

厚生労働科学研究費補助金  
ヒトゲノム・再生医療等研究事業

<研究課題名>  
骨髄非破壊的前処置療法を用いた同種造血幹細胞移植の  
確立と普及に関する研究

平成 15 年度～16 年度 総合研究報告書

主任研究者 高上 洋一  
(所属機関 国立がんセンター中央病院)

平成 17 (2005) 年 4 月

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【 目 次 】

- I. 総合研究報告  
P 1～3 高上 洋一 / 国立がんセンター中央病院  
『骨髄非破壊的前処置療法を用いた同種造血幹細胞移植の確立と普及に関する研究』
- II. 研究成果の刊行に関する一覧
- III. 研究成果の刊行物・別刷

『 骨髄非破壊的前処置療法を用いた同種造血幹細胞移植の確立と普及に関する研究 』

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**研究要旨** 本班研究においては、従来は同種造血幹細胞移植の対象とはならなかった50歳以上の患者を対象として、新たなプリン誘導体であるクラドリピンあるいはフルダラビンとブスルファンを併用する骨髄非破壊的前処置療法を用いたミニ移植の安全性と有効性を、単一施設の第Ⅰ相あるいは多施設共同無作為比較第Ⅱ相臨床試験を行って検討した。我が国の移植領域における臨床試験体制の基盤を確立するためにデータ管理は厳正に執り行った。特に多施設共同研究は新GCPに準拠して行い、そのデータを用いてミニ移植に用いる薬剤の適応拡大承認申請を行うことを検討中である。国立がんセンター中央病院における第Ⅰ相臨床試験には総計280名を登録し、若年者に対するミニ移植を施行して治療の安全性を確認した。同時に、治療の安全性を高める目的からリンパ球輸注療法(DLI)の標的分子の同定と適正化を検討し、さらに移植後の免疫能を解析することで、今後の研究の展開を図った。この研究の過程でミニ移植は治療の現場にも急速に導入されたため、全国の施設から国立がんセンター中央病院に研修医あるいは見学者として若手医師やコメディカルを積極的に受け入れ、合併症を含めた治療法、患者管理方法や試験管理法を具体的に教示した。研究期間中の長期任意研修医数は20名近くに及び、移植治療の均てん化と国民医療におけるがん医療水準の向上に大きく寄与した。

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A. 研究目的

本研究では、これまで同種移植を受けることができなかった高齢者などに対する画期的な治療として期待される骨髄非破壊的前処置療法を用いたミニ移植法を開発する。その安全性を国立がんセンター中央病院単独で行う第Ⅰ相臨床試験などで評価し、そのデータも参考にして有効性を評価する多施設共同無作為比較第Ⅱ相臨床試験を行う。また、全国の施設からの研修医あるいは見学者を積極的に受け入れることで、移植治療の均てん化と国民医療におけるがん医療水準の向上に寄与する。

その背景として、同種造血幹細胞移植は有効な治療法ではあるが、一方では治療関連合併症が多く発生する危険な治療でもある現実がある。特に患者の年齢が50歳を超える場合や移植前に既に臓器の働きが低下している場合には、現在の方法で移植を行うと早期死亡率が著しく高くなる。このため、根治的な治療を受けることができない患者は数多い。これを克服するために、移植前処置の抗がん剤量を減量して免疫抑制力

の強い薬剤を組み合わせることによって、副作用を抑えつつドナー造血幹細胞・リンパ球の生着を図り、抗腫瘍効果は主として移植後の同種免疫効果に期待するという考えに基づいて開発された移植法がミニ移植である。

本第Ⅱ相臨床試験の最大の特徴は、質の高いエビデンスを得るために公正で公開された厳正な新GCP 前方視的研究を実施することである。これにより、我が国の移植領域における臨床試験体制の基盤を確立すると同時に、得られたデータを用いて適応外医薬品の臨床承認申請を可能とする道を開く。ミニ移植の安全性と有効性をさらに高めるために、付随研究としてミニ移植やドナーリンパ球輸注療法 (donor lymphocyte infusion: DLI) の重要な合併症である graft-versus-host disease (移植片対宿主病: GVHD) やサイトメガロウイルス (CMV) 感染症を免疫学的側面から解明し、その対策を検討した。

