

存経過観察を得られたが、1例の stable disease 以外は progressive disease であり、本研究により安全性をまず確保したうえで奏効率を求める研究へと進めたい。

F. 健康危機情報

該当なし

G. 研究発表

該当なし

H. 知的財産権の出願・登録状況

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

研究成果の刊行に関する一覧

1. Hori S, Heike Y, Takei M, Maruyama M, Inoue Y, Lee JJ, Kim HJ, Harada Y, Kawai H, Shimosaka A, Kami M, Tanosaki MD, Wakasugi H, Saito S, Takaue Y, Kakizoe T: Freeze-thawing procedures have no influence on the phenotypic and functional development of dendritic cells generated from peripheral blood CD14+ monocytes. *J Immunother*, 27:27-35, 2004.
2. Hori A, Kami M, Kim SW, Murashige N, Sakiyama M, Kojima R, Hamaki T, Makimoto A, Miyakoshi S, Masuo S, Taniguchi S, Kunitoh H, Takaue Y: Urgent need for a validated tumor response evaluation system for use in immunotherapy. *Bone Marrow Transplant*, 33:255-256, 2004.
3. Kawano Y, Chan LL, Watanabe T, Abe T, Okamoto Y, Makimoto A, Nakagawa R, Watanabe H, Suzuya H, Takaue Y: Cryopreservation of mobilized blood stem cells at a higher cell concentration without the use of a programmed freezer. *Ann Hematology*, 83:50-54, 2004.
4. Yamasaki S, Ohno Y, Taniguchi S, Yoshida T, Hayashi S, Ogawa H, Shimazaki C, Takahashi S, Kasai M, Wake A, Nishimura M, Tokunaga K, Gondo H, Takaue Y, Harada M, Mineishi S: Allogeneic peripheral blood stem cell transplantation from 2- or 3-loci-mismatched related donors in adult Japanese patients with high-risk hematologic malignancies. *Bone Marrow Transplant*, 33:279-289, 2004.
5. Kamitsuji Y, Mori S, Kami M, Yamada H, Shirakawa K, Kishi Y, Murashige N, Kim SW, Heike Y, Takaue Y: Fatal deep vein thrombosis after allogeneic reduced-intensity hematopoietic stem-cell transplantation for the treatment of metastatic gastric cancer. *Ann Hematol*, 83:533-535, 2004.
6. Harashima N, Kurihara K, Utunomiya A, Tanosaki R, Hanabuchi S, Masuda M, Ohashi T, Fukui F, Hasegawa A, Masuda T, Takaue Y, Okamura J, Kannagi M: Graft-versus-human T-cell leukemia virus type-I response in adult T-cell leukemia patients following non-myeloablative hematopoietic stem cell transplantation. *Cancer Res*, 64:391-399, 2004.
7. Hori A, Kami M, Kim SW, Chizuka A, Kojima R, Imataki O, Sakiyama M, Hamaki T, Onishi Y, Usubuchi N, Kishi Y, Murashige N, Tajima K, Miyakoshi S, Heike Y, Masuo S, Taniguchi S, Takaue Y: Development of early neutropenic fever, with or without bacterial infection, is still a significant complication after reduced-intensity stem cell transplantation. *Biol Blood Marrow Transplant*, 10:65-72, 2004.
8. Mori T, Mori S, Kanda Y, Yakushiji K, Mineishi S, Takaue Y, Gondo H, Harada M, Sakamaki H, Yajima T, Iwao Y, Hibi T, Okamoto S: Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*, 33:431-434, 2004.
9. Kusumi E, Kami M, Yuji K, Hamaki T, Murashige N, Kim SW, Heike Y, Ueyama J, Miyakoshi S, Tanosaki R, Morinaga S, Mineishi S, Muto Y, Takaue Y: Feasibility of reduced-intensity hematopoietic stem cell transplantation from an HLA-matched unrelated donor. *Bone Marrow Transplant*, 33:697-702, 2004.
10. Hamaki T, Kami M, Kim SW, Onishi Y, Kishi Y, Murashige N, Hori A, Kojima R, Sakiyama M, Imataki O, Heike Y, Tanosaki R, Masuo S, Miyakoshi S, Taniguchi S, Tobinai K, Takaue Y: Reduced-intensity stem cell transplantation from an HLA-identical sibling donor in patients with myeloid malignancies. *Bone Marrow Transplant*, 33:891-900, 2004.

11. Lee JJ, Nam CE, Nam JH, Lee HC, Chung IJ, Park MS, Choi BH, Song WH, Lee IK, Park KS, Kook H, Hwang TJ, Takei M, Takaue Y, Kim HJ: Generation of cytotoxic donor CD8⁺ T cells against relapsing leukemic cells following allogeneic transplantation by stimulation with leukemic cell- or leukemic lysate pulsed donor cell-derived dendritic cells. *Leuk Res*, 28:517-524, 2004.
 12. Shimizu A, Sasaki H, Aoyagi K, Yoshida M, Kato K, Heike Y, Ikarashi Y, Shirakawa K, Takaue Y, Miyajima A, Terada M, Nagai H, Wakasugi H: The mouse natural killer T cell-associated antigen recognized by U5A2-13 monoclonal antibody is intercellular adhesion molecule-1. *Immunol Lett* 92:227-235, 2004.
 13. Imataki O, Kim SW, Kami M, Gotoh M, Komaba S, Kasai M, S Hashino S, Naito K, Masuda M, Anan K, Teshima H, Togitani K, Inoue T, Nishimura M, Adachi Y, Fukuhara T, Yamashita T, Uike N, Kobayashi Y, Hamaguchi M, Higuchi M, Kawakami K, Takaue Y: A nationwide survey of deep fungal infections and fungal prophylaxis after hematopoietic stem cell transplantation in Japan. *Bone Marrow Transplant*, 33:1173-1179, 2004.
 14. Tanimoto TE, Yamaguchi T, Tanaka Y, Saito A, Tajima K, Karasuno T, Kasai M, Kishi K, Mori T, Maseki N, Morishima S, Miyakoshi S, Kasai M, Ohno Y, Kim SW, Numata A, Kami M, Takaue Y, Mori S, Harada M: Comparative analysis of clinical outcomes after allogeneic bone marrow transplantation versus peripheral blood stem cell transplantation from a related donor in Japanese patients. *Br J Haematol*, 125:480-493, 2004.
 15. Hori A, Kami M, Kim SW, Makimoto A, Tanosaki R, Takaue Y: Balance between acute graft-versus-host disease and graft-versus-tumor effect after reduced-intensity transplantation for metastatic renal cell carcinoma. *Hematol J*, 5:276-278, 2004.
 16. Miyakoshi S, Yuji K, Kami M, Kusumi E, Kishi Y, Kobayashi K, Murashige N, Hamaki T, Kim SW, Ueyama J, Mori S, Morinaga S, Muto Y, Masuo S, Kanemaru M, Hayashi T, Takaue Y, Taniguchi S: Successful engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with advanced hematological diseases. *Clin Cancer Res*, 10:3586-3592, 2004.
 17. Sekiguchi N, Nishimoto J, Tanosaki R, Kubota N, Yokota Y, Kobayashi Y, Watanabe T, Kami M, Takaue Y, Matsuno Y, Tobinai K: EBV-positive Burkitt lymphoma as a late-onset posttransplantation lymphoproliferative disorder after allogeneic stem cell transplantation. *Int J Hematol*, 79:387-389, 2004.
 18. Nakagawa T, Kami M, Hori A, Kim SW, Murashige N, Hamaki T, Kishi Y, Fujimoto H, Matsuoka N, Okajima E, Komiyama M, Tobisu K, Wakayama T, Uike N, Tajima K, Makimoto A, Mori S, Tanosaki R, Takaue Y, Kakizoe T: Allogeneic hematopoietic stem cell transplantation with a reduced-intensity conditioning regimen for the treatment of metastatic renal cell carcinoma: single institution experience with a minimum 1-year follow-up. *Exp Hematol*, 32:599-606, 2004.
 19. Kishi Y, Miyakoshi S, Kami M, Ikeda M, Katayama Y, Murashige N, Kusumi E, Yuji K, Kobayashi K, Kato D, Hamaki T, Matsumura T, Kim SW, Morinaga S, Mori S, Kanemaru N, Hayashi T, Takaue Y, Taniguchi S: Early central nervous system complications after reduced-intensity stem cell transplantation. *Biol Blood Marrow Transplant*, 10:561-568, 2004.
 20. Kojima R, Kami M, Nannya Y, Kusumi E, Sakai M, Tanaka Y, Kanda Y, Mori S, Chiba S, Miyakoshi S, Tajima K, Hirai H, Taniguchi S, Sakamaki H, Takaue Y: Incidence of invasive aspergillosis after allogeneic hematopoietic stem cell transplantation with a reduced-intensity regimen compared with transplantation with a conventional regimen. *Biol Blood Marrow Transplant*, 10:645-652, 2004.
-

