ARTIFICIAL BLOOD

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To prepare for shortage of blood components and to avoid side effects such as blood borne infectious disease, blood substitutes such as artificial red cell (artificial oxygen carrier) and artificial platelet are being developed.

As for oxygen carriers, there are several candidates such as perfluorochemicals, modified hemoglobins and liposome encapsulated hemoglobins and albuimin heme. Perfluorochemicals have limited oxygen carrying capacity and oxygen inhalation is mandatory when they are used. Modified hemoglobins such as intermolecular or intramolecular cross linked hemoglobins have side effect to cause hypertension by scavenging nitro oxide (NO) which is produced by endothelial cells, because the size of these hemoglobins are small enough to go to the adjacent place near endothelial surface.

Hemoglobin vesicles (HbV) in which hemoglobins are encapsulated in liposome is most possible candidate for oxygen carrier. Usefulness and safety of the HbV is evidenced by animal shock model or exchange transfusion model and they are now being prepared for clinical trials as red blood substitutes or oxygen therapeutics. Albumin heme in which recombinant human serum albumin incorporating synthetic heme is thought an ideal resuscitation fluid as this material has colloid oncotic pressure.

Short time storage and viral infection are serious concern in platelet transfusion therapy for bleeding thrombocytopenic patients. Adhesion of the platelet to the collagen surface and aggregation at the bleeding sites to plug holes in blood vessels, and to facilitate the function of the remaining platelets is a starting point in developing platelet substitutes and several platelet substitutes have been proposed on this theory.

STEM CELLS AND DEVELOPMENT 13:315–322 (2004)
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Research Report

Cytotoxic Difference of T Cells Expanded with Anti-CD3 Monoclonal Antibody in the Presence and Absence of Anti-CD28 Monoclonal Antibody

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ABSTRACT

Bulk T cells can be expanded by CD3 stimulation alone (CD3-Ts) or by CD3/CD28 dual stimulation (CD3/CD28-Ts) of peripheral blood mononuclear cells (PBMC). However, few reports have described the difference of features between CD3-Ts and CD3/CD28-Ts. PBMC were stimulated with anti-CD3 monoclonal antibody (mAb) alone or co-stimulated with anti-CD3/CD28 mAbs immobilized on plastic plates, in the presence of rhIL-2 for 4 days, subsequently cultured in the presence of rhIL-2 with no antibody then analyzed. The expansion rate was significantly lower for CD3-Ts (965 + 510-fold, $n = 5$) than CD3/CD28-Ts (2263 + 856-fold, $n = 5$) ($p < 0.05$). The CD4/CD8 ratio, the percentage of $CD28^+$ cell, and the percentage of T cells with no ability to generate intracytoplasmic interleukin-4 (IL-4) or interferon-γ (IFN-γ) were all significantly higher, but, phenotypically, memory cells were lower in CD3/CD28-Ts than in CD3-Ts. The levels of activity of both natural killer (NK) and lymphocyte-activated killer (LAK) cells were lower in CD3/CD28-Ts than CD3-Ts. In comparison to CD3-Ts, CD3/CD28-Ts showed impaired migration toward RANTES. In conclusion, T cells expanded with anti-CD3 and anti-CD28 mAbs differ from those expanded with anti-CD3 alone with proliferation, cytotoxicity, chemotaxis, and phenotype. These differences may exert profound influences on the therapeutic potential of output cells.

INTRODUCTION

Antigen-specific T cell activation requires both a T cell receptor-mediated signal (1st signal) and a co-stimulatory molecule-mediated signal (2nd signal) (1,2). The events caused by CD28-mediated signals have been studied extensively in vitro using purified naive $CD4^+$ T cells, memory $CD4^+$ T cells, or $CD8^+$ T cells. It is believed that the role of the CD28-mediated signals is to enhance the signal intensity regulated through the T cell receptor (TCR) leading to the sufficient induction of cytokines such as interleukin-2 (IL-2) and expression of the IL-2 receptor. These events are believed to contribute to the enhancement of T cell proliferation and activation. CD28-mediated signals also have many other effects on T cell, for example, a reduced probability that lymphocytes all undergo apoptosis, possibly because of the induction of anti-apoptotic protein (3,4) or resistance to M tropic HIV (5). In addition, the 1st signal without the 2nd signal drives T cells into an anergic state (6). Together, these reports indicate that CD28 molecules and the signal transacted through them may influence the features of ex vivo-activated and proliferated T cells.

It has been reported that ex vivo expansion of peripheral blood T cells in numbers suitable for therapeutic use is possible by culturing T cells with a combination of

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anti-CD3 monoclonal antibody (mAb) and IL-2 (50–200 units/ml) and these bulk T cells have natural killer (NK)/lymphocyte-activated killer (LAK) activity (7–12). Therefore, they have been used for adoptive cellular immunotherapy in animal models and in clinical settings (13,14). In addition, T cells that are simultaneously activated by both anti-CD28 mAb and ant-CD3 mAb have been shown to proliferate better than T cells activated with anti-CD3 mAb alone (15,16). A clinical trial with doubly simulated bulk T cells as a tool for adoptive cellular immunotherapy has also been started (17,18).

Because of the recent clinical use of these T cells, it was important to elucidate the differences. Thus, we stimulated whole peripheral blood mononuclear cells (PBMNC) with mobilized anti-CD3 mAb alone or with immobilized anti-CD3 and immobilized anti-CD28 mAbs in the presence of rhIL-2 and analyzed the features of the output cells.

MATERIALS AND METHODS

Preparation of mAb-coated wells

Anti-CD3 mAb (OKT3) (Ortho Pharmaceutical Corp., NJ) and anti-CD28 mAb (PharMingen, San Diego, CA) were diluted at a concentration of 5 μg/ml in phosphate-buffered saline (PBS) (−). Twenty-four-well polystyrene plates (Sumitomo Bakelite Co., Tokyo, Japan) were coated overnight at 4°C with 150 μl of anti-CD3 mAb in combination with 150 μl of anti-CD28 mAb or PBS. The coated plates were washed several times with PBS(−) before use.

Preparation of cells and their ex vivo expansion

The ex vivo expansion of lymphocytes was performed as described elsewhere with some modification (7). After 4 days of culture in the presence of IL-2 and 10% fetal calf (FCS), cells were transferred to noncoated flasks and maintained by replenishing medium containing 175 units/ml of recombinant human (rh)IL-2 every 2 or 3 days. The total expansion rate was calculated as the product of expansion measured at each passage (7). Cells cultured for 12–14 days were used for experiments. The "bulk" T cells expanded by CD3 stimulation alone and CD3 and CD28 stimulation were designed by CD3-Ts and CD3/CD28-Ts, respectively. Informed consent was obtained from all donors.

Analysis of cell-surface markers

mAbs used for cell-surface marker analysis were as follows: FITC- or phycoerythrin (PE)-conjugated anti-CD3, anti-CD56, and anti-CD62L were from Immunotech (Marseille, France); anti-CD4, anti-CD8, anti-$\alpha\beta$TCR, anti-CD45RA, anti-CD45RO, anti-CD95, and anti-CD28 were from PharMingen; anti-CD18 and anti-$\gamma\delta$TCR were from Becton Dickinson (San Jose, CA); and anti-CD25 and anti-CD45RA were from DAKO A/S (Denmark). For staining the chemokine receptor, fluorescein isothiocyanate (FITC)- or PE-conjugated anti-CCR3, anti-CXCR-4, anti-CXCR-5 (DAKO) and anti-CCR5 (PharMingen) were used. Analysis was performed using Cytoron (Coulter, Tokyo, Japan) flow cytometer, and at least 4000 events were analyzed.

