JOURNAL OF HEMATOTHERAPY 2:259–261 (1993)
Mary Ann Liebert, Inc., Publishers

Purification, Expansion, and Multiple Fluorochrome Labeling of Cord Blood Hemopoietic Precursors: Preliminary Results

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ABSTRACT

CD34-positive cells were isolated from a total of 23 cords using CellPro Ceprate^{TM} columns. AIS MicroCellector^{TM} flasks, and panning. The cells were (1) expanded in serum-free culture supplemented with a variety of combinations of cytokines and (2) immunophenotyped using multiple fluorochrome labeling. The results indicated that the avidin column produced the highest purity of CD34-positive cells, and that immature blast cells could be expanded in serum-free culture. Preliminary results suggested that the four fluorochrome labeling technique may provide useful information on the lineage commitment of cord blood precursor and blast cells.

INTRODUCTION

THE OUTCOME OF HEMOPOIETIC TRANSPLANTATION may critically depend on the proliferative and differentiative properties of the transplanted precursors. Multiple fluorochrome labeling would be a powerful tool to evaluate cord blood concentrates and to study the lineage specificities of precursor amplification induced by combinations of cytokines. Extensive phenotyping of uncommitted stem cells and progenitors committed to the granulocytic, erythrocytic, thrombocytic, B or T lymphocytic lineages requires detection of up to eight antigens on individual cells. We present our first results on multiple immunofluorescence on purified CD34$^+$ cells.

METHODS AND RESULTS

A total of 23 cords from 26 to 40 week-old fetuses were collected in CPD and provided 75 ± 27 ml (range: 33–130) of blood. Samples were treated 5.5 ± 4 hours after birth. Light density mononuclear cells (MNC) were isolated with Ficoll–Hypaque sedimentation. A mean of $170.1 \pm 87.5 \times 10^6$ mononuclear cells (MNC) were obtained including $3.1 \pm 2.5 \times 10^6$ CD34$^+$ cells. Three purification methods were compared. In the first, avidin columns (Ceprate LC, CellPro, Botnell, WA) were used to separate CD34$^+$ cells from MNC suspensions previously stained with 12.8 (anti-CD34) monoclonal antibody (MAb) and a biotinylated goat anti-mouse IgM. Each kit could process up to 500×10^6 cells (Berenson *et al*, 1988). The second technique utilized flasks coated with ICH3 (anti-CD34) MAb (AIS MicroCELLector, Santa Clara, CA) for positive selection of CD34$^+$ cells. Each 25-cm^2 flask could be fed with 20×10^6 cells. Soybean agglutinin flasks were not used prior to the anti-CD34 flasks because soybean adsorption has been shown to cause the loss of certain progenitors types (Lebkowski *et al*, 1992). The third method was based on panning. A mean of $57.7 \pm 28.0 \times 10^6$ MNC were treated with My 10 (anti-CD34) MAb using 28-cm^2 Petri dishes coated with sheep F(ab′)$_2$ anti-mouse IgG (Saeland *et al*, 1989). The starting and enriched samples were analyzed using a FACSCAN flow cytometer. Table 1 shows that the avidin

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TABLE 1. ANALYSIS OF THE STARTING AND ENRICHED SAMPLES[a]

	N	*NC* $(\times 10^6)$	*MNC* $(\times 10^6)$		*CD34*$^+$ (%)		*CD34*$^+$ $(\times 10^6)$		*Yield* (%)	*Enrich* $(\times)$
		1	*1*	*2*	*1*	*2*	*1*	*2*		
Avidin column	10	742.2 ± 307.5	167.1 ± 91.1	1.1 ± 1.1	1.5 ± 0.6	61.1 ± 18.2	2.3 ± 1.2	0.6 ± 0.3	30.4 ± 11.5	47.2 ± 27.8
Coated flasks	6	118.3 ± 44.6	20.0	1.0 ± 0.8	2.3 ± 1.0	24.0 ± 3.3	0.5 ± 0.2	0.2 ± 0.2	52.8 ± 11.8	9.7 ± 4.1
Panning	7	348.0 ± 125.4	57.7 ± 28.0	1.7 ± 0.7	—	47.0 ± 5.3	—	0.8 ± 0.4	—	10.4 ± 2.5

[a] 1, start samples; 2, enriched samples; NC, nucleated cells; MNC, mononucleated cells.