21. Sakiyama M, Kami M, Hori A, Imataki O, Hamaki T, Murashige N, Kobayashi K, Kishi Y, Kojima R, Kim SW, Kusumi E, Yuji K, Miyakoshi S, Mori S, Tanosaki R, Taniguchi S, Takaue Y: Regimen-related toxicity following reduced-intensity stem-cell transplantation: comparison between Seattle criteria and National Cancer Center Common Toxicity Criteria (NCI-CTC) version 2.0. *Bone Marrow Transplant*, 34:787-794, 2004.
22. Kami M, Makimoto A, Heike Y, Takaue Y: Reduced-intensity hematopoietic stem cell transplantation (RIST) for solid malignancies. *Jap J Clin Oncol*, 34:707-716, 2004.
23. Hori A, Kanda Y, Goyama S, Onishi Y, Komeno Y, Mitani K, Kishi Y, Ogawa S, Imataki O, Chiba S, Kojima R, Hamaki T, Sakiyama M, Kami M, Makimoto A, Tanosaki R, Takaue Y, Hirai H: A prospective trial to evaluate the safety and efficacy of pravastatin for the treatment of refractory chronic graft-versus-host disease. *Transplantation*, 79:372-374, 2004.
24. Miyakoshi S, Kami M, Kishi Y, Murashige N, Yuji K, Kusumi E, Matsumura T, Onishi Y, Kobayashi K, Kim SW, Hamaki T, Takaue Y, Taniguchi S: Fatal hepatic failure associated with graft rejection following reduced-intensity stem-cell transplantation for chronic idiopathic myelofibrosis (CIMF). *Leuk Lymphoma*, 45:2513-2516, 2004.
25. Kojima R, Kami M, Hori A, Murashige N, Ohnishi M, Kim SW, Hamaki T, Kishi Y, Tsutsumi Y, Masauzi N, Heike Y, Mori S, Kobayashi K, Masuo S, Tanosaki R, Takaue Y: Reduced-intensity allogeneic hematopoietic stem-cell transplantation as an immunotherapy for metastatic colorectal cancer. *Transplantation*, 78:1740-1746, 2004.
26. Kishi Y, Kami M, Murashige N, Tanaka Y, Fujisaki G, Kusumoto S, Mori S, Takaue Y, Tanosaki R: Hyperacute GVHD and emergence of peripheral CD3+CD56+ T-cells and activated natural killer cells are useful markers for early diagnosis of post-transplant hemophagocytic syndrome. *Bone Marrow Transplant*, 35:415-417, 2005.
27. Hori A, Kanda Y, Goyama S, Onishi Y, Komeno Y, Mitani K, Kishi Y, Ogawa S, Imataki O, Chiba S, Kojima R, Hamaki T, Sakiyama M, Kami M, Makimoto A, Tanosaki R, Takaue Y, Hirai H; Japan Hematology and Oncology Clinical Study Group. A prospective trial to evaluate the safety and efficacy of pravastatin for the treatment of refractory chronic graft-versus-host disease. *Transplantation*, 79:372-374, 2005.
28. Chizuka A, Kami M, Kanda Y, Murashige N, Kishi Y, Hamaki T, Kim SW, Hori A, Kojima R, Mori SI, Tanosaki R, Gomi H, Takaue Y: Value of surveillance blood culture for early diagnosis of occult bacteremia in patients on corticosteroid therapy following allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*, 35:577-582, 2005.
29. Akiyama Y, Tanosaki R, Inoue N, Shimada M, Hotate Y, Yamamoto A, Yamazaki N, Kawashima I, Nukaya I, Takesako K, Maruyama K, Takaue Y and Yamaguchi K: Clinical response in Japanese metastatic melanoma patients treated with peptide cocktail-pulsed dendritic cells. *J Transl Med*, 3:4, 2005.
30. Kobayashi K, Kami M, Ikeda M, Kishi Y, Murashige N, Tanosaki R, Mori S, Takaue Y: Fulminant septicemia caused by *Bacillus cereus* following reduced-intensity umbilical cord blood transplantation. *Haematologica*, 90:ECR06, 2005.

Correspondence

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

Bone Marrow Transplantation (2004) 33, 255–256.
doi:10.1038/sj.bmt.1704371
Published online 15 December 2003

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 criteria² 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.

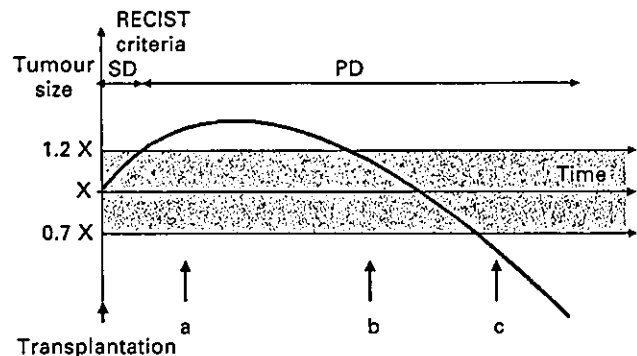


Figure 1 Course of tumor size after transplantation. Primary solid tumors are progressive, despite chemoradiotherapy prior to transplantation. (a) Most tumors continue their natural growth until the development of a GVT effect, which usually occurs several months after transplantation. (b) If the tumor 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.