Preparation of $CD3^+$, $CD4^+$, and $CD8^+$ T cell-rich fractions

$CD4^+$ and $CD8^+$ T cell subsets were enriched by negative selection using anti-CD8 and anti-CD4 magnetic beads (Dynal, Lake Success, NY), respectively, according to the manufacturer's protocol. The purity of the $CD4^+$ or $CD8^+$ T cell fraction was greater than 90%.

Assay for NK cell and LAK cell activities

A conventional 4-h ^{51}Cr release assay was performed on days 12–14 using K562 cells for the NK activity assay and Daudi cells for the LAK activity assay as described elsewhere (7). In some experiments, concanamycin A (CMA) (Sigma) was added to the well at a specific concentration to inhibit perforin-mediated cytotoxicity (19).

Intracellular cytokine and perforin staining

For cytokine detection at a single-cell level, the expanded cells (5×10^6) were incubated with or without 40 ng/ml of phorbol myristate acetate (PMA) (Sigma) and 4 μg/ml of ionomycin (Sigma) in the presence of 40 μg/ml of brefeldin A (Sigma) for 4 h in 5% CO_2 at 37°C in conditioned medium. Then, cell-surface molecules were stained using Pc5-conjugated anti-CD3, CD4, or CD8 mAbs (Immunotech). Cells were fixed and permeabilized using IntraPrep permeabilization reagent (Coulter, Tokyo, Japan). Subsequently, intracellular cytokine or perforin was stained using FITC-anti-interferon-γ (IFN-γ) and PE-anti-IL-4 mAbs (Immunotech) or FITC-anti-perforin mAb (PharMingen). Nonspecific fluorescence was analyzed using isotype-matched control mAbs. Analysis was performed using an Epics XL flow cytometer. $CD3^+$, $CD4^+$, or $CD8^+$ T cells were gated and up to 15,000 events were acquired for each analysis.

Extracellular cytokine selection

Expanded cells (2×10^6) were washed twice in PBS and cultured in 2 ml of culture medium with or without

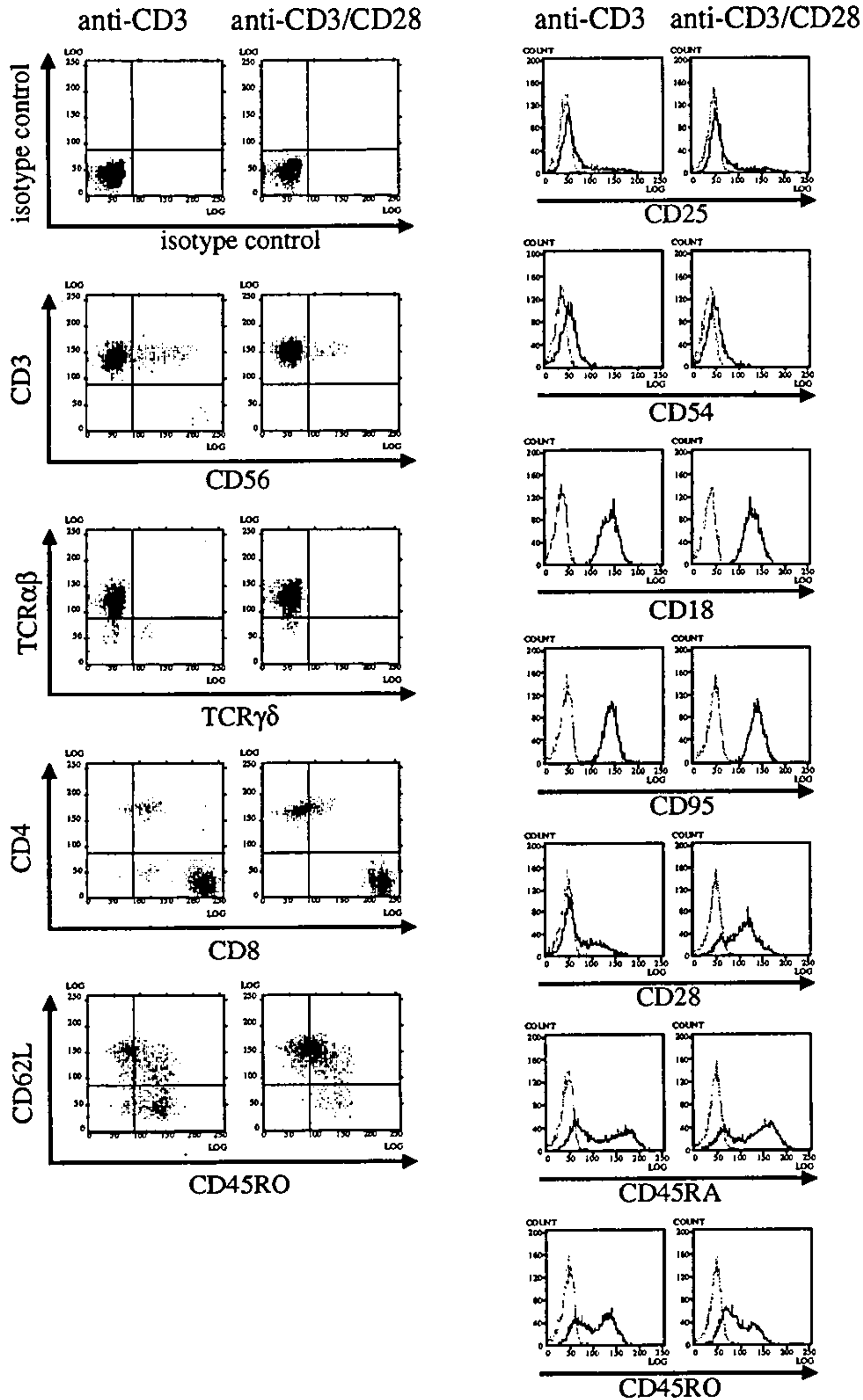

FIG. 1. Comparison of the expression of cell-surface molecules. CD3-Ts and CD3/CD28-Ts were collected on culture days 12–14 and assessed for the expression of various cell-surface molecules by flow cytometry (A, double staining; B, single-staining). Data shown are representative of five independent experiments.

TABLE 1. ANALYSIS OF CELL-SURFACE MARKERS AND INTRACYTOPLASMIC PERFORIN

	CD3-Ts	*CD3/CD28-Ts*	
$CD4^+$ T cells	19 ± 10 (10–36)	36 ± 4 (30–41)	$p < 0.05$
$CD8^+$ T cells	72 ± 9 (58–81)	59 ± 3 (56–63)	$p < 0.05$
CD4/CD8 ratio	0.28 ± 0.20	0.61 ± 0.09	$p < 0.05$
			Mean ± SD (range), $n = 5$

	Experiment 1			*Experiment 2*		
Subset	*Fresh PBMC*	*CD3-Ts*	*CD3/CD28-Ts*	*Fresh PBMC*	*CD3-Ts*	*CD3/CD28-Ts*
CD3	18.7	9.3	2.2	34.6	21.7	8.8
CD4	4	0.4	0.1	11.6	1.4	0.7
CD8	64.3	7.5	3.2	49.3	22.3	6.1

CD3-Ts and CD3/CD28-Ts were collected on culture days 12–14 and the percentage of $CD3^+$, $CD4^+$, and $CD8^+$ cells (upper) and intracytoplasmic $perforin^+$ cell in each subset (lower) was assessed by flow cytometry. Statistical analyses were performed using the paired *t*-test.

