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Effects of Cryopreservation on CD4+ CD25+ T Cells of HIV-1 Infected Individuals

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The role of T regulatory cells (Tregs) in human immunodeficiency virus (HIV)-1 infection, although not entirely clear, has recently been highlighted. Despite their lack of specificity, fluorochrome-labeled CD4 and CD25 antibodies are common flow cytometric reagents used to identify these cells with immunosuppressive potential. Cryopreservation has previously been shown to alter the proportions of lymphocytes with certain phenotypes expressed in peripheral blood mononuclear cells (PBMCs). The aim of this study was to assess the effect of cryopreservation on CD4+ CD25+ T cells in PBMCs from HIV-1+ individuals to guide the design of future studies on Tregs. We recruited 30 HIV-1+ individuals and nine healthy controls. CD25 expression in CD4+ T cells was compared between fresh and frozen/thawed PBMC samples from the same time point. In this study, cryopreservation significantly decreased the proportion of CD4 + CD25+ T cells in PBMC samples from HIV-1 infected subjects. This finding suggests that studies of CD4+ CD25+ T cells should be carried out on fresh samples to avoid bias introduced by cryopreservation. J. Clin. Lab. Anal. 22:153–158, 2008.

Key words: freezing; preservation; lymphocytes; T regulatory cells

INTRODUCTION

In recent years, the role of T regulatory cells (Tregs) in immune function has stimulated much interest and research (1). These cells are typically positive for surface expression of the CD4 molecule and can prevent the activation and expansion of self-reactive lymphocytes. Several autoimmune diseases, such as type I diabetes, multiple sclerosis, and allergic responses, are now thought to be associated with a reduction in number of Tregs, allowing for an expansion of self-reactive lymphocytes. Tregs are commonly identified by surface expression of both CD4 and CD25; CD25 is a marker for T cell activation and the $CD25^{hi}$ population appears particularly enriched with Tregs (2). These $CD25^{hi}$ cells in turn have higher proportions that stain with antibodies against the intracellular Forkhead activated transcription factor 3 (FoxP3), which is more specific for Tregs (3). This stain, the role of which in the identification of Tregs is still not entirely clear, was not yet available at the time of this study. With increased understanding of the effect of human immunodeficiency virus type 1 (HIV-1) on the immune system and the observation that HIV-1 downregulates the immune response, the role of Tregs in HIV-1 has been highlighted. Studies have shown that depletion of CD4+CD25+ T cells from peripheral blood mononuclear cells (PBMCs) of HIV-1-infected individuals results in an increased anti-HIV-1T cell response (4–6). However, in contrast, higher levels of Tregs are also correlated with lower viral loads in individuals with preserved CD4+ T cell counts (6). To facilitate large studies and batching of laboratory work on immune function in HIV-1+ individuals, cryopreservation of PBMCs has become increasingly important.

However, cryopreservation of PBMCs can change the proportions of certain lymphocyte subsets (7). Although

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previous studies have investigated the effects of cryopreservation on lymphocyte phenotypes, to our knowledge this is the first study to assess the effects of cryopreservation on CD4+ CD25+ T cells. To examine whether cryopreservation would be associated with a preferential loss of the CD4+ CD25+ T cell population of PBMCs, we directly compared fresh samples with frozen/thawed samples obtained from the same blood collection for HIV-1-infected individuals and healthy individuals.

MATERIALS AND METHODS

Study Population

This study was performed at the Medical Research Council (MRC) Laboratories, Fajara, Gambia. Individuals were recruited from the MRC Laboratories' longitudinal cohort study of immune function in HIV; in this study HIV+ individuals have previously been enrolled and have given written informed consent to have blood drawn routinely at 6-month intervals for storage of serum and PBMCs. The Gambia Government/MRC Ethics Committee has approved this cohort study. In our substudy we included a portion of the routinely collected PBMCs of 30 HIV-1+ individuals from the cohort, none of whom had received antiretroviral treatment. In addition, PBMCs were isolated from nine healthy volunteers who consented to serve as controls.