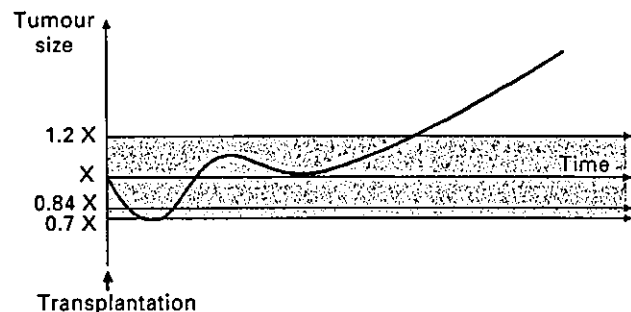


Figure 2 Fluctuation of tumor size after donor lymphocyte infusion or adjustment of immunosuppressive agents. It is difficult to handle patients in whom the tumor size fluctuates in response to post transplant immunotherapy, such as donor lymphocyte infusion and adjustment of immunosuppressive agents.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

A Hori¹
M Kami¹
S-W Kim¹
N Murashige¹
M Sakiyama¹
R Kojima¹
T Hamaki¹
A Makimoto¹
S Miyakoshi²
S Masuo³
S Taniguchi²
H Kunitoh⁴
Y Takaue¹

¹*Hematopoietic Stem Cell Transplant Unit, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 100-0045, Japan;*
²*Department of Hematology, Toranomon Hospital, Tokyo, Japan;*
³*Department of Hematology and Rheumatology, JR Tokyo General Hospital, Tokyo, Japan; and*
⁴*Department of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan*

References

- 1 Hentschke P, Barkholt L, Uzunel M *et al.* Low-intensity conditioning and hematopoietic stem cell transplantation in patients with renal and colon carcinoma. *Bone Marrow Transplant* 2003; 31: 253-261.
- 2 Therasse P, Arbuck SG, Eisenhauer EA *et al.* New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; 92: 205-216.
- 3 Childs R, Chernoff A, Contentin N *et al.* Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med* 2000; 343: 750-758.

Yoshifumi Kawano · Chan Lee Lee ·
Tsutomu Watanabe · Takanori Abe · Hiroko Suzuya ·
Yasuhiro Okamoto · Atsushi Makimoto ·
Ryuji Nakagawa · Hiroyoshi Watanabe ·
Yoichi Takaue

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

Received: 19 August 2003 / Accepted: 18 October 2003 / Published online: 13 November 2003
© Springer-Verlag 2003

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 $2\text{--}5 \times 10^7$ /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 $>1.0 \times 10^9$ /l, an absolute neutrophil count of $>0.5 \times 10^9$ /l, and a platelet count of $>50 \times 10^9$ /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

Y. Kawano (✉)
Department of Pediatrics,
Graduate School of Medical and Dental Sciences,
Kagoshima University,
8-35-1 Sakuragaoka, 890-8520 Kagoshima, Japan
e-mail: ykawano@m3.kufm.kagoshima-u.ac.jp
Fax: +81-99-2657196

Y. Kawano · T. Watanabe · T. Abe · H. Suzuya · Y. Okamoto ·
A. Makimoto · R. Nakagawa · H. Watanabe · Y. Takaue
Department of Pediatrics,
University of Tokushima,
Tokushima, Japan

C. L. Lee
Department of Pediatrics,
University of Malaya,
Kuala Lumpur, Malaysia

Y. Takaue
Stem Cell Transplant Unit,
National Cancer Center Hospital,
Tokyo

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 ($-1^\circ\text{C}/\text{min}$) 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 PBSC 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 PBSC 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 $>3 \times 10^9/l$ and a platelet count of $>100 \times 10^9/l$ in the recovery phase after chemotherapy. In some patients, PBSC were mobilized by G-CSF alone [13]. In these cases, patients received 10 $\mu\text{g}/\text{kg}$ 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 $2\text{--}5 \times 10^7/\text{ml}$ (Group I, n=20). Purified CD34+ cells were frozen at $2 \times 10^7/\text{ml}$ using the same method (Group III, n=22). Subsequently, a concentration of $2 \times 10^8/\text{ml}$ 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 $>300\text{ ml}$ [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 $200 \times G$ 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 $<5 \times 10^{10}$ 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 IgG₁-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 IgG₁) and phycoerythrin-conjugated CD34 monoclonal antibody (Anti-HPCA2 antibody, Becton Dickinson) at a concentration of 1 μg antibody/ 10^6 cells. 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 $\mu\text{g}/\text{ml}$ 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 N₂-O₂-CO₂ 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 $>1 \times 10^9/l$, an absolute neutrophil count (ANC) of $>0.5 \times 10^9/l$, and a platelet count of $>50 \times 10^9/l$. 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, $2.6 \times 10^6/kg$) was significantly lower than those in groups II ($5.9 \times 10^8/kg$) and I ($13 \times 10^8/kg$) ($p < 0.001$ each). The number of CD34+ cells in group II ($5.7 \times 10^6/kg$) was also significantly higher than that in group III ($2.2 \times 10^6/kg$) ($p = 0.002$). The number of CFU-GM in group II ($16 \times 10^5/kg$) was significantly higher than those in groups III ($4.2 \times 10^5/kg$) and I ($6.1 \times 10^5/kg$) ($p < 0.001$ each). However, there was no difference in the number of CFU-GM between groups III and I.

Table 2 Cell recovery rates and viabilities after thawing

	Group	MNC	Viability	CD34+ cell	CFU-GM
I	($2-5 \times 10^7/ml$) n=20	67 (26–298)	89 (84–95)	n.d.	88 (33–373)
II	($2 \times 10^8/ml$) n=44	72 (23–335)	87 (83–91)	103 (27–429)	64 (19–508)
III	($2 \times 10^7/ml$) 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 1 Numbers of frozen cells

	Group	MNC ($\times 10^8/kg$)	CD34+ cell ($\times 10^6/kg$)	CFU-GM ($\times 10^5/kg$)
I	($2-5 \times 10^7/ml$) n=20	13 (3.6–67.2)	n.d.	6.1 (1.5–22.0)
II	($2 \times 10^8/ml$) n=44	5.9 (0.8–35.5)	5.7 (0.13–83.2)	16 (0.4–73.2)
III	($2 \times 10^7/ml$) n=22	$2.6 \times 10^6/kg$ (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) $\times 10^5/kg$] was statistically identical to that in group II [9.1 (0.3–90) $\times 10^5/kg$] ($p = 0.086$). The number of infused CD34+ cells in group III [2.1 (0.43–7.0) $\times 10^6/kg$] was significantly lower than that in group II [5.3 (0.12–75) $\times 10^6/kg$] ($p = 0.001$), and the number of CFU-GM in group III [2.2 (0.3–12) $\times 10^5/kg$] was significantly lower than those in groups II [9.1 (0.3–90) $\times 10^5/kg$] and I [5.6 (1.5–20) $\times 10^5/kg$] ($p < 0.001$ each).

Table 3 Engraftment speed after autografting

Group	WBC ($>1 \times 10^9/l$)	ANC ($>5 \times 10^9/l$)	Platelets ($>50 \times 10^9/l$)
I ($2 \times 10^7/ml$) n=20	11 (8-29)	11 (8-22)	15 (10-88)
II ($2 \times 10^8/ml$) n=44	11 (8-18)	11 (8-19)	16 (10-37)
III ($2 \times 10^7/ml$) 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 $>1 \times 10^9/l$ and ANC of $0.5 \times 10^9/l$ 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 $>50 \times 10^9/l$ 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 $3.7 \pm 1.9 \times 10^8/ml$ [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 $2 \times 10^8/ml$ 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.

Acknowledgements The authors are grateful to Ms. Yasuda for her excellent technical assistance and to the nursing staff of the pediatric ward at the University Hospital of Tokushima.