10 ng/ml of PMA and 400 ng/ml of ionomycin for 24 h in 5% CO_2 at 37°C. Then, cytokine concentrations in the culture supernatant were assayed with an ELISA kit for IFN-γ, tumor necrosis factor-α (TNF-α), IL-4, IL-10, and IL-13 (BioSource International, CA) according to the manufacturer's directions. The detection limit of the ELISA kit for IFN-γ, TNF-α, IL-4, IL-10, and IL-13 was 8.6, 1.7, 2.0, 5.0 and 16.5 pg/ml, respectively.

Chemotaxis assays

Chemotaxis toward various concentration of SDF-1α and RANTES (R&D Systems, Inc., Minneapolis, MN) were assayed using transwell polycarbonate membranes (24-well format, 5-μm pore size) (Kurabo, Osaka, Japan). The expanded cells (5×10^5/200 μl) were added to the upper chamber. After incubation for 4 h at 37°C in 5% CO_2, the transmigrated cells in the lower chamber were enumerated. The percent migration was calculated as (the absolute number of cells from that population migrating in the presence of chemokine)/(the total number of cells) × 100.

Statistics

For the statistical analyses, the paired Student's *t*-test was used. The Mann–Whitney U test was used for comparison of the cumulative expansion rate.

RESULTS

Evaluation of cell proliferation and cell-surface markers and chemotaxis

The expansion rate on day 14 of CD3-Ts and CD3/CD28-T was 965 ± 510-fold (range 326–1551-fold, $n = 5$) and 2263 ± 856-fold (range 1428–3527-fold, $n = 5$), respectively. As can be expected, there was a significant difference ($p < 0.05$), and, regardless of CD28 co-stimulation, most of the expanded cells were positive for CD3 and $\alpha\beta$TCR and some (10–20%) co-expressed CD56. Substantial numbers of $\gamma\delta TCR^+$ T cells were also observed in some experiments (data not shown). The difference in the percentage of $CD4^+$ T cells, $CD8^+$ T cells, or CD4/CD8 ratio in CD3-Ts and CD3/CD28-Ts was significant ($p < 0.05$) (Fig. 1A, Table 1, upper). Some of the cells expressed CD25 (IL-2 receptor), and CD54 (ICAM-1), and most expressed CD95 (Fas) and CD18 (LFA-1), regardless of CD28 co-stimulation (Fig. 1B). More than 90% of CD3/CD28-Ts compared to approximately 60% of CD3-Ts were positive for CD28. The percentage of $CD45RA^+$ cells tended to be higher among CD3/CD28-Ts than CD3-Ts. In contrast, the percentage of $CD45RO^+$ T cells tended to be lower in the former than the latter (Fig. 1B). The percentage of $CD45RO^+CD62L^-$ (memory T) cells was significantly lower among CD3/CD28-Ts (14.7 ± 9.3%, $n = 5$) than CD3-Ts (28.5 ± 8.5%, $n = 5$)) ($p < 0.005$) (data not shown). Both bulk T cells equally migrated toward SDF-1. In contrast, CD3/CD28-Ts migrated significantly less than CD3-Ts in response to RANTES (data not shown).

Evaluation of killing activity and percentage of perforin-positive cell

Both NK and LAK activity was significantly weaker in CD3/CD28-Ts than CD3-Ts at all E/T ratios tested ($p < 0.05$) (Fig. 2A). To address whether or not this difference can be explained by the different CD4/CD8 ratio, we compared the CD4-rich population with the CD8-rich population of the same bulk T cells in terms of their killing activity. However, there was no significant difference

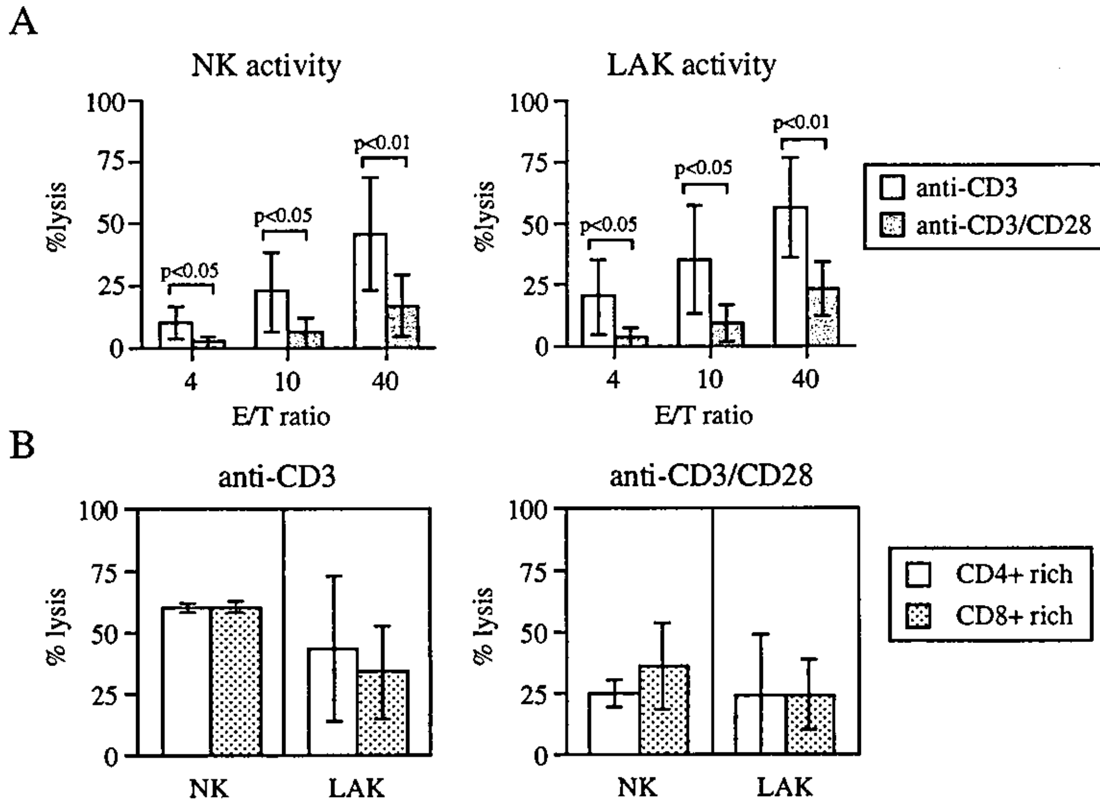

FIG. 2. Comparison of NK activity and LAK activity. (**A**) CD3-Ts and CD3/CD28-Ts were collected on culture days 12–14 and NK activity and LAK activity were measured at an E/T ratio of 4, 10, and 40. The results of seven independent experiments are shown as the mean ± SD. Both the NK and LAK activity were significantly higher in CD3-Ts than CD3/CD28-Ts ($p < 0.05$). (**B**) $CD4^+$ T cell and $CD8^+$ T cells were negatively selected from the bulk T cells, and their killing activities were measured at an E/T ratio of 40. The results of three independent experiments are shown as the mean ± SD. There was no significant difference between the NK (or LAK) activity of the $CD4^+$-rich fraction and $CD8^+$-rich fraction.

between the fraction (Fig. 2B). The killing activity of these bulk T cells was clearly blocked by CMA (data not shown), suggesting that it is mainly mediated by granule exocytosis (perforin/granzyme pathway). The percentage of perforin-positive cells in both bulk T cells populations was lower than that in fresh T cells. Furthermore, the percentage of perforin-positive cells was lower among CD3/CD28-Ts than CD3-Ts (Table 1, lower).