Recruited individuals were stratified according to CD4+ T cell percentages $<14\%$ or $\geq 14\%$, according to Centers for Disease Control (CDC) classification of individuals with low CD4+ T cell counts (8). CD4+ T cell percentages were determined immediately after blood collection. Mixed whole blood (minimum 50 μL) was stained with fluorochrome-conjugated monoclonal antibodies (MultiTest CD3/CD8/CD45/CD4 reagent; Becton-Dickinson [BD; Oxford, UK]) and analyzed in a Fluorescence Activated Cell Sorter (FACS) (FACSCalibur; BD) using MultiSet software (BD). The total white blood cell and differential count was obtained using a Beckman Coulter (High Wycombe, UK) counter. Results were calculated as a percentage of CD4 + T cells of the total lymphocyte population and as the absolute number of CD4+ T cells per μL of blood.

Separation of PBMCs

PBMCs were separated from heparinized whole blood within 3 hr of collection. The collected blood was centrifuged (400 *g*, 5 min), plasma removed and the volume of plasma replaced by RPMI 1640 medium referred to as R0 (Sigma-Aldrich Co. Ltd, Irme, Ayrshire, UK) before layering the diluted blood onto a Ficoll Hypaque gradient (Sigma). Samples were then centrifuged (400 *g*, brake off) for 25 min. The separated layer of PBMCs was removed, washed twice with R0, and resuspended in culture medium referred to as R10 (RPMI 1640, 10% fetal bovine serum [FBS]; GibcoBRL, Life Technologies]) L-glutamine 0.3 gm/L, 100 U/mL Penicillin G, 100 μg/mL Streptomycin sulfate). Cells were then counted using a hemocytometer. A total of three million cells were reserved for cryopreservation. A total of one million fresh cells were stored in culture flasks for staining within 12 hr of separation.

Cryopreservation

Cells for cryopreservation were resuspended in 100% filtered FBS in Sarstedt tubes (Aktiengesellschaft & Co., Numbrecht, Germany) and cooled at 4°C for 20 min. Precooled 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in FBS was added drop-by-drop to give a final concentration of 10% DMSO in FBS. Samples were placed in freezing boxes containing isopropanol and kept at –80°C until thawing.

Thawing

Within 2 weeks of freezing, samples were thawed rapidly by immersion in a water-bath at 37.5°C under continuous agitation, until only a small piece of ice remained in the vial. Immediately, 1 mL prewarmed FBS (to dilute the DMSO to 2%) was added drop-by-drop. The diluted sample was then transferred to 4 mL of FBS in a 25-mL Universal container and centrifuged (400 *g*, 5 min). The pellet was resuspended in R0 containing DNase I (type IV) (DN25; Sigma) at a 1:1,000 dilution, to protect against cell clumping (9), and left for 10 min with occasional agitation. The cells were then washed in 20 mL R10 culture medium, centrifuged (400 *g*, 5 min) and resuspended in R10 culture medium. Cells were counted with a hemocytometer and trypan blue dye exclusion was used to assess viability. Cells were then transferred to ventilated tissue culture flasks and incubated at 37.5°C, 5% CO_2 for 12 hr prior to staining.

Immunological Phenotype

PBMCs (fresh and thawed) were each divided into two FACS tubes (1/4 of the cells in the first, 3/4 in the second). They were then centrifuged (400 *g*, 5 min) and the supernatants were discarded. The larger proportion of PBMCs were stained with four fluorochrome conjugated anti-mouse anti-human monoclonal antibodies (all from Becton-Dickinson Pharmingen [BD], San Jose, CA); anti-CD25 fluorescein isothiocyanate (FITC;