TABLE 2. CELL NUMBER AND PERCENT BLASTS FOR VARIOUS CYTOKINE COMBINATIONS

	0	*IL-3*	*IL-3 IL-6*	*IL-3 SCF*	*IL-3 IL-6 SCF*	*IL-6*	*SCF*	*IL-6 SCF*
Cells/μl	3.0 ± 1.0	16.4 ± 5.0	36.8 ± 2.2	76.4 ± 3.7	125.0 ± 37.0	2.2 ± 1.5	15.1 ± 6.5	23.2 ± 0.1
Blasts (%)	—	45.0 ± 20.0	50.6 ± 16.4	75.8 ± 10.4	84.0 ± 3.2	—	46.0 ± 3.0	66.0 ± 4.0

column produced the greatest CD34$^+$ cell purity. However, some investigators have observed that MicroCELLector flasks used near expiry date have given poor cell recovery and purity (Hatzfeld, 1993). This could have been the case in some of our experiments.

To expand hematopoietic precursor populations, purified CD34$^+$ cells were cultured in 96-well plates, in a liquid serum-free medium (Salem *et al*, 1988) containing IL-3 (10 ng/ml), IL-6 (90 ng/ml), and stem cell factor (50 ng/ml) alone or in various combinations. Each 300 μl suspension contained 5000 cells of which 48.0 ± 20.0% were CD34$^+$. Using an IBAS image analyzer (Kontron, Germany) to discriminate and enumerate cultured cells, we compared at day 5 cell number and percent blasts for the various cytokine combinations. Table 2 shows that the combination of IL-3 + IL-6 + SCF resulted in the greatest proliferation of both total cells and blasts.

Purified CD34$^+$ cells were used for multiparameter phenotyping. An IBAS image analyzer was connected to a light microscope equipped with a Dage MTI ISIT camera. This setup allows enumeration of cells, excitation of fluorochrome-labeled cells in defined spectral regions, and measurement of light intensity in selected emission bands. In a first set of experiments, the validity of quadruple immunofluorescence was evaluated using Cascade Blue (CB; emission max 420 nm), FITC (max 520 nm), R-phycoerythrin (R-PE; max 575 nm), and allophycocyanine (APC; max 660 nm). Using T lymphocytes treated with unlabeled anti-CD45, the relative emission intensity of goat anti-mouse IgG labeled with CB, FITC, R-PE, and APC was 0.64, 1.00, 2.48, and 2.25, respectively. Spectral overlap, which was less than 9% in all cases, could be corrected by the image analyzer. This method was validated by the demonstration in suspensions of purified cord blood progenitors of cells binding biotinylated anti-CD34 coupled to streptavidin-APC together with monoclonals recognizing erythroid (anti-CD36-CB), megakaryocytic (anti-CD61-FITC), and granulomonocytic (anti-CD13-R-PE) progenitors.

We then evaluated by spectrofluorometric analysis of protein-conjugated dyes the usefulness of aminomethylcoumarin (AMCA), Lucifer Yellow (LY), Bodipy, the algal chromoprotein PerCP, Texas Red (TxR), Red 613 (a covalent complex between R-PE and TxR), Cyanine 5 (CY 5), and C-phycocyanin (C-PC). The data suggest that it is possible to discriminate the emissions of eight fluorochromes using only three excitation bands: the first one, at 400 nm, includes CB (emission max 420 nm) and LY (max 520 nm); the second one, at 485 nm, includes FITC (max 520 nm), R-PE (max 575 nm), Red 613 (max 610 nm), and PerCP (max 675 nm); the third one, at 590 nm, comprises TxR (max 610 nm) and APC (max 660 nm). Work is in progress to quantify the spectral overlaps between adjacent dyes in order to introduce adequate corrections in videomicroscopic measurements.

Our results show that purified CD34$^+$ cells can be expanded substantially by short-term culture in a defined serum-free medium and that immature blast cells are preferentially expanded under these conditions. The multiparameter labeling technique presented here could be useful to determine the "lineage formula" of cord blood precursors and amplified blast cells.

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