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Technical note

A simple flow-cytometry method to evaluate peripheral blood contamination of bone marrow aspirates

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ABSTRACT

Bone marrow (BM) aspirates used for flow-cytometry (FCM) studies are usually obtained from a second aspiration, as the primary aspirate is used for morphological assessment. For this reason, the FCM samples unavoidably contain some blood; although, good-quality samples contain only a small amount. It is of utmost importance to assess the quality of samples prior to FCM analysis; yet, contamination with peripheral blood (PB) is not evaluated in most laboratories, possibly because the methods available are either qualitative or too complex for daily practice. Here, we propose a simple FCM method to quantitatively evaluate PB contamination in BM aspirates, by analyzing the percentage of plasma cells and CD34$^+$ cells – two cell populations nearly absent from PB – and CD10$^+$ granulocytes, which comprise the majority of the PB granulocyte population. We analyzed these three populations in 122 BM aspirates from subjects without hematological disease, and identified samples with PB contamination by performing a hierarchical cluster analysis. A discriminant analysis yielded a function, which we named the PB contamination index (PBCI). This index value gives a quantitative indication about the degree of hemodilution of a given sample. A threshold was identified that discriminates low-quality samples. The method and the threshold proved to be useful in BM aspirates infiltrated with malignant cells, with the exception of cases where hematological disease altered two of the three parameters included in the index. We have easily implemented the PBCI calculation in our daily routine, and find it very helpful for an accurate interpretation of FCM results in a large proportion of BM specimens. Limitations of the technique are discussed.

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1. Introduction

Bone marrow (BM) aspirates are routinely used in flow-cytometry (FCM) laboratories for the diagnosis and follow-up of hematological malignancies. There is a certain degree of hemodilution with this kind of specimen, since it is usually obtained from a second aspiration with a larger volume, as the first-pull is used entirely for morphological assessment. For this reason, the percentages of BM cell populations obtained from morphologic analysis are often different from those based on FCM analyses (Smock et al., 2007; Rawstron et al., 2008). Although a certain amount of peripheral blood (PB) is unavoidable, there is a limit for the sample to be representative of the BM. Low-quality BM aspirates should be discarded, especially if they are to be used for some specific FCM applications, such as minimal residual disease (MRD) quantitation (Rawstron et al., 2008; Brooimans et al., 2009; Gupta et al., 2009).

Despite its importance, PB contamination of BM aspirates is not routinely evaluated in most laboratories. Some consensus protocols recommend a morphological analysis of the sample, while other guidelines do not mention this issue when addressing the quality of BM aspirates (Rawstron et al., 2008; Kalina et al., 2012; Johansson et al., 2014). The morphological approach poses two difficulties. First, many FCM laboratories do not have morphological techniques available. Second, it is a qualitative approach that can identify BM aspirates with a high level of hemodilution, but it does not provide quantitative information about lesser degrees of PB contamination. A system to estimate the level of PB contamination of samples would be very helpful, to be

Abbreviations: B-CLPD, B-cell chronic lymphoproliferative disorders; BM, bone marrow; CONT, contaminated group; FCM, flow-cytometry; GC, good-quality group; MGUS, Monoclonal gammopathy of undetermined significance; MM, Multiple myeloma; MRD, minimal residual disease; PB, peripheral blood; PBCI, peripheral blood contamination index; PC, plasma cells; SSC/FSC, side scatter/forward scatter.

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aware of the degree of underestimation in the quantitated BM populations.

To the best of our knowledge, only one described method quantitatively estimates PB contamination in BM samples (Holdrinet et al., 1980). Some authors have found this method reliable (Brooimans et al., 2009); however, it is not widely used, possibly because a paired sample of PB is needed. In the present study, we propose a simple FCM method for quantitative evaluation of the degree of PB contamination in BM aspirates. This method does not require complex calculations or additional samples.

2. Materials and methods

2.1. Samples

A total of 188 BM aspirates were included in this study, 122 from subjects without hematological disease (BM1-122) and 66 from patients with hematological disease showing BM infiltration: 11 with Monoclonal Gammopathy of Undetermined Significance (MGUS) (BM123-133); 35 with Multiple Myeloma (MM) (BM134-168); and 20 with B-Cell Chronic Lymphoproliferative Disorders (B-CLPD) (6 Follicular Lymphoma, 5 Diffuse Large B-Cell Lymphoma, 3 Chronic Lymphocytic Leukemia, 2 Marginal Zone Lymphoma, and 4 Lymphoplasmacytic Lymphoma) (BM169-188). All samples corresponded to the material remaining after a routine study for the diagnosis or follow-up of hematological malignancies. The samples were the second aspiration of BM, since in our hospital the first pull is used entirely to make smears for diagnostic morphologic evaluation (May Grünwald-Giemsa). The leukocyte count of samples was obtained in a Sysmex XN-1000™ Hematology Analyzer.