Evaluation of cytokine production

To determine if the expanded T cells become Th1 (Tc1)-type or Th2 (Tc2)-type cells, we evaluated the cytokine production at a single-cell level (Fig. 3). Neither IFN-γ- nor IL-4-positive cells were detected without restimulation (data not shown). When $CD3^+$ T cells were gated, $IFN\text{-}\gamma^+$ $IL\text{-}4^-$ (Th1/Tc1) cells were dominant in both bulk T cells. However, the percentage of Th1/Tc1 cells were significantly lower among CD3/CD28-Ts (53.7 ± 13.9%) than CD3-Ts (71.4 ± 9.4%) ($p < 0.05$). In contrast, the percentage of $IFN\text{-}\gamma^-/IL\text{-}4^-$ (Th0/Tc0) cells was significantly higher among CD3/CD28-Ts (43.2 ± 15.6%) than CD3-Ts (21.6 ± 7.4%) ($p < 0.05$). The same results were observed when $CD4^+$ or $CD8^+$ T cells were gated. Even if the incubation time with PMA and ionomycin was extended to 24 h, the results were the same. Next, we measured the cytokine levels in the cultured supernatant of expanded bulk T cells. Without restimulation, the levels of the cytokine measured were all below or near the detection limit (Table 2). After stimulation, the production of Th1 cytokines (IFN-γ and TNF-α) was remarkably enhanced compared with the production of Th2 cytokines (IL-4, IL-10, and IL-13) in both of the bulk T cells, although the data varied among experiments. This is basically consistent with the observation at a single cell level.

DISCUSSION

CD28 co-stimulation affected the characteristics of expanded cells at day 14

The expansion rate, the CD4/CD8 ratio or percentage of $CD4^+$ T or $CD28^+$ T cells on day 14 was significantly

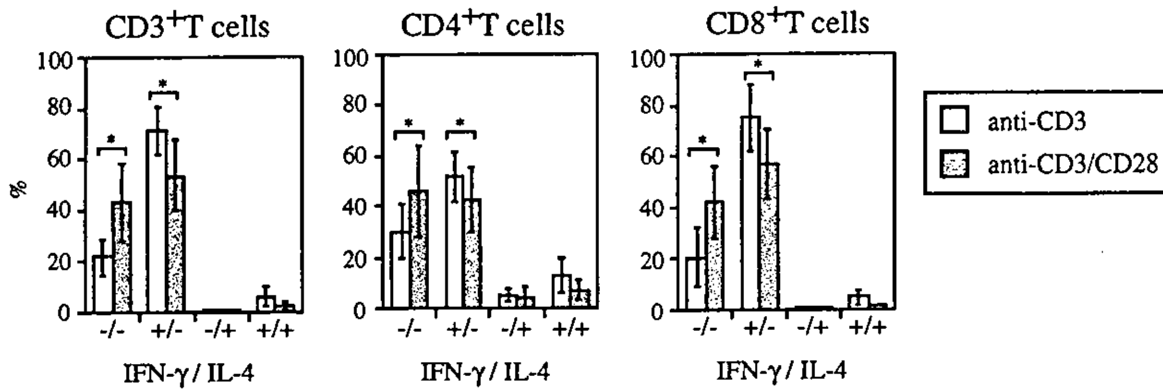

FIG. 3. Comparison of the expression of intracellular IFN-γ and IL-4. CD3-Ts and CD3/CD28-Ts were collected on culture days 12–14 and restimulated with PMA and ionomycin for 4 h. Then, intracellular IFN-γ and IL-4 were analyzed by flow cytometry as described in Materials and Methods. The results of four independent experiments are shown as the mean ± SD. Statistical analyses were performed using the paired t-test. (*) The difference is significant ($p < 0.05$). The percentage of IFN-γ^-IL-4^- T cells was higher ($p < 0.05$) in CD3/CD28-Ts than in CD3-Ts.

higher in CD3/CD28-Ts than CD3-Ts. Furthermore, several additional differences were revealed. That is, compared with CD3-Ts, the percentage of phenotypically memory T cells, (CD45RO$^+$CD62L$^-$T cells) (20,21) is lower in CD3/CD28-Ts, and the percentage of T cells that produce neither IL-4 nor IFN-γ (nonpolarized T cells) (22) is significantly higher in CD3/CD28-Ts (Fig. 3). In addition, CD3/CD28-Ts showed impaired migration to RANTES compared with CD3-Ts. These differences indicate that CD28 costimulation affects the characteristics of CD3-Ts. However, whether this difference was due to CD28 co-stimulation itself or merely reflects the difference in nature between CD28$^+$ T cells (which are abundant in CD3/CD28-Ts) and CD28$^-$ T cells (which are abundant in CD3-Ts) remains to be addressed.

As far as the cytokine production pattern is concerned, both bulk T cells were demonstrated to have the ability to secrete huge amounts of tumoricidal cytokine (IFN-γ and TNF-α). Consistent with this intracytoplasmic cytokine analysis showed that Th1 (or Tc1) type cells were predominant in both bulk T cell populations (Fig. 3).

CD28 co-stimulation affected the NK/LAK activity at day 14

The percentage of perforin$^+$ cells decreased in both bulk T cells compared with fresh-CD3$^+$ T cells (Table 1, lower). This suggests that CD3 and/or CD28 co-stimulation may not enhance the production of perforin. It

TABLE 2. COMPARISON OF CYTOKINE PRODUCTION

Restimulation	Cytokines	1st stimulation: Anti-CD3 mAb	1st stimulation: Anti-CD3/CD28 mAbs
−	IFN-γ	9.8 ± 2.1	8.7 ± 0.2
−	TNF-α	8.0 ± 2.4	7.8 ± 3.0
−	IL-4	2.2 ± 0.2	2.4 ± 0.5
−	IL-10	<5.0	<5.0
−	IL-13	22.1 ± 9.7	<16.5
+	IFN-γ	51.009 ± 30.748	51.346 ± 43.148
+	TNF-α	12.050 ± 6.348	13.863 ± 12.741
+	IL-4	235.4 ± 101.8	121.5 ± 90.6
+	IL-10	29.1 ± 25.1	27.6 ± 26.2
+	IL-13	1161.5 ± 362.7	693.9 ± 499.0
			Mean ± SD, n = 3

CD3-Ts and CD3/CD28-Ts were collected on culture days 12–14 and restimulated with or without PMA and ionomycin for 24 h as described in Materials and Methods. The concentration of cytokine in the culture supernatant was measured by ELISA. The results of three independent experiments are shown as the mean ± SD (pg/ml). There were no significant differences in cytokine production ability between CD3-Ts and CD3/CD28-Ts.

should be stressed that the NK/LAK activity of CD3/CD28-Ts was significantly weaker than that of CD3-Ts, because it is believed to be important for the in vivo antitumor effect. This may not reflect the difference in CD4/CD8 ratio between them, because when the killing activity of the CD4-rich fraction and CD8-rich fraction in CD3/CD28-Ts was compared, no significant difference was found (Fig. 2B). The same result was obtained when CD3-Ts were analyzed. In addition, we could not see any apparent difference in cell surface expression of CD18 and CD54, which are supposed to be involved in nonspecific killing (Fig. 1B). Based on the fact that the NK/LAK activity was clearly blocked by CMA, the killing activities of these bulk T cells must mainly be mediated by granule exocytosis (perforin/granzyme pathway) (19). In this sense, the fact that the percentage of perforin$^+$ cells was lower in CD3/CD28-Ts than in CD3-Ts may explain, to some extent, why CD3/CD28-Ts killed the target less effectively than CD3-Ts. It is possible that some other factor, such as granulysin, plays a role in the killing activity because the percentage of perforin$^+$ cells (especially in the case of CD4$^+$ T cells) was less than 1%, while these CD4$^+$ T cells still showed NK/LAK activity (23).

