR primers and the probe were selected from the US17 region of CMV AD169. The Taqman probe selected between the primers was dual-labeled with 6-carboxyfluorescein (FAM) and with 6-carboxy-teremethyl-rhodamine (TAMRA). PCR reaction was performed by using TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA). CMV quantification was carried out with a serially diluted standard ranging from 10 to 1×10^7 copies/well, and the gene copy numbers were calculated by Sequence Detection System ver.1.6.3. software (PE Biosystems). The minimum detection level was 20 copies/100μl of plasma. A high-level copy number was defined as 1000 or more copies of CMV-DNA per ml of plasma.

Definition of CMV-GI disease

CMV-GI disease was defined as gastrointestinal symptoms such as diarrhea, nausea, and epigastralgia, accompanied by histologic demonstration of CMV on biopsy materials obtained by endoscopy.

Results

Study population

Nineteen patients with histologically diagnosed CMV-GI disease were collected, and then subjected to retrospective review. Patient characteristics are shown in Table 1. Except for one case of aplastic anemia, all these patients had undergone allogeneic HSCT for the treatment of hematological malignancies. Conditioning regimens included total body irradiation (TBI)-based regimens ($n=13$), busulfan-based regimens ($n=3$), a fludarabine-based regimen ($n=2$), and total lymphoid irradiation plus cyclophosphamide ($n=1$). A total of 10 patients received a transplant from an unrelated donor. All but one patient developed acute graft-versus-host disease (GVHD) of grade II–IV, and received high-dose glucocorticoids in addition to the ongoing cyclosporin A or tacrolimus. No patient developed CMV disease in any organs outside the gastrointestinal tract.

Onset of CMV-GI disease and CMV antigenemia/real-time PCR

The median onset of CMV-GI disease was 31 days post transplant (range: days 19–62). Only four (21%) of the 19 patients yielded positive CMV antigenemia test before developing CMV-GI disease. Although these four patients were pre-emptively treated with ganciclovir, they subsequently developed CMV-GI disease. The remaining 15 patients developed CMV-GI disease before CMV antigenemia was identified, and were therapeutically

Table 1 Patient characteristics

Case	*Gender*	*Age*	*Disease*	*Donor*	*Conditioning*	*GVHD prophylaxis*	*CMV serology recipient/donor*	*aGVHD*
1	Male	26	AA	Related	TLI-regimen	CSA + MTX	Pos/Pos	III
2	Female	54	ALL	Related	TBI-regimen	CSA + MTX	Pos/Pos	I
3	Male	36	ALL	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	II
4	Male	39	CML	Unrelated	BU-CY-TLI	CSA + MTX	Pos/Pos	II
5	Female	42	MDS	Related	BU-CY	CSA + MTX	Pos/Pos	II
6	Female	54	ATL	Related	TBI-regimen	CSA + MTX	Pos/Pos	III
7	Male	22	ALL	Unrelated	TBI-regimen	CSA	Pos/Pos	III
8	Male	50	CML	Unrelated	BU-CY	Tacrolimus + MTX	Pos/Pos	II
9	Male	50	MDS	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	IV
10	Male	20	ALL	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	II
11	Female	44	MDS	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	II
12	Male	24	AML	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	III
13	Male	46	CML	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
14	Male	41	AML	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
15	Male	30	ALL	Unrelated	TBI-regimen	Tacrolimus + MTX	Pos/Pos	II
16	Female	25	ALL	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
17	Male	22	ALL	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
18	Female	37	MM	Related	FLU-Melphalan	CSA + MTX	Pos/Pos	III
19	Female	53	NHL	Unrelated	FLU-Melphalan	Tacrolimus + MTX	Pos/Pos	II

CMV = cytomegalovirus; aGVHD = acute graft-versus-host disease: AA = aplastic anemia; ALL = acute lymphoblastic leukemia; CML = chronic myeloid leukemia; MDS = myelodysplastic syndrome; ATL = adult T-cell leukemia; AML = acute myeloid leukemia; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma; TLI = total lymphoid irradiation; TBI = total body irradiation; BU = busulfan; CY = cyclophosphamide; FLU = fludarabine; CSA = cyclosporin A.

treated with ganciclovir. Of these patients, however, all became CMV antigenemia positive after the diagnosis of CMV-GI disease. Among the 14 patients with evaluable real-time PCR results, seven (50%) yielded positive results before the development of CMV-GI disease. As well as positivity for CMV antigenemia, the remaining seven patients yielded positive real-time PCR results after the diagnosis of CMV-GI disease.

Quantitative significance of CMV antigenemia/real-time PCR in CMV-GI disease

The quantitative significance of CMV antigenemia and real-time PCR in association with CMV-GI disease was evaluated by the maximum values of each assay (Table 2). The values of CMV antigenemia remained at low levels in nine (47%) of the 19 patients, whereas maximum viral load evaluated by real-time PCR was high in all patients.