References

- Goldman JM, Schmitz N, Niethammer D, Gratwohl A (1998) Allogeneic and autologous transplantation for hematological disease, solid tumors and immune disorders: current practice in Europe in 1998. *Bone Marrow Transplant* 21:1-7
- Goldman JM, Th'ng KH, Park DS, Spiers AS, Lowenthal RM, Ruutu T (1979) Collection, cryopreservation and subsequent viability of haemopoietic stem cells intended for treatment of chronic granulocytic leukemia in blast-cell transformation. *Br J Haematol* 40:185-195
- Stroncek DF, Fautsh SK, Lasky LC, Hurd DD, Ramsay NK, McCullough J (1991) Adverse reactions in patients transfused with cryopreserved marrow. *Transfusion* 31:521-526
- Okamoto Y, Takaue Y, Saito S, Shimizu T, Suzue T, Abe T, Sato J, Hirao A, Watanabe T, Kawano Y, Kuroda Y (1993) Toxicities associated with cryopreserved and thawed peripheral blood stem cell autografts in children with cancer. *Transfusion* 33:578-581
- Al Alessandrino P, Bernasconi P, Caldera D, Colombo A, Bonfichi M, Malcovati L, Klersy C, Martinelli G, Maiocchi M, Pagnucco G, Varettoni M, Perotti C, Bernasconi C (1999) Adverse events occurring during bone marrow or peripheral blood progenitor cell infusion: analysis of 126 cases. *Bone Marrow Transplant* 23:533-537
- Rowley SD (1992) Hematopoietic stem cell cryopreservation: a review of current techniques. *J Hematother* 1:233-250

7. Makino S, Harada M, Akashi K, Taniguchi S, Shibuya T, Inaba S, Niho Y (1991) A simplified method for cryopreservation of peripheral blood stem cells at -80°C without rate-controlled freezing. *Bone Marrow Transplant* 8:239-244
8. Takaue Y, Abe T, Kawano Y, Suzue T, Saito S, Hirao A, Sato J, Makimoto A, Kawahito M, Watanabe T, Shimokawa T, Kuroda Y (1994) Comparative analysis of engraftment after peripheral blood stem cell autografts cryopreserved by controlled vs uncontrolled-rate method. *Bone Marrow Transplant* 13:801-804
9. Rowley SD, Bensinger WI, Gooley TA, Buckner CD (1994) Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. *Blood* 83:2731-2736
10. Cabezedo E, Dalmases C, Ruz M, Sanchez JA, Torrico C, Sola C, Querol S, Garcia J (2000) Leukapheresis components may be cryopreserved at high cell concentrations without additional loss of HPC function. *Transfusion* 40:1223-1227
11. Takaue Y, Kawano Y, Kuroda Y (1995) Application of recombinant granulocyte colony-stimulating factor in peripheral blood stem-cell transplantation: a pediatric experience. In: Levitt D, Mertelsmann R (eds) *Hematopoietic stem cells*. Marcel Dekker, New York, pp 611-630
12. Makimoto A, Kawano Y, Abe T, Okamoto Y, Sato J, Nakagawa R, Watanabe H, Watanabe T, Kuroda Y, Sweet L, Takaue Y (1999) Comparative evaluation of procedures with a Baxter CS-3000 cell separator for collecting peripheral blood cells from children. *J Hematother* 8:305-310
13. Kawano Y, Takaue Y, Watanabe T, Abe T, Okamoto Y, Iwai A, Watanabe A, Ito E, Makimoto A, Nakagawa R, Watanabe H, Sato J, Suenaga K, Suzuya H, Ohnishi T, Kanamaru S, Kaneko S, Kuroda Y (1999) Efficacy of the mobilization of peripheral blood stem cells by granulocyte colony-stimulating factor in pediatric donors. *Cancer Res* 59:3321-3324
14. Takaue Y, Kawano Y, Watanabe A, Eguchi H, Abe T, Makimoto A, Okamoto Y, Kuroda Y (1996) Transplantation with purified or unmanipulated mobilized blood stem cells in children. In: Ikehara S, Takaku F, Good RA (eds) *Bone marrow transplantation: basic and clinical studies*. Springer, Berlin, Heidelberg, New York, pp 246-249
15. Kawano Y, Takaue Y, Watanabe T, Saito S, Abe T, Hirao A, Sato J, Ninomiya T, Suzue T, Koyama T, Shimokawa T, Yokobayashi A, Asano S, Masaoka T, Takaku F, Kuroda Y (1993) Effects of progenitor cell dose and preleukapheresis use of human recombinant granulocyte-macrophage colony-stimulating factor on the recovery of hematopoiesis after blood stem cell autografting in children. *Exp Hematol* 21:103-108
16. Takaue Y, Kawano Y, Abe T, Okamoto Y, Suzue T, Shimizu T, Saito S, Sato J, Makimoto A, Nakagawa R, Watanabe T, Ito M, Kuroda Y (1995) Collection and transplantation of peripheral blood stem cells in very small children weighing 20 kg or less. *Blood* 86:372-380
17. To LB, Roberts MM, Haylock DN, Dyson PG, Branford AL, Thorp D, Ho JQ, Dart GW, Horvath N, Davy ML, Olweny LM, Juttner CA (1992) Comparison of haematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone marrow transplants and allogeneic bone marrow transplants. *Bone Marrow Transplant* 9:277-284
18. Takaue Y, Watanabe T, Kawano Y, Koyama T, Huq M, Suzue T, Abe T, Sato J, Shimokawa T, Kosaka M, Shimizu M, Ogura T, Ninomiya T, Kuroda Y (1989) Isolation and storage of peripheral blood hematopoietic stem cells for autotransplantation into children with cancer. *Blood* 74:1245-1251
19. Keung YK, Cobos E, Morgan D, Park M, Dixon S, Wu K, Park CH (1996) High cellular concentration of peripheral blood progenitor cells during cryopreservation adversely affects CFU-GM but not hematopoietic recovery. *J Hematother* 5:73-77
20. Balint B, Ivanovic Z, Petakov M, Taseski J, Jovicic G, Stojanovic N, Milenkovic P (1999) The cryopreservation protocol optimal for progenitor recovery is not optimal for preservation of marrow repopulating ability. *Bone Marrow Transplant* 23:613-619

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

Nanae Harashima,¹ Kiyoshi Kurihara,¹ Atae Utsunomiya,² Ryuji Tanosaki,³ Shino Hanabuchi,¹ Masato Masuda,⁴ Takashi Ohashi,¹ Fumiyo Fukui,¹ Atsuhiko Hasegawa,¹ Takao Masuda,¹ Yoichi Takaue,³ Jun Okamura,⁵ and Mari Kannagi¹

¹Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, Tokyo; ²Department of Hematology, Inamura Bun-in Hospital, Kagoshima; ³National Cancer Center Hospital, Tokyo; ⁴Second Department of Internal Medicine, University of the Ryukyus, Okinawa; and ⁵National Kyushu Cancer Center, Fukuoka, Japan

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-leuke-

mia (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

Received 5/22/03; revised 9/18/03; accepted 10/28/03.

Grant support: Ministry of Health, Welfare, and Labor of Japan, the Ministry of Education, Science, Culture and Sports of Japan, and Japan Science and Technology Corporation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Mari Kannagi, Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81-3-5803-5798; Fax: 81-3-5803-0235; E-mail: kann.impt@tmd.ac.jp.

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/m² 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-#97 were 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 (Dyna, 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% CO₂ for over 2 months. An EBV-transformed B-cell line, LCL-#36, was established by main-

taining 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 anti-human 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* 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

* 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 ⁵¹Cr-release assay at various E:T cell ratios as described previously (27, 40). Specific cytotoxicity was calculated as [(experimental ⁵¹Cr release - spontaneous ⁵¹Cr release) / (maximum ⁵¹Cr release - spontaneous ⁵¹Cr 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 ⁵¹Cr-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 (MAIP54510; 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 × 10⁴ 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% CO₂. 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 × 10⁶) 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).