Overall, in both CD3-Ts and CD3/CD28-Ts, Th1 (and Tc1)-type cells were dominant and showed no difference of cytokine production ability. However, they were different in terms of expansion rate and cell-surface marker phenotype, such as CD4/CD8 ratio, percentage of cells with memory phenotype, percentage of cells with Th0/Tc0-type cells, chemotaxis toward RANTES, and NK/LAK activity. These facts, at least, the difference in killing activity between them, should be taken into consideration, because the difference may have influence on their therapeutic potential in antitumor immunotherapy.

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Received June 30, 2003; accepted April 17, 2004.

Epstein-Barr virus 特異的 CD4 陽性 bulk cytotoxic T lymphocytes の *ex vivo* 増幅と解析

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日本輸血学会雑誌　第50巻　第4号　別刷

Japanese Journal of Transfusion Medicine, Vol. 50. No. 4 50(4): 588—595, 2004

原 著

Epstein-Barr virus 特異的 CD4 陽性 bulk cytotoxic T lymphocytes の *ex vivo* 増幅と解析

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(平成 15 年 12 月 8 日受付)
(平成 16 年 3 月 4 日受理)

Ex vivo EXPANSION AND CHARACTERIZATION OF EPSTEIN-BARR VIRUS-SPECIFIC CD4-POSITIVE BULK CYTOTOXIC T LYMPHOCYTES

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Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (CTLs) were induced from PBMCs of a sero-positive healthy donor by stimulation with an autologous EBV-transformed B-lymphoblastoid cell line (EBV-LCL). CD4$^+$ lymphocytes with high cytotoxic activities were found in the cultured fraction. The purified CD4$^+$ bulk CTLs were expanded with immobilized anti-CD3 and anti-CD28 antibodies in the presence of IL-2. The CD4$^+$ CTLs showed vigorous proliferation, up to 6,000-fold cumulative expansion. They maintained high killing activity against autologous target cells while exhibiting no NK and LAK activities, even after expansion. These data indicate that *ex vivo* expansion with immobilized antibodies is useful to obtain a large number of CTLs without losing their specific activities. The cytotoxity mediated by the CD4$^+$ bulk CTLs seemed to depend mainly on the perforin/granzyme pathway because a H$^+$-ATPase inhibitor, concanamycin A, strongly inhibited the killing. The CD4$^+$ CTLs proliferated in response to autologous monocyte-derived dendritic cells pulsed with one of the EB viral proteins, EBNA 1. This finding suggests the existence of EBNA 1-recognizing clone(s) in the cell fractions. When stimulated with autologous EBV-LCL or activated with PMA and ionomycin, the CD4$^+$ CTLs predominantly produced IFN-γ, not IL-4, indicating that they belong to the T helper 1 (Th1) type.

Key words: CD4$^+$ CTL, *ex vivo* expansion, EBV, EBNA-1

はじめに

Epstein-Barr virus (EBV) はヒトヘルペスウィルスのひとつで，我が国の成人では 95% 以上に不顕性感染がみられる．感染の主な標的はＢリンパ

球で[1]，正常な個体内では，細胞傷害性T細胞（CTL）が細胞表面上のウィルス抗原を認識して排除することにより[2]，感染Bリンパ球の増殖が抑制されている．しかしながら，移植後の患者のような強度の免疫抑制状態におかれている個体ではEBVが再燃し，リンパ球増多症，日和見リンパ腫等をひきおこす[3]．EBVが潜伏感染している細胞では，限られたウィルス遺伝子の発現により，核抗原（EBV-nuclear antigen；EBNA）1，2，3A，3B，3C，LPと潜伏感染膜蛋白（latent infection membrane protein；LMP）1，2A，2Bが検出される．これらの発現は宿主細胞によって異なり，EBNA 1のみが発現しているI型，さらにLMP 1と2とが発現しているII型，上記の分子全てが見られるIII型とに分けられる[1][4]．

近年，再燃EBVに対する治療法として，選択的攻撃性のあるCTLの応用が期待されている．その際には，特異性の高い細胞傷害活性と，有効な数の輸注細胞の確保が必須である．通常CTLはCD8陽性のリンパ球であるが，しかしながらヘルパーT細胞（Th）と呼ばれるCD4陽性Tリンパ球のなかに細胞傷害活性を有する一群が存在するという報告が相次いでなされてきた[5]~[7]．CD4陽性Tリンパ球の細胞傷害機序にはCD8陽性Tリンパ球と同様，Fas-Fas ligand（FasL）と，perforin/granzymeの二経路が存在する．後者の経路はvacuolar type H^+-ATPaseの選択的阻害剤であるconcanamycin Aにて阻害される[8][9]．

我々はEBVに対するCTLの臨床応用を目的とし，*ex vivo*での誘導を行った．その過程で1名のドナー由来CD4陽性画分に細胞傷害活性が検出された．この細胞集団を抗CD3抗体ならびに抗CD28抗体を固相化したプレートにて増幅し，*in vitro*での解析を行ったので報告する．

材料と方法

1. ヒト末梢血検体

インフォームドコンセントの得られたEBV陽性健常人よりヘパリン加採取した末梢血をFicollに重層し，定法に従って末梢血単核球（PBMC）を得た．下記の「2. 細胞株と培養条件」，「3. 抗EBV-LCL CD4陽性CTLの誘導と固相化抗体による増幅」に記載した実験を行う都度，繰り返し採血した．全ての実験は同一ドナー由来末梢血から誘導したCTLを用いて行ったものである．

2. 細胞株と培養条件

Bリンパ芽球様細胞株（EBV-LCL）HA-2は，定法に従い0.1μg/mlサイクロスポリン存在下，EBV産生株B95-8培養上清とPBMCを培養して樹立した．B細胞マーカーCD19, CD20, CD22, CD80, CD86, CD19陽性，T細胞マーカーCD2, CD3, CD4, CD8陰性であった．HA-2, K562, Daudiは10% fetal calf serum（FCS）を含むRPMI-1640にて継代培養した．ヒト巨核芽細胞UT7/TPOは10% FCSと10ng/mlトロンボポエチン（キリンビール株式会社，東京）を含むIscove's Modified Dulbecco's Mediumにて継代培養した．

3. 抗EBV-LCL CD4陽性CTLの誘導と固相化抗体による増幅

CTLの誘導は，EBV-LCLを刺激細胞としたautoの系で行った．γ線照射（20 Gy）したHA-2と自己PBMCを10% FCSを含むRPMI-1640＋7（日研生物医学化学，京都）培地中にて共培養を繰り返すことによって得た．初回刺激はPBMCとHA-2との比率を20：1で行い10日間培養した．2回目は比率を10：1とし，50U/mlのIL-2（藤沢薬品，大阪）を添加した．3回目以降は5：1の比率で刺激を行い，175U/mlのIL-2濃度で培養を続けた．得られたリンパ球画分からCD8陽性細胞をDynabeads M-450 CD8（DYNAL A.S., Oslo, Norway）にて除き，CD4陽性画分を得た（Fig. 1）．