Discussion

The introduction of the CMV antigenemia assay, a sensitive and quantitative assay to detect viral reactivation, has contributed to the successful outcome of preemptive therapy for CMV disease in allogeneic HSCT recipients. However, we and other investigators have pointed out that the assay has some disadvantages.[7,12,13] For example, it is relatively time-consuming, involves subjective components in the interpretation of slides, and requires sufficient granulocytes. Furthermore, despite its high sensitivity in detecting viral reactivation before the onset of CMV pneumonitis, CMV antigenemia does not necessarily precede the onset of other CMV diseases; thus CMV-GI disease or CMV retinitis can occur in allogeneic HSCT recipients receiving CMV antigenemia-guided pre-emptive therapy.[4–7] In this retrospective study, we evaluated the clinical significance of CMV antigenemia in predicting and diagnosing CMV-GI disease in allogeneic HSCT recipients. Among 19 cases of histologically diagnosed CMV-GI disease, CMV antigenemia preceded the onset of disease in only four (21%) patients, although the remaining patients subsequently became CMV antigenemia positive after developing CMV-GI disease. These findings strongly suggested that CMV antigenemia is of little clinical value in predicting and diagnosing CMV-GI disease. In addition, these four patients developed CMV-GI disease after starting pre-emptive therapy, suggesting that CMV antigenemia can confirm viral reactivation before CMV-GI disease develops in some cases, but not early enough for therapy to be preventive.

In addition to the CMV antigenemia assay, PCR is a useful technique for detecting CMV reactivation.[13,14] There have been several reports of using PCR for pre-emptive therapy in allogeneic HSCT recipients.[15–17] Although it has been reported that PCR is highly sensitive in the detection of viral reactivation and that PCR-guided pre-emptive therapy is effective in preventing the development of CMV diseases, conventional PCR, as compared to the CMV antigenemia assay, is of less clinical use because of lack of quantification. The quantitative real-time PCR is in clinical use, and has become recognized as one of the standard assays for evaluating viral reactivation both qualitatively and quantitatively.[12,13] In this study, we investigated the clinical significance of real-time PCR and that of the CMV antigenemia assay in assessing viral reactivation in cases of CMV-GI disease. Comparatively, PCR was more effective in predicting and diagnosing CMV-GI disease, although its incidence of preceding disease onset was only 50%. Thus, even real-time PCR, which is considered more sensitive than the CMV antigenemia assay, could not satisfactorily detect CMV reactivation before the onset of CMV-GI

Table 2 CMV-GI disease and the results of CMV antigenemia/real-time PCR

Case	*Onset of CMV-GI disease*	*Antibodies for CMV Ag*	*First day of CMV Ag +tive*	*Maximum value of CMV Ag*	*First day of PCR+tive*	*Maximum value or PCR*
1	30	HRP-C7	38	96 (H)	ND	ND
2	49	HRP-C7	31[a]	9 (L)	ND	ND
3	39	HRP-C7	45	52 (H)	ND	ND
4	29	HRP-C7	32	124 (H)	ND	ND
5	62	HRP-C7	69	2 (L)	ND	ND
6	30	HRP-C7	16[a]	44 (H)	12[a]	2.0 × 104(H)
7	51	HRP-C7	28[a]	31 (H)	34[a]	5.0 × 104(H)
8	26	HRP-C7	26	10 (H)	19[a]	3.0 × 103(H)
9	34	C 10/11	38	141 (H)	26[a]	1.9 × 105(H)
10	20	C 10/11	14[a]	1 (L)	14[a]	1.1 × 104(H)
11	31	C 10/11	45	22 (H)	31	4.5 × 104(H)
12	27	C 10/11	27	6 (L)	27	1.9 × 104(H)
13	30	C 10/11	34	1 (L)	27[a]	3.7 × 103(H)
14	31	C 10/11	38	117 (H)	27[a]	2.6 × 105(H)
15	19	C 10/11	24	7 (L)	19	2.8 × 104(H)
16	42	C 10/11	45	7 (L)	45	1.2 × 105(H)
17	20	C 10/11	27	2 (L)	27	6.0 × 103(H)
18	39	C 10/11	45	54 (H)	39	7.0 × 105(H)
19	31	C 10/11	39	2 (L)	39	3.0 × 103(H)

Maximum value of CMV antigenemia was described as positive cells per 50 000 for HRP-C7 and per one slide for C10/11 (150 000 cells applied). High-level CMV Ag and real-time PCR was defined as 10 or more positive cells and 1 × 103 copies or more, respectively (H = high level; L = low level).
[a]Patients showing positive CMV Ag or PCR before disease occurrence.