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Immunomagnetic Bead Separation of Mononuclear Cells from Contaminating Granulocytes in Cryopreserved Blood Samples

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Abstract

Density gradient centrifugation usually allows efficient separation of mononuclear cells from granulocytes using fresh human blood samples. However, we have found that with cryopreserved blood samples, density gradient centrifugation fails to separate granulocytes from mononuclear cells and have explored using immunomagnetic anti-CD15 microbeads as an alternate method to separate these cell populations. Using cryopreserved blood samples from 10 healthy donors we have shown that granulocytes express a significantly higher level of CD15 antigen than monocytes and lymphocytes, which allows for their efficient separation from mononuclear cells using anti-CD15 microbeads. This procedure is critical for purification of individual cell populations from cryopreserved leukocyte samples and could also potentially be applied to avoid granulocyte contamination of mononuclear cells isolated from stored blood and from patients with sepsis or thermal injury.

Keywords

Cryopreservation; Granulocytes; Mononuclear cells; Density gradient; centrifugation; Immunomagnetic beads; Human blood

Isolation of mononuclear cells from peripheral blood leukocytes is a common procedure in different research and clinical settings. This is most frequently performed by density gradient centrifugation [5]. Using Ficoll or other mixtures of polysaccharide and radiopaque contrast medium with a density of 1.077 to isolate mononuclear cells from such samples usually provides high purity of mononuclear cells with about 1-2% of contaminating granulocytes [1]. However, we have found that when this procedure was used to isolate mononuclear cells from cryopreserved blood leukocytes, most of the granulocytes were not separated from mononuclear cells. Since we were not able to find any published data regarding isolation of mononuclear cells from cryopreserved blood cell samples, we developed a novel immunomagnetic bead technique that allows isolation of mononuclear cells and elimination of granulocytes from cryopreserved blood samples.

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Leukocytes were isolated from blood obtained from 10 healthy volunteers using ammonium chloride lysing procedure as previously described [8]. Cells were frozen at -80°C using Nalgene Cryo 1°C Freezing Container with isopropyl alcohol, and were stored at -80°C for no longer than 3 months. After thawing and washing with 0.3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) in Dulbecco's modified phosphate buffer solution (DPBS, Sigma-Aldrich, St. Louis, MO), aliquots of the samples were stained for 15 minutes at room temperature with FITC-anti-CD15 (16 µg/ml; Cat. # 340703; Becton Dickinson, Franklin Lakes, NJ) and ECD-anti-CD45 (Beckman Coulter, Fullerton, CA) antibodies for flow cytometric analysis. The rest of the sample was divided in half, and one half was layered over mononuclear cells isolation reagent HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, MO) and mononuclear cells were isolated as recommended by manufacturer. The rest of the cells were incubated with magnetic CD15 Microbeads (Miltenyi Biotec, Auburn, CA) and mononuclear cells and granulocytes were separated following manufacturer's recommendations. After isolation, cells were stained with ECD-anti-CD45 antibody as described above. Percentage of lymphocytes, monocytes, and granulocytes in each sample was measured using FC-500 Cytomics flow cytometer (Beckman Coulter, Fullerton, CA) with CD45/log side scatter gating [7] and analyzed using Venturi One (Applied Cytometry, Sheffield, UK), or FCS Express (De Novo Software, Los Angeles, CA) software.

As shown in Table 1, cryopreserved granulocytes have considerably higher levels of CD15 antigen compared to lymphocytes and monocytes in all tested samples, which is critical for efficient isolation with immunomagnetic microbeads. Histograms presented in Fig. 1 illustrate typical staining with anti-CD45-ECD antibody and percentage of granulocytes in original cryopreserved leukocyte sample (Fig. 1A), and in samples isolated with Histopaque (Fig. 1B) or CD15 microbead (Fig. 1C) procedures. This figure clearly demonstrates that with Histopaque procedure granulocytes are not separated from mononuclear cells, while treatment with magnetic microbeads almost completely removes contaminating granulocytes. Using the same Histopaque technique in fresh blood samples yielded clean separation of granulocytes from mononuclear cells (not shown). The results obtained with 10 samples isolated from different healthy donors are presented in Table 2. In all tested samples the percentage of granulocytes after Histopaque isolation (range 66-84%) is even higher than in the original sample (Range 37-68%), while after microbead isolation in all samples contamination with granulocytes is very low (range 1-8%).

Separation of mononuclear cells from contaminating granulocytes is a critical step in any study requiring a highly-purified population of lymphocytes or monocytes. Although density gradient technique is often used, several reports have indicated that under certain clinical conditions granulocytes cannot be efficiently separated with gradient centrifugation. For example, mononuclear cell samples separated with ficoll gradient centrifugation from patients with sepsis have been reported to contain 48-89 % granulocytes [10], samples from patients with thermal injury contained about 40 % [2], and normal blood samples stored at room temperature contained up to 55% [4;9]. If changes of granulocytes in patients with sepsis and thermal injury are similar to modifications during cryopreservation, the procedure described in this paper can be used for the isolation of highly-purified mononuclear cell population from the blood of such patients.

Density gradient centrifugation is also used as a method for separation of dead and viable cells [3;6]. According to our data, isolation of viable cells in cryopreserved leukocytes will result in contamination of viable cell fraction with granulocytes; however, we have found that this procedure can still be effective if granulocytes are removed with magnetic beads before gradient centrifugation (data not shown).

In conclusion, we present evidence that in cryopreserved blood samples, mononuclear cells cannot be efficiently separated with density gradient, but can be effectively separated from contaminating granulocytes using an immunomagnetic microbead procedure. This method can also potentially be used for the elimination of contaminating granulocytes from mononuclear cells in patients with sepsis and burn injury.

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Figure 1.

Typical histograms indicating percentage of granulocytes in original cryopreserved normal human blood leukocytes (A) and after separation with Histopaque (B) or anti-CD15 microbead procedure (C). See details of cell isolation and staining in the text.

Table 1

Relative expression of CD15 in cryopreserved blood cells measured by flow cytometry

Sample	Lymphocytes	Monocytes	Granulocytes
		FITC-CD15 Mean Fluorescence Intensity (AU)	
Donor 1	0.88	20.24	293.46
Donor 2	0.71	17.49	370.02
Donor 3	1.11	49.47	520.28
Donor 4	1.68	25.62	552.97
Donor 5	1.08	31.64	528.60
Donor 6	0.69	29.19	347.06
Donor 7	1.18	33.24	381.59
Donor 8	1.16	39.73	562.56
Donor 9	0.64	15.55	445.58
Donor 10	0.99	23.12	400.87

AU indicates arbitrary units.

Table 2

Percentage of various blood cells in normal cryopreserved blood cell samples before and after separation with Histopaque or anti-CD15 microbead procedure

Sample	Before Separation	Lymphocytes Histopaque	Microbeads	Before Separation	Monocytes Histopaque	Microbeads	Before Separation	Granulocytes Histopaque	Microbeads
Donor 1	25	18	79	8	5	12	62	72	2
Donor 2	29	18	81	9	7	13	57	68	1
Donor 3	34	18	60	3	3	19	54	69	8
Donor 4	23	11	62	3	3	21	66	84	6
Donor 5	32	13	75	7	3	15	53	79	2
Donor 6	16	17	67	8	4	17	53	66	1
Donor 7	12	8	53	6	5	15	57	71	2
Donor 8	36	25	68	7	1	15	40	68	1
Donor 9	32	15	54	9	1	16	37	80	7
Donor 10	19	10	65	4	6	12	68	79	2