## B. 研究方法

新たなプリン誘導体であるクラドリピンあるいはフルダラビンとブスルファンを併用するミニ移植を開発し、国立がんセンター中央病院において第Ⅰ相臨床試験を行った。対象となったのは造血器腫瘍患者のうち、他の治療では治癒や長期生存を期待できないような疾患や病状であるにもかかわらず、年齢制限 (55 歳) や各種の臓器機能障害があるために通常の血縁/非血縁者間同種造血幹細胞移植を行うことができない患者である。ドナーは HLA 一致あるいは一座不一致の血縁ドナーとし、G-CSF 5  $\mu$ g/kg を 1 日 2 回連日皮下注射し、4 日目から CD34 陽性細胞として  $3 \times 10^6$  個/kg の採取を目標にしてアフレーシスを行った。主要評価項目は、移植後 100 日以内の早期移植関連死亡とドナー型完全キメラの達成とした。またミニ移植における最適の GVHD 予防法を検討する目的で、同様の治療プロトコルを用いて多施設共同無作為比較第Ⅱ相臨床試験を開始し、移植した後にシクロスポリン単剤と、シクロスポリンとメトトレキサートの併用による方法の 2 群に無作為に割り付ける (各群 30 例、計 60 例)。この試験は、ミニ移植に必要な薬剤の適応拡大承認を得る必要性からも新 GCP に準拠して行う必要がある。治療の安全性を高めるためにも DLI の適正化を行い、またミニ移植後の免疫機能の評価として同種移植で最も重要な感染症である CMV 感染を指標とし、その発症

頻度および CMV 特異的 T 細胞の再構築について経時的に検討した。

## <倫理面への配慮>

対象患者については、いずれも患者本人に説明同意文書の内容を極力分かり易い言葉で説明し、説明同意文書 2 部を作製して本人に渡したうえで文書による同意を得た。説明同意文書に本人の自由意志で同意の署名がなされた後に、この文書の 1 部を本人に提供し、個人情報に厳重に保護することで倫理性も確保した。本研究は、参加各施設の倫理審査委員会における審査を受けて承認されることが条件となる。

## C. 研究結果及び D. 考察

国立がんセンター中央病院における第Ⅰ相臨床試験には、2004 年 12 月末現在で合計 280 名の患者を登録した。高リスク患者が対象となったにもかかわらず、HLA 一致血縁者間移植の場合には移植後 1 年以内の移植関連死亡の発生は 5% 以内に留まり治療の安全性が確認された。しかし、急性 GVHD の合併率は通常移植と同程度であったことから、その改善を目指した第Ⅱ相臨床試験を行うことの意義は大である。多施設共同第Ⅱ相臨床試験に関しては、移植領域における医師主導試験としては初めて、新 GCP に基づいた厳正で透明性のある臨床試験を遂行した。このデータベースの基本構造の開発を本分担研究の主業務としたが、これは、今後行われる移植領域における各種の臨床試験に汎用が可能であり、実際に厚生労働科学研究の効果的医療技術の確立推進臨床研究事業における研究活動の推進に大きく寄与した (「固形がんに対する同種細胞免疫療法を用いた標準的治療法の確立に関する研究」)。小児患者におけるミニ移植の妥当性や新たな DLI 戦略を検証し、臍帯血を用いたミニ移植の開発研究に着手した。付随研究として、移植後免疫の標的分子の同定やミニ移植後の CMV 特異的 T 細胞動態などを検討し、ミニ移植の特徴を免疫学的側面から解明する手がかりを提供した。移植治療の均てん化と国民医療におけるがん医療水準の向上に寄与する目的で、全国の施設からの研修医あるいは見学者を積極的に受け入れた。その結果、研究期間中の長期任意研修医数は 20 名近くに及んだ。

## E. 結論

厳正な臨床試験を行った本研究の結果、ミニ移植後の副作用は通常の移植と比較して比較的軽微であるため、従来は根治的治療法を持たなかった多くの高齢患者を救済できる可能性を示した。この過程で、我が国の移植領域における臨床試験基盤を構築し、医師主導臨床試験に基づいた適応外医薬品の申請を行う道を模索し、移植治療の均てん化にも大きく貢献した。移植後期に発生する GVHD や CMV 感染の解明と対策確立を目指した付随研究も推進した。

## F. 健康危機情報

現在までのところ、試験の中止に関わるような重大な有害事象の発生は認めていない。医薬品の適応拡大を目的とした医師主導臨床試験を計画・実施するうえでは、安全性情報の取扱いが最も重要な課題となる。このような質の高い臨床試験で得られる安全性情報は、将来的に国内の共有情報としてデータベース化することにより、規制当局への報告の際の重要なデータソースになると共に、我々自身に重要な意味を持って還元される貴重なデータとなり得る。また、これは、抗癌剤を含めた医薬品、医療機器、及び生物由来製品などの臨床開発、適正使用に大きく寄与すると考えられ、海外との情報交換も可能な形式での蓄積が必要不可欠であるため、本研究の重要課題の一つとして取り組んだ。