抗CD3/CD28抗体刺激による増幅は[10]，抗CD3抗体（Ortho Pharmaceutical Corp., Raritan, NJ, USA）と，抗CD28抗体（BD Bioscience, San Jose, CA, USA）を固相化したプレートにCD4陽性CTL，それと同数のγ線照射自己PBMCとを加え，175U/mlのIL-2を含む培地中で培養することによって行った．培養4日目に抗体を固相化していないプレートに移し，IL-2存在下で引き続き培養を続けた．

4. 細胞傷害活性の測定

細胞傷害活性は，^{51}Crにてラベルした細胞を対

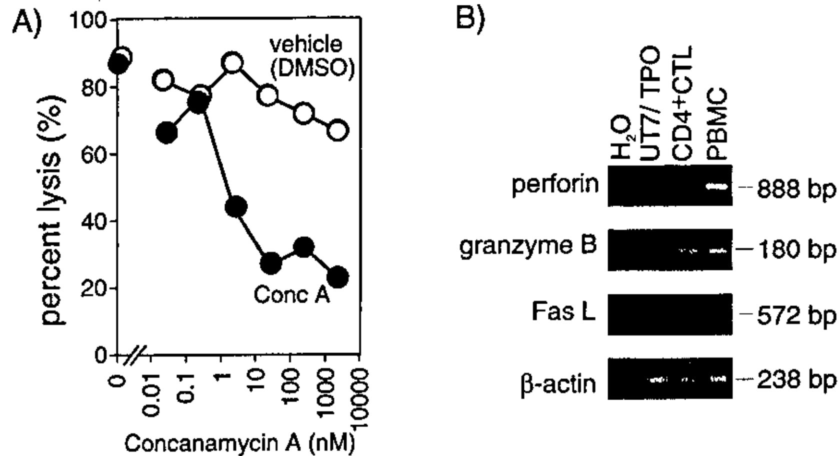

Fig. 4 Mechanism of cytotoxicity mediated by CD4$^+$ bulk CTL
(A) ^{51}Cr-releasing assay was performed in the presence of various concentrations of concanamycinA (ConcA). (B) RT-PCR analysis was investigated on perforin, granzymeB and FasL, and the PCR product was 888 bp, 180 bp and 572 bp respectively. β-actin mRNA was used as an internal control (238 bp PCR products). PCR primers were as follows : perforin sense 5'-GAG GCC CAG GTC AAC ATA GGC ATC-3' and antisense 5'-TCA CCA CAC GGC CCC ACT CCG GTT-3' ; GranzymeB sense 5'-TGC AGG AAG ATC GAA AGT GCG-3' and antisense 5'-GAG GCA TGC CAT TGT TTC GTC-3' ; FasL sense 5'-GGA TTG GGC CTG GGG ATG TTT CA-3' and antisense 5'-GAG CTT ATA TAA GCC GAA AAA CG-3 ; β-actin sense 5'-GGG TCA GAA GGA TTC CTA TG-3' and antisense 5'-GGT CTC AAA CAT GAT CTG GG-3'. PCR thermal cycles were as described in *Materials and Methods*.

concanamycin A 存在下で CTL assay を行った．細胞傷害活性は concanamycin A の濃度依存的に著しく低下し（Fig. 4A），concanamycin A 非存在時の活性（89%）に比して，その 1/4 程度（23%）まで低下した．対照（DMSO）では活性の顕著な低下は見られなかった．以上のことから，得られた CD4 陽性 bulk CTL では，perforin/granzyme 系が主たる細胞傷害メカニズムと考えられた．RT-PCR を行ったところ，CD4 陽性 CTL では perforin，granzyme さらに FasL の mRNA の発現が確認された（Fig. 4B）．他方，巨核球系の細胞である UT7/TPO では，細胞傷害活性を担うこれらの分子の発現は見られなかった．

3. 認識しているウィルス抗原同定の試み

EBV 核抗原のひとつである EBNA1 の組み換えタンパクをパルスした自己樹状細胞と，CD4 陽性 CTL 画分とを共培養したところ，パルスした濃度依存的に［^{3}H］TdR の取り込み量が増し，対照に比して 2.8 倍まで上昇した（Fig. 5）．このことから bulk CD4 陽性 CTL 画分に class II MHC 上に提示された EBNA 1 由来ペプチド断片を認識するクローンの存在が示唆された．

4. 刺激によって Th1 タイプのサイトカインの産生が見られる

増幅した bulk CD4 陽性 CTL を HA-2 にて刺激し，その際に産生されるサイトカインを検討した（Fig. 6）．HA-2 と共培養後の細胞内サイトカイン染色を行ったところ（Fig. 6A，CTL＋HA-2），11.3% が IFN-γ 陽性，3.14% の細胞が IL-4 陽性となった．PMA と ionomycin による活性化の際（Fig. 6A，activated CTL）には 67.3% が IFN-γ 陽性，15.8% の細胞が IL-4 陽性と，IFN-γ 陽性細胞の占める割合が高かった．細胞外に分泌されたサイトカインの定量（Fig. 6B）においても同様の結果

が得られた．以上のことから本実験によって得られたCD4陽性CTL画分ではTh1タイプの細胞が優位であると考えられた．

考　察

EBV-LCL刺激にて自己CTLを誘導した．1名のドナーよりCTL活性を有するCD4陽性細胞を得た．

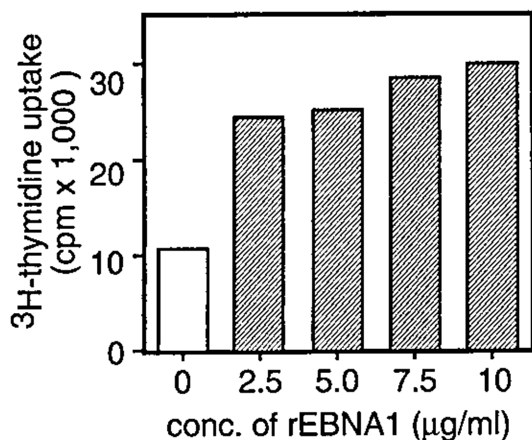

Fig. 5　EBNA 1-specific proliferation
Proliferative response of EBV-specific CD4^{+} bulk CTLs was measured by ^{3}H-thymidine incorporation. Responses to EBNA 1 (0-10μg/ml) were observed. Representative results of three similar experiments are shown.