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. 2A, 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

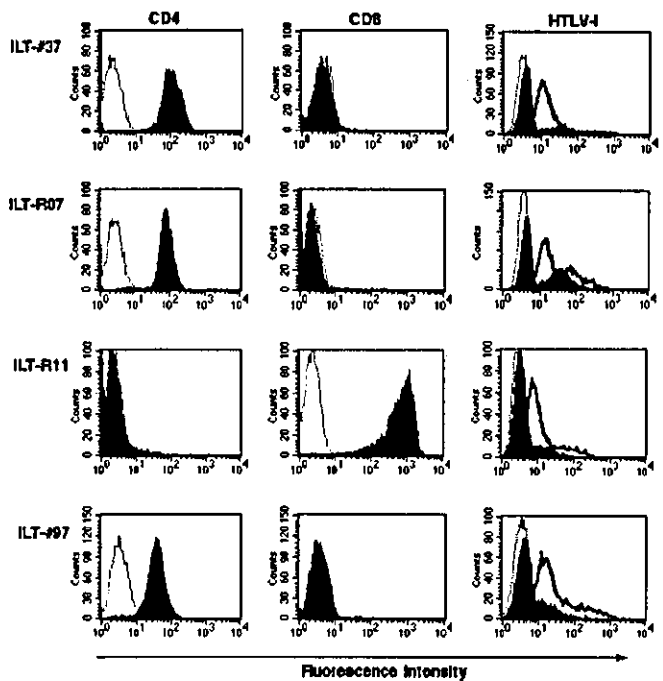


Fig. 1. Surface phenotype and human T-cell leukemia virus type I (HTLV-I) expression in T-cell lines spontaneously established from prehematopoietic stem cell transplantation adult T-cell leukemia (ATL) patients. ILT-#37, ILT-R07, ILT-R11, and ILT-#97 cells that were cultured for >2 months in the presence 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).

⁶ Internet address: http://bimas.dcrf.nih.gov/molbio/bla_bind/.

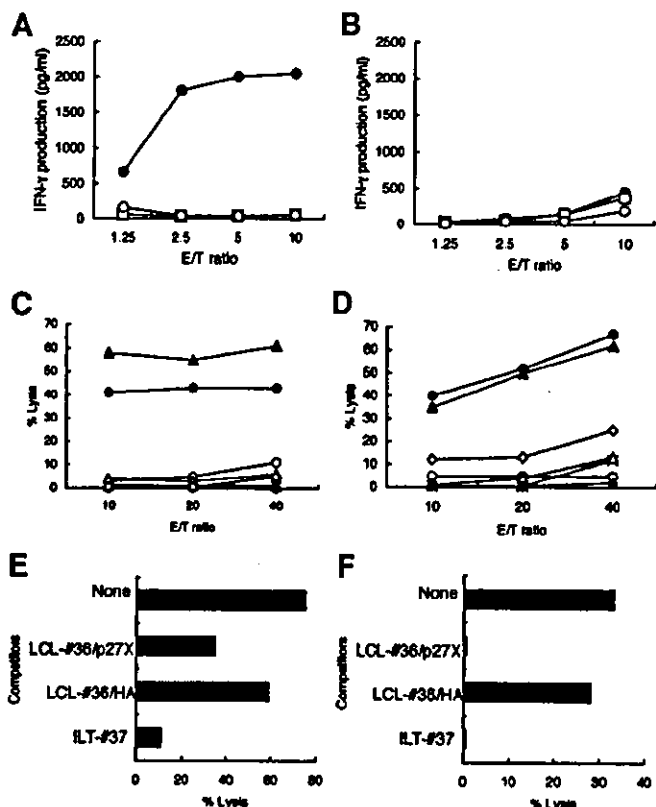


Fig. 2. Induction of and human T-cell leukemia virus type I (HTLV-I)-specific CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37 and donor #36 in response to ILT-#37 cells. Peripheral blood mononuclear cells (PBMCs) from patient #37 (+183 days post-HSCT) (A, C, and E) and donor #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 target cells by ELISA and 6-h ^{51}Cr -release assay, respectively. The target cells used were formalin-fixed ILT-#37 (\bullet), K562 (\square), or none (\circ) in A and B, HLA-identical ILT-#37 (\bullet), LCL-#36 (\circ), and PHA-activated PBMCs of pre-HSCT patient #37 (\times), HLA-A2- and B46-matched TCL-Kan (\blacktriangle) and LCL-Kan (\triangle), and HLA-mismatched ILT-As-2 (\blacklozenge) and LCL-As (\blacklozenge) 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. 2B), although the activities increased at later time points of culture (Fig. 2D).

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. 2C) and donor #36 (Fig. 2D) 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. 2E). The cytotoxicity of CTL-#36 for ILT-#37 was almost completely inhibited by unlabelled LCL-#36/p27X as well as ILT-#37 cells (Fig. 2F). 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.

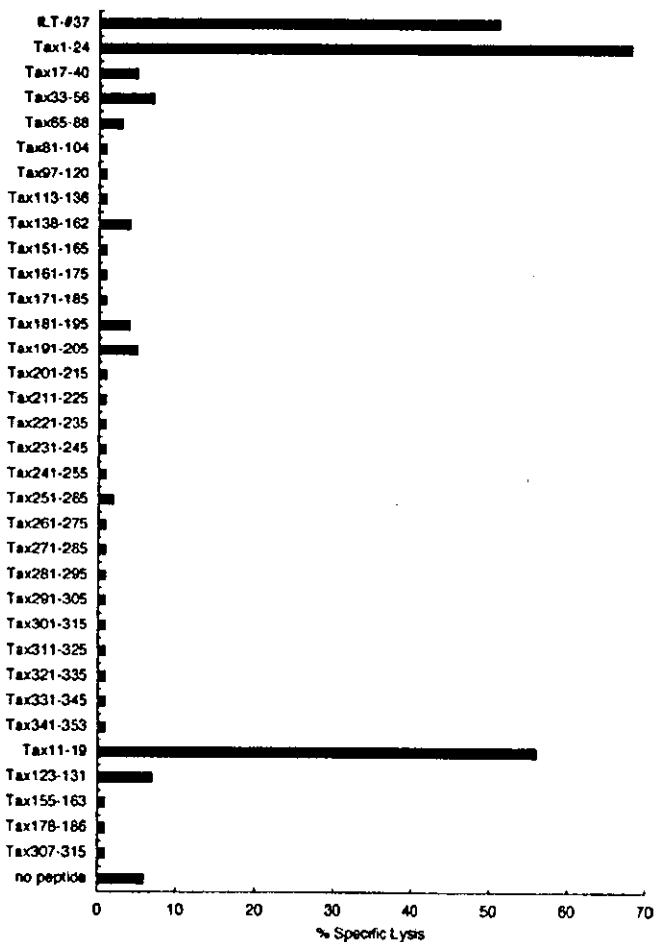


Fig. 3. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37. LCL-#36 cells were pulsed with 10 nmol of 33 kinds of 9-24 mer synthetic oligopeptides corresponding to the Tax amino acid sequence, and their susceptibility 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 (MAHFPGF-GQSLFLGYPVYVFGDCV) 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. 4A, 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. 4B, 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. 4B), 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-

