Establishing the Purity of Mononuclear Cell Preparations Using Morphology and Flow Cytometry

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Abstract

Context:

Simple tandem repeat loci are used to track bone marrow engraftment using mononuclear buffy coat cells and T-cells. Poor isolation purity of these subpopulations can result in lower analytical sensitivity of the bone marrow engraftment assay by diluting the cell population in question with other nucleated cells. Validation of the mononuclear cell preparation can be performed by flow cytometry or by counting cell populations on the slide.

Design:

Peripheral blood samples were taken from 5 random study subjects. White blood cell counts were performed to ensure that the samples were within the reference range prior to mononuclear cell isolation. The Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) density gradient was used to isolate mononuclear cells. Morphologic analysis consisted of identifying and classifying 400 nucleated cells per subject on 2 peripheral blood smears stained with Wright-Giemsa. By flow cytometry, cell populations were gated using side scatter and CD45. Expression of the CD2, CD20, and CD33 markers were also analyzed.

Results:

Morphologically, one granulocyte was counted in 400 cells from one of 5 samples. By flow cytometry, between 0.9% and 2.0% were non-mononuclear cells (mean 1.4%; coefficient of variation, 28.88%). The samples had between 74.3% and 84.8% lymphocytes (mean, 80.8%; coefficient of variation, 5.08%) and between 14.3% and 23.6% monocytes (mean, 17.7%; coefficient of variation, 21.33%).

Conclusions:

Our results show that the purity of the Histopaque-1077 mononuclear cell preparation is excellent and that morphology may be sufficient to validate the mononuclear cell isolation method if flow cytometry is not available.

Introduction:

Bone marrow transplantation is the standard of care for patients with a variety of hematologic malignancies, and engraftment monitoring is of paramount importance in tracking the status of bone marrow transplantation. Periodic bone marrow aspirates and peripheral blood samples are assessed for residual or recurrent disease using molecular techniques. Isolation of mononuclear cells (MNC) can increase the sensitivity of the detection of recurrent recipient cells by excluding an abundance of granulocytes from the preparation. Centrifugation over a density gradient is a common separation technique that accomplishes blood cell subpopulation isolation based on density and size differences between blood cell populations Histopaque®-1077 medium facilitates rapid recovery of viable mononuclear cells from small volumes of blood and bone marrow which is accomplished due to its adjusted density of 1.077 +/- 0.001 g/ml. When venous blood anticoagulated with EDTA is layered onto Histopaque-1077 and subjected to centrifugal forces, erythrocytes and granulocytes are aggregated by polysucrose, which causes rapid sedimentation, whereas lymphocytes and other mononuclear cells remain at the plasma-Histopaque-1077 interface. Following subpopulation isolation using Histopaque-1077, MNC isolation purity can be assessed using Wright-Giemsa stained smears or Flow Cytometry. The purpose of this study is to compare counting Wright-Giemsa stained smears to Flow Cytometric analysis for assessing the purity of the MNC isolation.

Methods:

MNC isolation was performed on five venous peripheral blood samples (each 5-10 ml total) collected in four 3mL EDTA tubes each. Complete blood counts were performed to ensure the samples had cell counts within the normal reference range. Additionally, two Wright-Giemsa slides were prepared from each sample and were used for comparison with the MNC cell isolate slides.

MNC isolation:

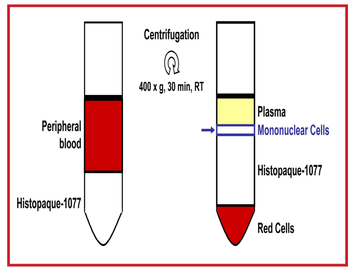

Figure 1: Schematic of density gradient

1. Pipet 3ml Histopaque-1077 in 15ml conical centrifuge tube
2. Carefully layer 3ml EDTA blood atop the Histopaque-1077 layer
3. Centrifuge at 400xg for 30min at room temperature
4. Remove top layer of plasma down to 0.5cm from MNC layer
5. Aspirate MNC layer and transfer to 15ml conical centrifuge tube containing 10ml Phosphate Buffered Saline (PBS)
6. Centrifuge at 250 x g for 10min at room temperature
7. Discard supernatant, resuspend pellet in 700µl PBS and transfer to microcentrifuge tube
8. Centrifuge at 250 x g for 5min at room temperature
9. Discard supernatant, resuspend pellet in 200µl PBS with 10% Bovine Serum