この細胞集団を抗CD3抗体ならびに抗CD28抗体を固相化したプレートにて増幅したところ，増幅後も高い細胞傷害活性を示し，他方NK活性（K562に対する細胞傷害活性），LAK活性（Daudiに対する細胞傷害活性）は検出されなかった．以上の結果から，選択的攻撃性の高いCTLを誘導した後に固相化抗体での増幅を行うという手法は，臨床応用に必要な細胞数のCTLを簡便に得る手法として適しているものと考えられた．

結果には示さないが，抗DR抗体と抗DQ抗体存在下ではHA-2に対する細胞傷害活性が低下した．また自己EBV-LCLであるHA-2のclass II HLAタイプ（DRB1*0403/0901，DQB1*0301/0302）とDRB1*0901のみが共通のアロEBV-LCLや，DQB1*0301が一致しているアロEBV-LCLとの反応性も見られたことから（CTLアッセイ，細胞外IFN-γ産生），得られたCTL画分中には異なるclass II HLA拘束性のクローンが含まれるものと推測された．

本研究で得られたCD4陽性bulk画分では，perforin/granzyme系が主たる細胞傷害機序との結果が得られたが，concanamycin Aを高濃度にしても活性が完全には阻害されなかったこと，またFasLのmRNA発現もRT-PCRにて確認され

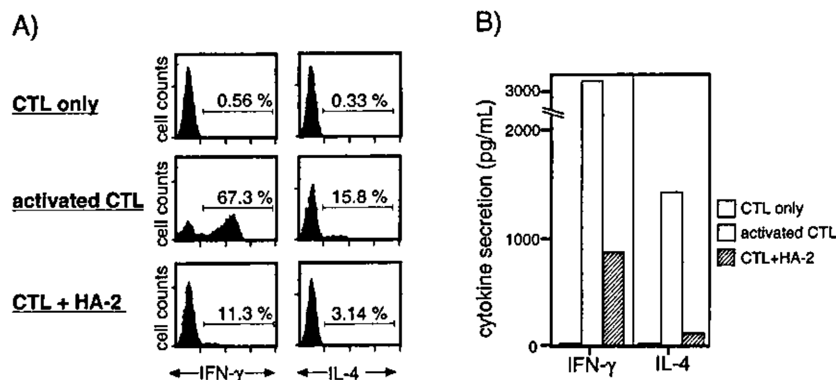

Fig. 6　Th1-type behavior of EBV-specific CD4^{+} bulk CTLs
Expression of IFN-γ and IL-4 was examined. (A) Intracellular cytokines were analysed by flow cytometry. Cells were gated on CD4^{+} T lymphocytes and the results were displayed as histograms. (B) Secreted cytokine concentrations were determined by ELISA.

た (Fig. 4B) ことなどから，Fas-FasL経路による細胞傷害の可能性も否定することはできない．また，本研究では検討していないが，TRAIL[11]，granulyisin[12][13]といった近年新たに報告されてきた細胞傷害性分子の関与も考えられる．

自己EBV-LCLでの刺激，あるいはPMAとionomycinによる活性化の際には，Th1タイプサイトカインであるIFN-γの産生が，Th2タイプサイトカインのIL-4に比較して優位であった．この結果はEBVに対するCD4陽性CTLで得られてきた結果[14)~16]と同様のものである．

これまでにもEBNA 1を認識するCD4陽性CTLは報告されているが[14)~17]，本研究にて得られたbulk CD4陽性CTL画分にもウィルス核抗原EBNA 1反応性のクローンの存在を示唆する結果が得られた．冒頭で触れたとおり，EBNA 1は全ての不顕性感染細胞において発現しているウィルスタンパクであるが，分子N末端にあるGly/Alaリピートドメインの存在によりclass I MHC上へ抗原提示が抑制されているため[18][19]，CD8陽性CTLの免疫応答から免れている[19]．よってEBVに対する生体防御，特にバーキットリンパ腫のようにEBNA 1のみを発現している細胞に対する免疫応答においてはCD4陽性CTLが重要な働きを担っている可能性が考えられる．細胞内抗原であるEBNA 1がclass Iではなくclass II MHC上にcross-presentationされるということは，EBV-LCL[14][17]やバーキットリンパ腫細胞[14][16][17]でも報告されており，本論文中のHA-2細胞でも同様のメカニズムで抗原提示されているものと推測される．

本手法にて試みた限りでは，報告したドナー以外にこのような細胞集団が誘導されたドナーはなかったが，異なる刺激法を用いるなどして他ドナーからCD4陽性CTLが得られる可能性も考えられる．

骨髄移植後患者でのEBVによるリンパ腫発症の回避を目的とし，ドナーPBMCからEBV特異的なポリクローナルCTLを誘導し予防的に投与する試みもなされている[20]．今回報告したCD4陽性bulk CTLも*in vivo*で有効な細胞傷害活性を示すことが期待される．

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臍帯血凍結保存状態に関する保管検体による評価の有用性の検討

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日本輸血学会雑誌　第50巻　第4号　別刷

Japanese Journal of Transfusion Medicine, Vol. 50. No. 4 50(4)：620—625, 2004

原　著

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(平成16年3月5日受付)
(平成16年4月7日受理)

QUALITY ASSURANCE OF CRYOPRESERVED CORD BLOOD UNITS BY EXAMINATION OF TEST SAMPLES

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Quality control of cryopreserved cord blood has assumed greater importance as the number of unrelated donor umbilical cord blood transplantations has increased. At present, we examine cryopreserved samples before cord blood transplantations, but it is unknown whether their results adequately reflect the quality of cord blood units. In this study, we tested the viability and recovery of colony forming cells (CFC) of 30 cord blood units (CB) and 2 kinds of samples. As test samples, we used segments (Seg) and tubes (Tube). With regard to viability, there was a high correlation between CB and sample type (CB vs Seg：$r=0.938$, CB vs Tube：$r=0.939$). Recovery of CFC of CB also correlated with sample type (CB vs Seg：$r=0.879$, CB vs Tube：$r=0.817$). These results suggested that the quality of CB could be determined by the testing of samples. Based on these results, criteria for cord blood transportation can be established.

Key words：cord blood, quality assurance, cryopreservation, viability, colony forming cells

はじめに

近年，非血縁者間臍帯血移植の実施数は急速に増加しており[1)~5)]，日本国内でも1,000例を越える移植が施行されている[6)]．これに伴い，臍帯血の品質管理と安全性の向上は重要な問題となっている．日本さい帯血バンクネットワークでは，臍帯血出庫前に保管検体による移植前検査を義務づけているが[7)]，その結果が実際移植に使用される臍帯血本体の品質を反映しているか否かは明らかではない．そこで我々は，保管検体と臍帯血本体の生細胞率およびコロニー形成細胞回収率を比較し，移植前検査の有用性について検討したので報告する．

材料と方法

1　臍帯血および検査用検体の凍結保存と解凍方法

出産前に同意を得た妊婦から，臍帯血を専用バッグ(ニプロパックC，ニプロ，大阪)で採取した．臍帯血バンクでは，採取から24時間以内に細胞処理を開始すると規定されているが[9]，今回の検討では種々の生細胞率を得るため，採取から細胞処理までの放置時間に差をつけた（10～140時間）．細胞処理法をFig. 1に示した．臍帯血に抗凝固剤（CPD液）を含めた血液量の40%容量の6% hydroxy-ethylstarch (HES40，菱山製薬，大阪）を添加し，10℃，60Gで5分間遠心後，多白血球血漿を回収し，赤血球の大部分を除去した．10℃，400Gで10分間遠心後，上清を除去し，多白血球血漿21.8mlとなるよう調整後，0.2mlを凍結前検体として採取した．凍害保護液5.4mlを最終濃度10% DMSO（Cryoserv，Reseach Industries，Salt Lake City)/1% dextran（Dextran40，小林製薬工業，東京）となるように添加し，細胞浮遊液25.5 mlを凍結バック（F-025A，ニプロ，大阪)，1.0 mlを凍結チューブ（Nunc Cryo Tube，Nunc，Denmark）に入れ（Tube)，前者のうち，25.0mlを臍帯血本体（CB）とし，残り0.5mlでセグメントを作成した（Seg)．本体，セグメントは専用の金属製キャニスター（Thermogenesis，CA）に，凍結チューブは簡易凍結容器（バイセル，日本フリーザー，東京）に入れ，－80℃ディープフリーザーで2時間以上静置後，液体窒素中に保存した．