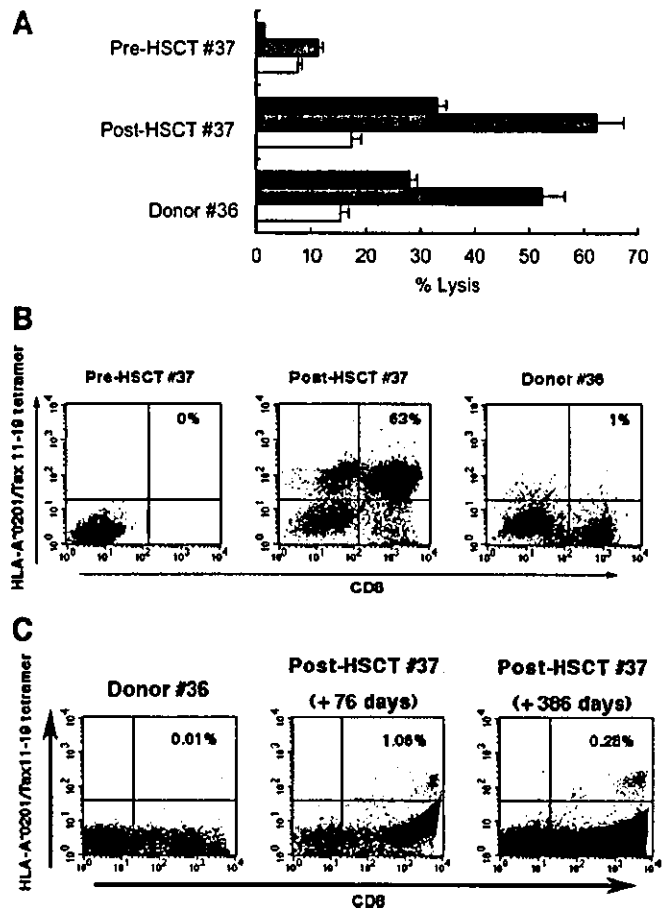


Fig. 4. Selective expansion of Tax11-19-specific CTL in peripheral blood mononuclear cell (PBMC) cultures from patient #37 after hematopoietic stem cell transplantation (HSCT). *A*, PBMCs from pre-HSCT patient #37 at 40 days of culture which were stimulated with formalin-fixed ILT-#37 cells two times, 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 11-19 tetramer⁺ cells in the PBMCs are indicated in the *top right quadrants*.

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 cytotoxicity 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.

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 \pm 67	N.T. ^d	
			ILT-As-2	Positive	A24	866 \pm 117	N.T.	
			TOK	Negative	A24	160 \pm 7	N.T.	
	Day 24	ILT-R07	None			36 \pm 12	N.T.	
			ILT-R07	Positive	Identical	778 \pm 55	N.T.	
			ILT-Myj-3	Positive	A24, B60, DR4	355 \pm 0	N.T.	
			TOK	Negative	A24	62 \pm 9	N.T.	
			None			25 \pm 19	N.T.	
			None			N.T.	39 \pm 9	
	Day 63	ILT-R07	ILT-R07	Positive	Identical	N.T.	48 \pm 5	
			ILT-As-2	Positive	A24	N.T.	5 \pm 6	
			TOK	Negative	A24	N.T.	27 \pm 11	
			TOK/p27X ^e	pX products	A24	N.T.	11 \pm 4	
			TOK/pEnv ^f	Envelope	A24	N.T.	6 \pm 10	
			TOK/pGag ^g	Core	A24	N.T.		
R11 (+153)	Day 25	ILT-R11	ILT-R11 ^h	Positive	Identical	11 \pm 1	N.T.	
			ILT-Nkz-2	Positive	A2, A26	12 \pm 2	N.T.	
			LCL-Nkz	Negative	A2, A26	19 \pm 6	N.T.	
			None	Negative		14 \pm 0	N.T.	
			None			477 \pm 34	N.T.	
			None			415 \pm 2	N.T.	
#97 (+104)	Day 21	ILT-#97	ILT-#97	Positive	Identical	104 \pm 15	N.T.	
			ILT-Har	Positive	A2, B51, DR4	63 \pm 4	N.T.	
			LCL-Har	Negative	A2, B51, DR4	63 \pm 4	N.T.	
	Day 50	ILT-#97	None			128 \pm 36	29 \pm 2	
			ILT-#97	Positive	Identical	111 \pm 10	23 \pm 3	
			ILT-Har	Positive	A2, B51, DR4	10 \pm 1	16 \pm 3	
			LCL-Har	Negative	A2, B51, DR4	1 \pm 0	N.T.	
			None					
			None					

^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 \pm SD of duplicate determinations by ELISA.

^c Specific cytolysis at an E:T ratio of 40 measured by 6-h ⁵¹Cr-release assays was shown as the mean \pm 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 ⁵¹Cr-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 (SFHSLHLLFEEYTN) 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 HSCT, 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. 4C, >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-HSCT patient #37 decreased to 0.28% at a later time point (+386 days after HSCT) 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

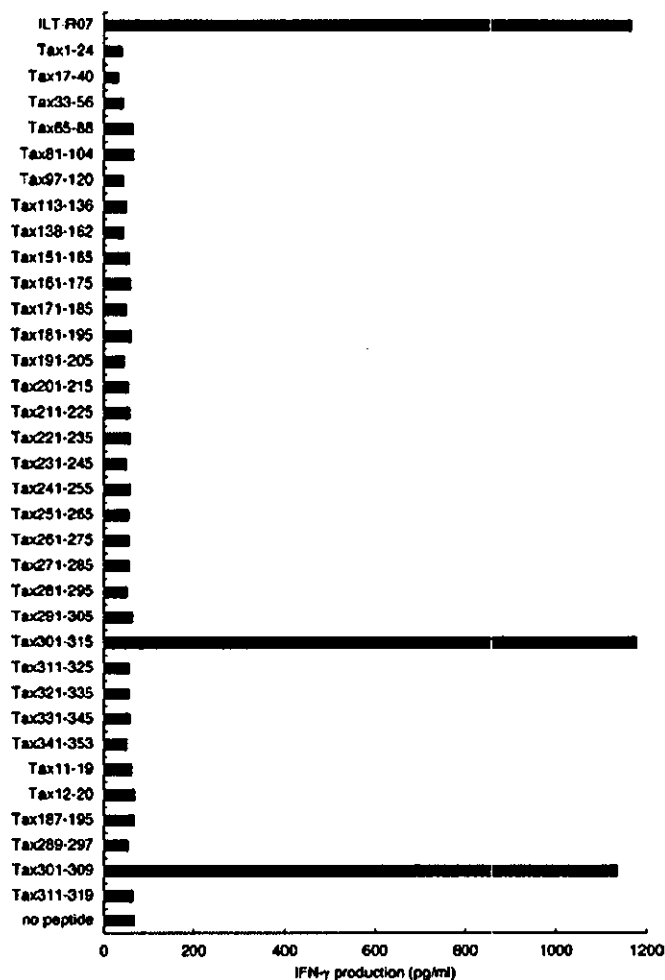


Fig. 5. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by the CTLs induced from post-hematopoietic stem cell transplantation patient R07. CD8⁺ cell-enriched peripheral blood mononuclear cell that had been cultured for 32 days with three stimulations with formalin-fixed ILT-R07 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. 4B). Although CTLs induced from donor #36 also recognize Tax as shown in Fig. 2F, 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. 4C). 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 has 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 \times 10 ⁴ PBMC ^b	
	Pre-HSCT R07	Post-HSCT R07
ILT-R07	5 \pm 6	44 \pm 16
Tax301-309 (SFHSLHLLF)	1 \pm 1	5 \pm 1
Tax11-19 (LLFGYPVYV)	1 \pm 1	0 \pm 1
Medium	0 \pm 0	0 \pm 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 \times 10⁴/well as described in "Materials and Methods." Values represent the mean \pm SD of triplicate assays.