Cat. #555431), anti-CD4 allophycocyanin (APC; Cat. #555349), anti-CD8 Peridinin-chlorophyll-protein complex (PerCP; Cat. #345774) and anti-cytotoxic T lymphocyte-associated antigen (CTLA)-4 R-phycoerythrin (PE; Cat. #555853). The smaller proportion of PBMCs was stained with anti-CD4 APC alone. Following incubation in the dark for 30 min at room temperature, the PBMCs were resuspended and washed in 3 mL FACS wash buffer (5 mM ethylene diamine tetraacetic acid [EDTA; Sigma-Aldrich, St. Louis, MO], 1% albumin from bovine serum [BSA, Sigma-Aldrich, St Louis, MO], in phosphate-buffered saline [PBS]). Stained cells were fixed following centrifugation by resuspension in 2% paraformaldehyde (in PBS). Cells were acquired with four-color flow cytometry using FACSCalibur with Cell Quest Software. A minimum of 18,000 gated events were obtained for analysis. Anti-CD4+ APC staining was used to set the gates for determination of the CD25+ population. The analysis was done using FCS express Version 2 software (De Novo Software, Thornhill, Ontario, Canada). $CD25^{hi}$+ populations were determined setting a gate such that the CD25+CD4– population was equal to 0.05% of total PBMCs (6). This gating is shown in Fig. 1 with a representative sample of a control individual's fresh and frozen/thawed FCS plots. The proportion of CD4+ CD25+ T cells (of all CD4+ T cells) was calculated for each individual for both fresh and frozen/thawed samples.

Statistical Analysis

Results were analyzed for the difference in CD4+ CD25+ and CD8+ CD25+ T cell populations in fresh and frozen/thawed samples, using the Wilcoxon signed rank test (Stata 8; Stata Corporation, College Station, TX). Results with a P value of <0.05 were considered significant.