解凍は37℃恒温槽内で速やかに行った．洗浄処理は行わず，検査用血液を採取し，細胞評価を行った．

2　検査法

臍帯血本体および検査用検体につき以下の検査を実施した．

①白血球数　凍結前および解凍後の検体を自動血球装置COULTER AcT diff（ベックマン・コー

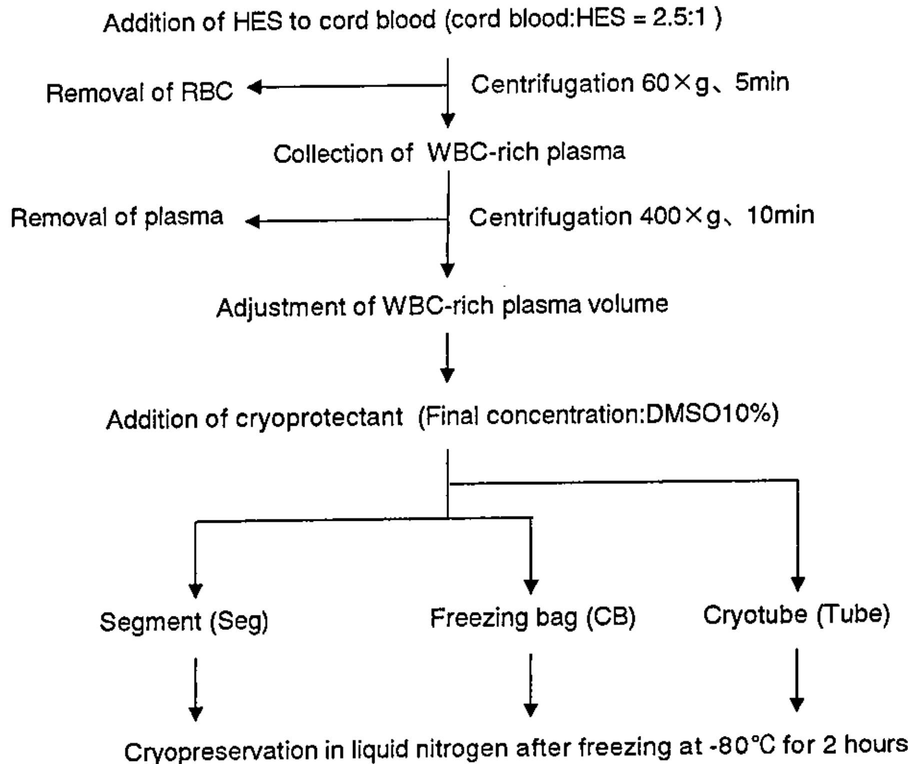

Fig. 1　Method for processing cord blood cells

ルター，東京)で測定した．解凍後白血球数/凍結前白血球数×100(%)にて白血球回収率を算出した．

②生細胞率 チュルク試薬（シグマアルドリッチジャパン，東京）および0.2%トリパンブルー(Gibco BRL, New York)で細胞染色し，計算板を用いて前者で総有核細胞数，後者で死細胞数を顕微鏡により計測した．総有核細胞数から死細胞数を引いた値を生細胞数とし，生細胞数/総有核細胞数×100（%）にて生細胞率を算出した．

③コロニー形成細胞回収率 凍結前および解凍後の検体をそれぞれ培地（MethoCult GF H4434V，ベリタス，東京)に加え，35mm培養皿4枚に播種し，37℃，5%CO2下で2週間培養後，顕微鏡でコロニー数を計測した．コロニーとして，顆粒球-マクロファージコロニー（CFU-GM），赤芽球バースト（BFU-E）および混合コロニー(CFU-Mix)を判定し，その合計をコロニー形成細胞（CFC）とした．コロニー形成細胞回収率は，CFC（解凍後)/CFC（凍結前)×100（%）にて算出した．

3 統計学的解析方法

臍帯血本体と保管検体の生細胞率，コロニー回収率はt-testで有意差検定を行い，また相関係数と回帰式を求めた．これらの解析にはStatView 4.0（Abacus concepts, Inc. CA）を用いた．

結 果

1 臍帯血本体と保管検体の生細胞率の比較

臍帯血30件について検討した．臍帯血本体(CB)，セグメント(Seg)，チューブ(Tube)の白血球回収率の中央値(範囲)は，CB 99.1% (75.5～130.5)，Seg 93.2%（69.9～128.5），Tube 89.7% (65.2～127.3)で，各群間に有意差はなかった(p>0.05)．生細胞率の中央値（範囲）は，CB 70.3%（29.1～87.4），Seg 69.1%（19.6～93.9），Tube 69.6%（25.0～87.9）であった．臍帯血本体と保管検体の相関係数は，CB vs Seg（Fig. 2A），CB vs Tube（Fig. 2B）でそれぞれr=0.938，r=0.939となり，どちらの保管検体も臍帯血本体と非常に良い相関性を示した．

2 臍帯血本体と保管検体のコロニー形成細胞(CFC）回収率の比較

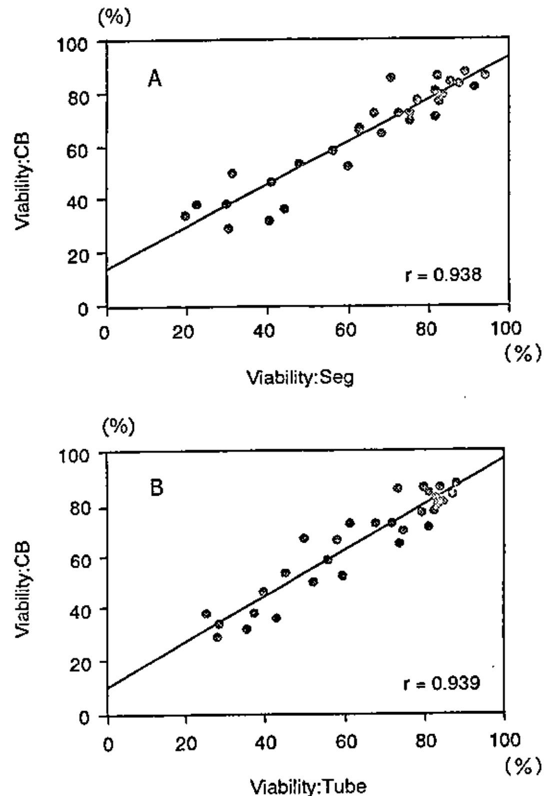

Fig. 2 Comparison of viability between cord blood units and test samples

CFC回収率の中央値（範囲）はCB 89.6% (20.9～146.0)，Seg 85.4%（22.9～138.8），Tube 75.2%（9.5～124.4）で，各群間に有意差はなかった（p>0.05)．臍帯血本体と保管検体の相関係数は，CB vs Seg（Fig. 3A），CB vs Tube（Fig. 3B）それぞれr=0.879，r=0.817となり，Segの方が若干良い傾向はあるものの，どちらの保管検体も臍帯血本体と良い相関性を示した．また，CFC回収率の回帰式は，CB vs Seg：Y=19.635+0.845X，CB vs Tube：Y=17.8+0.838Xであった．

3 生細胞率とCFC回収率の関係

生細胞率とコロニー形成細胞回収率の関係を検討した（Fig. 4)．CB，Seg，Tubeの生細胞率とCFC回収率の相関係数は，それぞれr=0.782，r=0.808，r=0.688となり，いずれも弱いながら正の相関を示した．また，大部分の例で生細胞率よ