^b Results of IFN- γ -enzyme-linked immunospot assay are expressed as spot-forming cells (SFCs)/5 \times 10⁴ 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. 2E), 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*.

ACKNOWLEDGMENTS

We thank Drs. Hisatoshi Shida (Hokkaido University) and Yuetsu Tanaka (Okinawa-Asia Research Center, University of the Ryukyus) for providing recombinant vaccinia viruses and HTLV-I-specific monoclonal antibodies, respectively. We also thank the National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University Vaccine Center at Yerkes for providing HLA-A*0201/Tax11-19 tetramer.

REFERENCES

- Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K. I., Shirakawa, S., and Miyoshi, I. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA*, **78**: 6476–6480, 1981.
- Tajima, K. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of, A. T. L., and its geographical and clinical features. The T- and B-cell Malignancy Study Group. *Int. J. Cancer*, **45**: 237–243, 1990.
- Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K., and Uchino, H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*, **50**: 481–492, 1977.
- Shimoyama, M., Ota, K., Kikuchi, M., Yunoki, K., Konda, S., Takatsuki, K., Ichimaru, M., Ogawa, M., Kimura, I., Tominaga, S., *et al.* Chemotherapeutic results and prognostic factors of patients with advanced non-Hodgkin's lymphoma treated with VEPA or VEPA-M. *J. Clin. Oncol.*, **6**: 128–141, 1988.
- Shimoyama, M., Ota, K., Kikuchi, M., Yunoki, K., Konda, S., Takatsuki, K., Ichimaru, M., Tominaga, S., Tsugane, S., Minato, K., *et al.* Major prognostic factors of adult patients with advanced T-cell lymphoma/leukemia. *J. Clin. Oncol.*, **6**: 1088–1097, 1988.
- Tsukasaki, K., Maeda, T., Arimura, K., Taguchi, J., Fukushima, T., Miyazaki, Y., Moriuchi, Y., Kuriyama, K., Yamada, Y., and Tomonaga, M. Poor outcome of autologous stem cell transplantation for adult T-cell leukemia/lymphoma: a case report and review of the literature. *Bone Marrow Transplant.*, **23**: 87–89, 1999.
- Utsunomiya, A., Miyazaki, Y., Takatsuka, Y., Hanada, S., Uozumi, K., Yashiki, S., Tara, M., Kawano, F., Saburi, Y., Kikuchi, H., Hara, M., Sao, H., Morishima, Y., Kodera, Y., Sonoda, S., and Tomonaga, M. Improved outcome of adult T-cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.*, **27**: 15–20, 2001.
- Goulmy, E., Schipper, R., Pool, J., Blokland, E., Falkenburg, J. H., Vossen, J., Grathwohl, A., Vogelsang, G. B., van Houwelingen, H. C., and van Rood, J. J. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N. Engl. J. Med.*, **334**: 281–285, 1996.
- Wang, W., Meadows, L. R., den Haan, J. M., Sherman, N. E., Chen, Y., Blokland, E., Shabanowitz, J., Agulnik, A. I., Hendrickson, R. C., Bishop, C. E., *et al.* Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. *Science (Wash. DC)*, **269**: 1588–1590, 1995.
- den Haan, J. M., Meadows, L. M., Wang, W., Pool, J., Blokland, E., Bishop, T. L., Reinhardt, C., Shabanowitz, J., Offringa, R., Hunt, D. F., Engelhard, V. H., and Goulmy, E. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science (Wash. DC)*, **279**: 1054–1057, 1998.
- Behar, E., Chao, N. J., Hiraki, D. D., Krishnaswamy, S., Brown, B. W., Zehnder, J. L., and Grumet, F. C. Polymorphism of adhesion molecule CD31 and its role in acute graft-versus-host disease. *N. Engl. J. Med.*, **334**: 286–291, 1996.
- Balduini, C. L., Noris, P., Giorgiani, G., Martinetti, M., Klersy, C., Spedini, P., Belletti, S., MacCarlo, R., Gusberri, L., and Locatelli, F. Incompatibility for CD31 and human platelet antigens and acute graft-versus-host disease after bone marrow transplantation. *Br. J. Haematol.*, **106**: 723–729, 1999.
- Maruya, E., Saji, H., Seki, S., Fujii, Y., Kato, K., Kai, S., Hiraoka, A., Kawa, K., Hoshi, Y., Ito, K., Yokoyama, S., and Juji, T. Evidence that CD31, CD49b, and CD62L are immunodominant minor histocompatibility antigens in HLA identical sibling bone marrow transplants. *Blood*, **92**: 2169–2176, 1998.
- Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H. J., Rimm, A. A., Ringden, O., Rozman, C., Speck, B., *et al.* Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, **75**: 555–562, 1990.
- Mutis, T., Verdijk, R., Schrama, E., Esendam, B., Brand, A., and Goulmy, E. Feasibility of immunotherapy of relapsed leukemia with *ex vivo*-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood*, **93**: 2336–2341, 1999.
- Pinilla-Ibarz, J., Cathcart, K., Korontsvit, T., Soignet, S., Bocchia, M., Caggiano, J., Lai, L., Jimenez, J., Koltz, J., and Scheinberg, D. A. Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. *Blood*, **95**: 1781–1787, 2000.
- Gaiger, A., Reese, V., Disis, M. L., and Cheever, M. A. Immunity to WT1 in the animal model and in patients with acute myeloid leukemia. *Blood*, **96**: 1480–1489, 2000.
- Kannagi, M., Matsushita, S., Shida, H., and Harada, S. Cytotoxic T cell response and expression of the target antigen in HTLV-I infection. *Leukemia (Baltimore)*, **8** (Suppl. 1): S54–S59, 1994.
- Kannagi, M., Sugamura, K., Kinoshita, K., Uchino, H., and Hinuma, Y. Specific cytolysis of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient. *J. Immunol.*, **133**: 1037–1041, 1984.
- Jacobson, S., Shida, H., McFarlin, D. E., Fauci, A. S., and Koenig, S. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I-associated neurological disease. *Nature (Lond.)*, **348**: 245–248, 1990.
- Kannagi, M., Harada, S., Maruyama, I., Inoko, H., Igarashi, H., Kuwashima, G., Sato, S., Morita, M., Kidokoro, M., Sugimoto, M., *et al.* Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. *Int. Immunol.*, **3**: 761–767, 1991.
- Yoshida, M., Osame, M., Usuku, K., Matsumoto, M., and Igata, A. Viruses detected in HTLV-I-associated myelopathy and adult T-cell leukaemia are identical on DNA blotting. *Lancet*, **1**: 1085–1086, 1987.
- Tsukahara, T., Kannagi, M., Ohashi, T., Kato, H., Arai, M., Nunez, G., Iwanaga, Y., Yamamoto, N., Ohtani, K., Nakamura, M., and Fujii, M. Induction of Bcl-x(L) expression by human T-cell leukemia virus type I Tax through NF- κ B in apoptosis-resistant T-cell transfectants with Tax. *J. Virol.*, **73**: 7981–7987, 1999.
- Hanabuchi, S., Ohashi, T., Koya, Y., Kato, H., Takemura, F., Hirokawa, K., Yoshiki, T., Yagita, H., Okumura, K., and Kannagi, M. Development of human T-cell leukemia virus type I-transformed tumors in rats following suppression of T-cell immunity by CD80 and CD86 blockade. *J. Virol.*, **74**: 428–435, 2000.
- Ohashi, T., Hanabuchi, S., Kato, H., Koya, Y., Takemura, F., Hirokawa, K., Yoshiki, T., Tanaka, Y., Fujii, M., and Kannagi, M. Induction of adult T-cell leukemia-like lymphoproliferative disease and its inhibition by adoptive immunotherapy in T-cell-deficient nude rats inoculated with syngeneic human T-cell leukemia virus type I-immortalized cells. *J. Virol.*, **73**: 6031–6040, 1999.
- Ohashi, T., Hanabuchi, S., Kato, H., Tateno, H., Takemura, F., Tsukahara, T., Koya, Y., Hasegawa, A., Masuda, T., and Kannagi, M. Prevention of adult T-cell leukemia-like lymphoproliferative disease in rats by adoptively transferred T cells from a donor immunized with human T-cell leukemia virus type I Tax-coding DNA vaccine. *J. Virol.*, **74**: 9610–9616, 2000.
- Hanabuchi, S., Ohashi, T., Koya, Y., Kato, H., Hasegawa, A., Takemura, F., Masuda, T., and Kannagi, M. Regression of human T-cell leukemia virus type I (HTLV-I)-associated lymphomas in a rat model: peptide-induced T-cell immunity. *J. Natl. Cancer Inst.* (Bethesda), **93**: 1775–1783, 2001.
- Hinuma, Y., Gotoh, Y., Sugamura, K., Nagata, K., Goto, T., Nakai, M., Kamada, N., Matsumoto, T., and Kinoshita, K. A retrovirus associated with human adult T-cell leukemia: *in vitro* activation. *Gann*, **73**: 341–344, 1982.