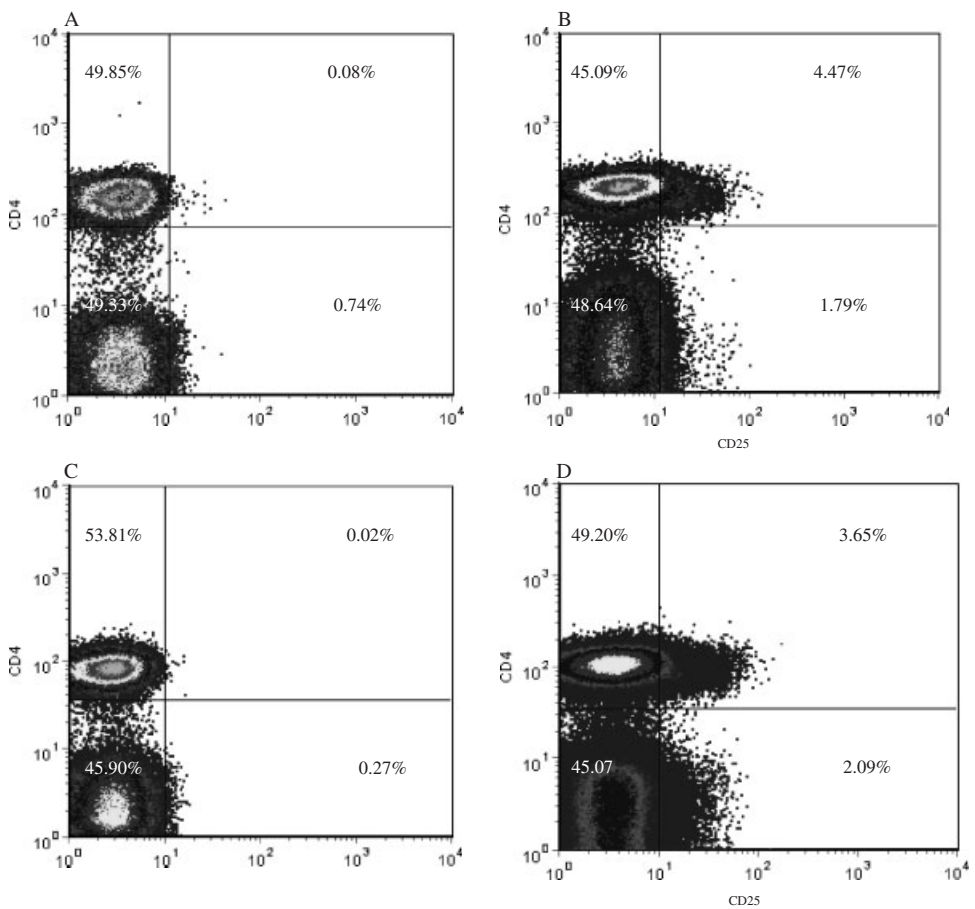

Fig. 1. Representative sample: analysis of fresh and frozen/thawed FCS dot plots. One individual's FCS dot plots are shown: (A–C) are from fresh cells; and (D–F) are from frozen/thawed cells. To set gates around the CD4+ population, a small proportion of cells were stained CD4+ only (A,C). These gates were then used in analysis of cells stained for both CD4 and CD25 (B,D). Proportions of cells staining CD25+ of total CD4+ T cells were calculated from plots; these decreased in frozen/thawed samples when compared to fresh.

TABLE 1. Subject characteristics and peripheral blood mononuclear cell recovery after cryopreservation*

	CD4<14%	CD4≥14%	Controls
Number of individuals (n)	16	14	9
Age in years, mean (range)	34 (18–50)	35 (6–57)	34 (25–48)
CD4+ T cell %, mean (range)	7.75 (1–11)	20.7 (14–36)	40.5 (27–50)
CD4+ T cells/μL, mean (range)	128 (20–300)	440 (180–860)	
% Viability, mean (range)	98.95 (97.2–100)	98.6 (94.9–100)	98.4 (95.9–100)
Viable cells recovered X106, mean (range)	3.03 (1.71–4.95)	3.13 (1.56–4.66)	2.93 (1.86–4.26)

*Samples are divided into HIV-1+ individuals (grouped according to CD4+ T cell percentage), and controls. For each, the number of people in each group, their mean age, CD4% T cell count, and absolute CD4 T cell count (CD4+T cells/μL) are shown. The final two columns indicate percentage and absolute counts of viable cells recovered from samples after freezing/thawing.

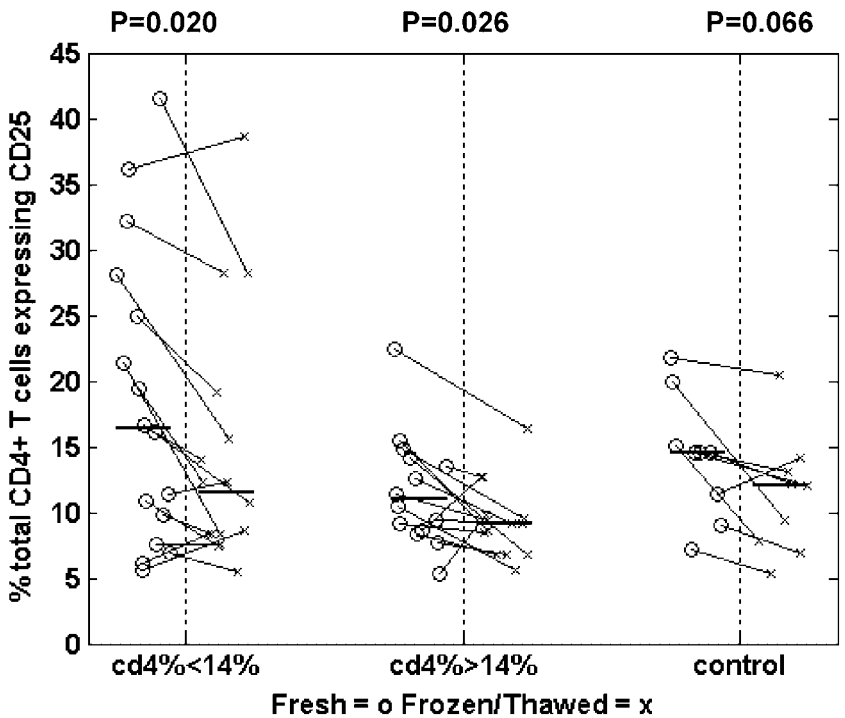

Fig. 2. CD4+CD25+ T cell survival after freezing/thawing. Survival of CD25+CD4+ T cells as a percentage of total CD4+ T cells, fresh vs. frozen/thawed. Data is stratified into HIV-1+ individuals with a CD4T cell count <14%, those with a CD4T cell count ≥14% and controls. Horizontal bars indicate the mean.