## G. 研究発表

一覧を別添する

## 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

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### 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

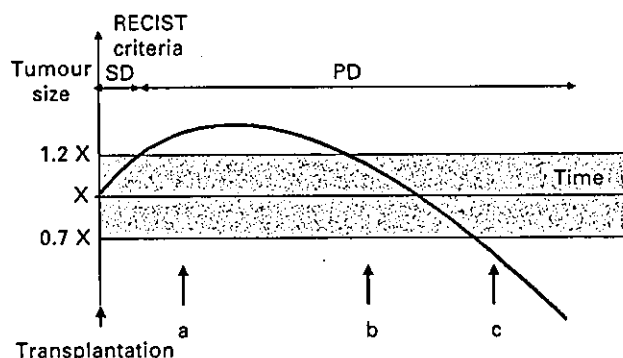
Hentschke *et al* recently reported a detailed case series on reduced-intensity stem-cell transplantation (RIST) for the treatment of renal cell and colon cancers.<sup>1</sup> While they provided important information on the feasibility of RIST and its possible antitumor effect, we would like to comment 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,<sup>2</sup> 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.<sup>3</sup> Some responses and GVHD effects, in general, occur during the late period of RIST. Thus, most tumors continue their natural growth until the manifestation of effective alloimmunity to restrain tumor growth. If the original RECIST criteria<sup>2</sup> are applied to patients undergoing RIST for solid tumors, most of the GVT effects would be evaluated as progressive disease (PD), which would preclude subsequent evaluation (Figure 1a). Therefore, RECIST may underestimate the efficacy of RIST. Furthermore, while there is no concept of spontaneous regression in the field of chemotherapy, this is quite common in RIST.

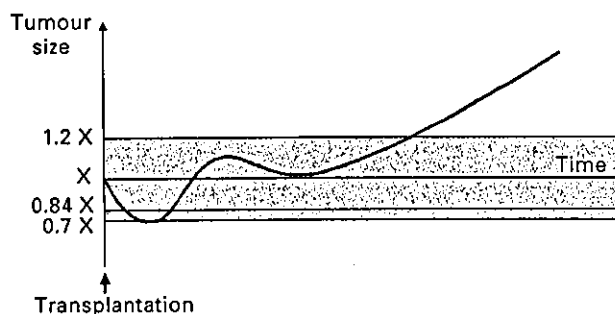
Second, the proper time to measure the tumor size as a baseline for evaluating a subsequent tumor response has not been clearly defined. In contrast to the results with chemotherapy, the tumor often temporarily increases in size following RIST. Some metastases initially progress slowly, while others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

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

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



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



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

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## 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  
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**Abstract** Cryopreservation of peripheral blood stem cells (PBSC) mobilized by chemotherapy combined with or without granulocyte colony-stimulating factor (G-CSF) is an essential part of procedure for anti-cancer strategies. We evaluated whether a higher cell concentration ( $2 \times 10^8$ /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  $2 \times 10^8$ /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  $2 \times 10^7$ /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 PBSC 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 ( $2 \times 10^8$ /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

### Introduction

Peripheral blood stem cell transplantation (PBSC) 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 ( $2 \times 10^7$ /ml) and rate control ( $-1^\circ\text{C}/\text{min}$ ) are critical in cell freezing procedure [2]. In case of PBSC, the traditionally recommended concentration of  $2 \times 10^7$ /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

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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  $\mu\text{g/kg}$  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  $2\text{--}5 \times 10^7/ml$  (Group I, n=20). Purified CD34+ cells were frozen at  $2 \times 10^7/ml$  using the same method (Group III, n=22). Subsequently, a concentration of  $2 \times 10^8/ml$  was adopted (Group II, n=44). In all methods, cells were transferred to 5-ml polypropylene cryo-tubes (MS4605 W, Sumitomo Bakelite, Osaka), placed directly into a  $-80^\circ\text{C}$  electric

freezer, and then transferred to  $-135^\circ\text{C}$  on the following day. The cells were stored in the same freezer until use.