29. Kannagi, M., Matsushita, S., and Harada, S. Expression of the target antigen for cytotoxic T lymphocytes on adult T-cell leukemia cells. *Int. J. Cancer*, *54*: 582-588, 1993.
30. Kinoshita, T., Shimoyama, M., Tobinai, K., Ito, M., Ito, S., Ikeda, S., Tajima, K., Shimotohno, K., and Sugimura, T. Detection of mRNA for the tax1/ret1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, *86*: 5620-5624, 1989.
31. Sugamura, K., and Hinuma, Y. *In vitro* induction of cytotoxic T lymphocytes specific for Epstein-Barr virus-transformed cells: kinetics of autologous restimulation. *J. Immunol.*, *124*: 1045-1049, 1980.
32. Sugamura, K., Fujii, M., Kannagi, M., Sakitani, M., Takeuchi, M., and Hinuma, Y. Cell surface phenotypes and expression of viral antigens of various human cell lines carrying human T-cell leukemia virus. *Int. J. Cancer*, *34*: 221-228, 1984.
33. Kannagi, M., Shida, H., Igarashi, H., Kuruma, K., Murai, H., Aono, Y., Maruyama, I., Osame, M., Hattori, T., Inoko, H., *et al.* Target epitope in the Tax protein of human T-cell leukemia virus type I recognized by class I major histocompatibility complex-restricted cytotoxic T cells. *J. Virol.*, *66*: 2928-2933, 1992.
34. Lozzio, C. B., and Lozzio, B. B. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood*, *45*: 321-334, 1975.
35. Tanaka, Y., Yoshida, A., Takayama, Y., Tsujimoto, H., Tsujimoto, A., Hayami, M., and Tozawa, H. Heterogeneity of antigen molecules recognized by anti-tax1 monoclonal antibody Lt-4 in cell lines bearing human T cell leukemia virus type I and related retroviruses. *Jpn. J. Cancer Res.*, *81*: 225-231, 1990.
36. Tanaka, Y., Lee, B., Inoi, T., Tozawa, H., Yamamoto, N., and Hinuma, Y. Antigens related to three core proteins of HTLV-I (p24, p19, and p15) and their intracellular localizations, as defined by monoclonal antibodies. *Int. J. Cancer*, *37*: 35-42, 1986.
37. Parker, K. C., Bednarek, M. A., Hull, L. K., Utz, U., Cunningham, B., Zweerink, H. J., Biddison, W. E., and Coligan, J. E. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J. Immunol.*, *149*: 3580-3587, 1992.
38. Parker, K. C., Bednarek, M. A., and Coligan, J. E. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol.*, *152*: 163-175, 1994.
39. Kubo, R. T., Sette, A., Grey, H. M., Appella, E., Sakaguchi, K., Zhu, N. Z., Amott, D., Sherman, N., Shabanowitz, J., Michel, H., *et al.* Definition of specific peptide motifs for four major HLA-A alleles. *J. Immunol.*, *152*: 3913-3924, 1994.
40. Ohashi, T., Hanabuchi, S., Suzuki, R., Kato, H., Masuda, T., and Kannagi, M. Correlation of major histocompatibility complex class I down-regulation with resistance of human T-cell leukemia virus type I-infected T cells to cytotoxic T-lymphocyte killing in a rat model. *J. Virol.*, *76*: 7010-7019, 2002.
41. Siomi, H., Shida, H., Nam, S. H., Nosaka, T., Maki, M., and Hatanaka, M. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. *Cell*, *55*: 197-209, 1988.
42. Herr, W., Schneider, J., Lohse, A. W., Meyer zum Buschenfelde, K. H., and Wolfel, T. Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor α in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J. Immunol. Methods*, *191*: 131-142, 1996.
43. Lalvani, A., Brookes, R., Hambleton, S., Britton, W. J., Hill, A. V., and McMichael, A. J. Rapid effector function in CD8+ memory T cells. *J. Exp. Med.*, *186*: 859-865, 1997.
44. Bieganski, K., Hollsberg, P., Buckle, G. J., Lim, D. G., Greten, T. F., Schneck, J., Altman, J. D., Jacobson, S., Ledis, S. L., Hanchard, B., Chin, J., Morgan, O., Roth, P. A., and Hafler, D. A. Direct analysis of viral-specific CD8+ T cells with soluble HLA-A2/Tax1-19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. *J. Immunol.*, *162*: 1765-1771, 1999.
45. Daenke, S., Kermodé, A. G., Hall, S. E., Taylor, G., Weber, J., Nightingale, S., and Bangham, C. R. High activated and memory cytotoxic T-cell responses to HTLV-I in healthy carriers and patients with tropical spastic paraparesis. *Virology*, *217*: 139-146, 1996.
46. Furukawa, K., Mori, M., Ohta, N., Ikeda, H., Shida, H., and Shiku, H. Clonal expansion of CD8+ cytotoxic T lymphocytes against human T cell lymphotropic virus type I (HTLV-I) genome products in HTLV-I-associated myelopathy/tropical spastic paraparesis patients. *J. Clin. Investig.*, *94*: 1830-1839, 1994.
47. Elovaara, I., Koenig, S., Brewah, A. Y., Woods, R. M., Lehky, T., and Jacobson, S. High human T-cell lymphotropic virus type I (HTLV-I)-specific precursor cytotoxic T lymphocyte frequencies in patients with HTLV-I-associated neurological disease. *J. Exp. Med.*, *177*: 1567-1573, 1993.