RESULTS

Characteristics of Subjects

A total of 30 individuals were recruited; 16 HIV-1 infected subjects with a CD4T cell count of less than 14%, 14 HIV-1 infected subjects with a CD4+ T cell count of 14% or greater, and nine healthy controls. There were no significant differences in age or sex among the three groups. CD4+ T cell percentage is similar to absolute CD4+ T cell counts (CD4+/μL) as shown (Table 1).

Cryopreservation: Cell Recovery and Viability

There were no significant differences in the viability of thawed cells or in the mean absolute number recovered between controls and patient groups (Table 1). In all groups, the mean number of PBMCs recovered was within 5% of total fresh PBMCs frozen (3.0×10^6).

Phenotypic Changes Following Cryopreservation

Despite good recovery, the proportion of CD4+ T cells staining CD25+ decreased following freezing/

thawing, as shown in the representative sample (Fig. 1). Setting the $CD25^{hi}$ population within the quadrant such that the CD25+CD4– population was equal to 0.05% of total PBMCs (according to Kinter et al. (6)) led to a shift to the right in the $CD25^{hi}$ gate for frozen and thawed samples due to an increase in CD4–CD25+ staining in the bottom right quadrant (Fig. 1D). We therefore only compared the CD25+ populations as defined by the CD4+ population, considering only the upper quadrants. Figure 2 shows the reduction in the proportion of CD4+ T cells staining CD25+ in both HIV-1+ groups and controls after freezing/thawing. These reductions were significant ($P<0.05$) in both HIV-1+ groups (those with CD4+ T cell count percentages $<14\%$ and CD4+ $\geq 14\%$) and showed the same trend in controls ($P = 0.066$). Frequencies of CD25+CD8+ staining cells were very low and no significant differences were observed in any of the groups in the proportions of these cells following cryopreservation (data not shown).

DISCUSSION

Our findings suggest that cryopreservation decreases the proportion of CD4+CD25+ T cells in PBMCs from both HIV-1-infected individuals and controls. This population is highly enriched for Tregs (2). Despite the obvious advantages cryopreservation offers, such as the ability to conduct nested case-control studies and to batch samples for analysis, these findings suggest that use of fresh blood is preferable for an unbiased quantification of Tregs. The mechanism for the loss of certain lymphocytic populations after cryopreservation is unknown. Earlier studies have suggested that the changes observed are due to downregulation of specific markers, particularly when cells are stained soon after thawing (7,10). We therefore thawed the PBMCs 12 hr prior to staining. Cell recovery was high despite differing effects of cryopreservation on cell phenotypes in different groups.

Length of freezing, particularly in liquid nitrogen, may relate to changes in lymphocyte phenotype (11). While prolonged storage in liquid nitrogen may affect phenotypic expression further, we show that freezing to –80°C for brief periods is sufficient to induce the changes in phenotypic expression described above. Surface expression of CD25 is a marker of T cell activation and it is possible that activated T cells are more susceptible to freezing-induced apoptosis. However, freshly isolated CD4+CD25+FoxP3+ Tregs were shown to be highly sensitive toward CD95-mediated apoptosis, but not to T-cell receptor (TCR)-mediated cell death (12). This increased susceptibility to CD95 ligand might explain the loss of CD25T cells after freezing/thawing. Our study does not assess the function of the CD4+CD25+ or the $CD4+CD25^{hi}$ T cells, and therefore provides no evidence that these cells have suppressive capabilities. Even in combination with FoxP3 staining, however, the $CD4+CD25^{hi}$ phenotype remains a cornerstone in the quantification of Tregs and the preferential loss we describe after cryopreservation introduces an immeasurable amount of bias. In summary, this study suggests that cryopreservation can induce significant changes in surface expression of CD25+ markers on CD4+ T cells, suggesting that the use of fresh cells is preferable in order to avoid bias. As further markers for Tregs are identified, comparisons of fresh and frozen/thawed cells can clarify whether the loss of CD4+CD25+ T cells after freezing constitutes mainly activated T cells or indicates a true loss of Tregs.

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