Preparation of Smears for Microscopy (2 slides per sample):

1. Pipet 10µl of the MNC cell suspension (200µl PBS/10% Bovine serum prepared as described above) next to the frosted margin of the slide
2. Add 10µl of a 10% formalin solution to the drop of the MNC cell suspension on the slide
3. Prepare smear using a clean glass slide
4. Perform standard Wright-Giemsa stain
5. Microscopically score 200 cells per slide (400 cells per sample) as MNCs or non-MNCs using 1000X magnification

Flow Cytometry:

1. Add 50 µl of cell suspension to each tube.
2. Incubate with 20ul of labeled primary antibody (Becton Dickinson monoclonal mouse anti-human antibodies CD45, CD2, CD20, CD33) for 30 min at room temperature in the dark.
3. Add 2ml of 1x FACS lysing solution to each tube and incubate for 10 minutes at room temperature in the dark
4. Centrifuge for 1 minute on high speed, aspirate the supernatant carefully, and wash cells once with 1ml of 1X Dulbecco's PBS with 0.1% Sodium Azide
5. Centrifuge for 1 minute on high speed, aspirate the supernatant carefully and resuspend pellet in 0.5 ml of 1% paraformaldehyde
6. Analyze on FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software. Gate cell populations of interest using the CD45 stain and side scatter. The same gate is applied when calculating percentages for CD2, CD20 and CD33

Results:

MNC preparations of high purity were obtained as assessed with both methods, scoring Wright-Giemsa stains and using Flow Cytometry.

Wright-Giemsa staining revealed presence of one granulocyte in 400 cells in one of five samples (Fig 2). No non-mononuclear cells were identified in the other four samples. Scoring the slides manually is time consuming and cumbersomeand does not allow for discrimination of different MNC subpopulations if that is desired.

After exclusion of debris, flow cytometry showed between 0.9% and 2.0% non-mononuclear cells for all the samples (mean 1.4%; coefficient of variation, 28.88%). The samples had between 74.3% and 84.8% lymphocytes (mean, 80.8%; coefficient of variation, 5.08%) and between 14.3% and 23.6% monocytes (mean, 17.7%; coefficient of variation, 21.33%) (Fig. 3, Tab. 1) which is expected for healthy individuals and correlated with the WBC prior to the MNC isolation.

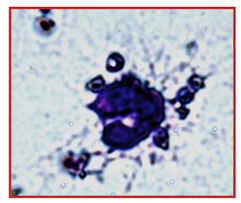

Figure 2: single granulocyte identified by morphology

Table 1: Breakdown of flow cytometric analysis

	Lymphocyte (%)	Monocyte (%)	Non-MNC (%)
Specimen 1	84.1	14.3	1.6
Specimen 2	83.9	14.9	1.2
Specimen 3	82.3	16.7	0.9
Specimen 4	79.3	19.1	1.5
Specimen 5	74.3	23.6	2
Average	80.8	17.7	1.4
Median	82.3	16.7	1.5
Standard Deviation	4.1	3.8	0.41
Coefficient of Variation	5.1	21.3	28.9

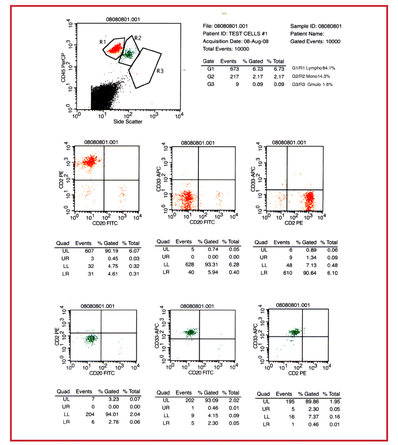

Figure 3: Flow cytometric analysis following Histopaque-1077 mononuclear cell isolation

Conclusions:

- Using Histopaque-1077 results in excellent purity of the MNC preparation demonstrated on peripheral blood samples from healthy individuals
- Analysis of a Wright-Giemsa stained smear appears to be sufficient to assess the purity of the preparation in peripheral blood samples from healthy individuals
- Flow cytometry provides an efficient analysis of MNC isolation quality and allows for subtyping of cell populations, however, the equipment may not be available in all laboratories

References:

1. Slifkin M, Cumbie R. J Clin Microbiol. 1992;30 (10):2722-2724.
2. Sigma-Aldrich HISTOPAQUE-1077 procedure guide.
3. Thiele, J et al. Virchows Arch. 2000;437:160-166.