Cells were thawed rapidly in a water bath maintained at  $37^\circ\text{C}$ . 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 IgG<sub>1</sub>-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  $\mu\text{l}$  of cell suspension were added to a test tube (Falcon 2052, Becton Dickinson, Lincoln Park, NJ, USA) containing isotype control (phycoerythrin-mouse IgG<sub>1</sub>) and phycoerythrin-conjugated CD34 monoclonal antibody (Anti-HPCA2 antibody, Becton Dickinson) at a concentration of 1  $\mu\text{g}$  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)  $\text{NH}_4\text{Cl}$ , 0.1%  $\text{KHCO}_3$ , and 0.004% EDTA-4Na.

### Hematopoietic progenitor assay

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

previously determined optimal concentration in our laboratory. Triplicate or quadruplicate cultures were plated in volumes of 0.4 ml in 24-well tissue culture plates (Corning 258201, New York, NY) that were then placed in an ESPEC N<sub>2</sub>-O<sub>2</sub>-CO<sub>2</sub> 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 PBSC.

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.

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

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### ABSTRACT

Adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) is characterized by poor prognosis after chemotherapy. Recent clinical trials have indicated, however, that allogeneic but not autologous hematopoietic stem cell transplantation (HSCT) for ATL can yield better clinical outcomes. In the present study, we investigated cellular immune responses of ATL patients who obtained complete remission after nonmyeloablative allogeneic peripheral blood HSCT from HLA-identical sibling donors. In the culture of peripheral blood mononuclear cells (PBMCs) from a post-HSCT but not pre-HSCT ATL patient, CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and CD25<sup>+</sup> 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.

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

## MATERIALS AND METHODS

**Recipient/Donor Pairs and Blood Samples.** Four acute type ATL patients, #37 (case 1), R07 (case 2), R11 (case 3), and #97 (case 4) and their corresponding HLA-identical sibling donors, #36, D07, D11, and #98, respectively, donated peripheral blood samples under written informed consent. The patients were participants in the clinical trial protocol for allogeneic HSCT for ATL with a reduced-intensity conditioning regimen that was supported by the Ministry of Health, Welfare, and Labor of Japan. After cyclophosphamide, doxorubicin, vincristine, prednisolone therapy, patient #37 at the beginning of recurrence, patients R07 and #97 in partial remission, and patient R11 in complete remission received conditioning treatment consisting of fludarabine (30 mg/m<sup>2</sup> 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<sup>+</sup> 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% CO<sub>2</sub> for over 2 months. An EBV-transformed B-cell line, LCL-#36, was established by main-

taining positively separated CD19<sup>+</sup> PBMCs from donor #36 in RPMI 1640 with 10% FCS after infection with an EBV-containing culture supernatant of the B95-8 cell line (31). TCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9; Ref. 32), ILT-As-2 (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), ILT-Myj-3 (HLA-A2/A24, B54/B60, Cw1/Cw3, and DR4/DR5; Ref. 21), ILT-Nkz-2 (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and ILT-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are HTLV-I-infected T-cell lines, and LCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9), LCL-As (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), TOK (HLA-A24/-, B52/-, and DR2/-; Ref. 33), LCL-Nkz (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and LCL-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are EBV-transformed B-cell lines. An erythroblastoid cell line, K562 (34), was also used.

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

**Induction of HTLV-I-Specific CTL.** One million whole PBMCs from post-HSCT patient #37 were stimulated with 1 µg/ml PHA-P and then mixed with the same number of ILT-#37 cells, derived from pre-HSCT patient #37, and pretreated with 1% formaldehyde/PBS. These T cells were maintained in AIM-V medium (Invitrogen-Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 0.5 mg/ml streptomycin, 10% heat-inactivated FCS, and 100 units/ml recombinant human IL-2 with periodic stimulation with formaldehyde-fixed respective ILT cells at 10-14-day intervals. PBMCs from donor #36 and pre-HSCT patient #37 were similarly stimulated with PHA and subsequently with formaldehyde-fixed ILT-#37 in cultures for CTL induction. CTL induced from CD8<sup>+</sup> 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<sup>+</sup> 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 <sup>a</sup> infection	HLA	In vitro immune analysis of PBMC <sup>b</sup>			HTLV-I proviral DNA (copies/1000 PBMC) <sup>d</sup>		Clinical outcome after HSCT
								Sampling date (days after HSCT)	Stimulated with	Induction of CTL <sup>c</sup>	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

<sup>a</sup> 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.

<sup>b</sup> 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."

<sup>c</sup> Culture in which CTL specific for autologous ILT cells grew is indicated as (+) and that without CTL induction is indicated as (-).

<sup>d</sup> 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.