2.2. Immunophenotyping of samples

We analyzed $CD34^+$ cells, plasma cells (PC), and neutrophils ($CD10^+$ granulocytes). Since $CD34^+$ cells and PC are nearly absent in PB, contamination of BM samples with blood results in diminished BM percentages of both populations. On the contrary, neutrophils are the main granulocytes in PB; thus, PB contamination of BM samples would increase the percentage of $CD10^+$ granulocytes ($CD10^+$G).

The monoclonal antibody (mAb) combination used was: anti-$CD38^{FITC}$/anti-$CD34^{PE}$/anti-$CD138^{PerCP\text{-}Cy5.5}$/anti-$CD10^{APC}$/anti-$CD45^{APC\text{-}H7}$. The mAbs used were: anti-$CD38^{FITC}$, clone HIT2 (Pharmingen, Becton-Dickinson (BD), San Jose, CA), anti-$CD34^{PE}$, clone 8G12 (BD), anti-$CD138^{PerCP\text{-}Cy5.5}$, clone MI15 (BD), anti-$CD10^{APC}$, clone HI10a (BD), and anti-$CD45^{APC\text{-}H7}$, clone 2D1 (BD). Two million leukocytes in 100 μl of BM suspension were stained with mAbs for 15 min at room temperature in the dark. After staining, FACS lysing solution (2 ml, BD) was added. After 10 min at room temperature, samples were centrifuged and resuspended in 500 μl of phosphate buffered saline. The samples were immediately acquired in a FACSCanto II flow cytometer (BD) equipped with FACSDiva software (BD) (see Supplementary material for calibration details). A minimum of 3×10^5 leukocytes were acquired. Events acquired during the first 10–15 s for each tube were not recorded to avoid turbulence in the leading edge of samples. Data were analyzed using Infinicyt™ software, version 1.6 (Cytognos, Salamanca, Spain).

Leukocytes were gated on side scatter/forward scatter (SSC/FSC) and SSC/CD45 dot plots. The $CD34^+$ cells were identified as $CD34^+$ $CD45^{+lo}$ cells and PC as $CD38^{++}$ $CD138^+$ cells. Both cell populations were expressed as a percentage of leukocytes. The granulocyte population was gated on SSC/FSC and SSC/CD45 dot plots ($CD45^+$ SSC^{hi} cells). Then, $CD10^+$ neutrophils were identified and expressed as a percentage of the granulocyte population ($CD10^+$G) (Domingo et al., 2010; Sandes et al., 2013) (see Supplementary material for dot plots).

2.3. Statistical analyses

Statistical analyses were performed with SPSS Statistics software, version 20 (IBM). Differences between groups of samples were evaluated by an unpaired two-tailed *t*-test. The Mann-Whitney *U*-test was used to compare the ratio M/C between groups. $P < 0.05$ was considered statistically significant in all cases.

Once normal samples were classified into the two groups, values of the three parameters from all samples were analyzed by discriminant analysis, a multivariant technique that identifies differences in independent variables between groups, and gives a canonical function to discriminate these differences. This discriminant function was obtained using unstandardized coefficients without missing data imputation, and was termed the PB contamination index (PBCI). The PBCI values from MGUS and MM samples were calculated without including the percentage of PC in the formula, since it is increased in these diseases.

3. Results

3.1. Classification of normal BM aspirates according to the quality of samples

We analyzed the percentage of $CD10^+$G, $CD34^+$ cells, and PC in 122 BM aspirates from subjects without hematological disease (Fig. 1). We compared the results with the reference values of our laboratory, which are consistent with published reference ranges (Ocqueteau et al., 1998; Sandes et al., 2013) and classified samples in two groups. Twenty-seven samples simultaneously showed an increased percentage of $CD10^+$G and a decreased percentage of PC and $CD34^+$ cells and were considered contaminated with PB (CONT). The rest of the samples (n = 95) formed the good-quality (GC) group. The differences between groups were statistically significant in the three parameters analyzed ($P < 0.0001$).

As expected, CONT samples showed significantly lower leukocyte counts than GC specimens (Fig. 2A). In the samples in which morphological data were available (n = 57), we analyzed the disparity between the percentage of PC obtained from FCM and morphological studies. The disparity between both techniques was significantly higher in the group of contaminated samples (Fig. 2B).

3.2. Quantitative analysis of PB contamination in BM aspirates

To develop a formula for classification of any given sample as GC or CONT, we performed a discriminant analysis with the three parameters analyzed, as explained in the Materials and methods. The function obtained, named the PBCI (PB contamination index), was as follows:

$$\text{PBCI} = -3.052 + 0.065 * (\%\text{CD10}^{+}\text{G}) - 0.609 * (\%\text{CD34}^{+}) - 2.008 * (\%\text{PC}).$$

As shown in Fig. 3, the PBCI function performed in a linear fashion when an increasing amount of PB was added to a given BM sample.

A BM aspirate with the three parameters values falling in the average range of reference values would show a PBCI value about −1.00; whereas, the value for PB is approximately 3.4 (Fig. 3). As is usual in this kind of analysis, the mean between both values (1.2) was arbitrarily chosen as the cutoff for excessive PB in a BM aspirate. The validity of this cutoff was tested in the 122 samples analyzed. All samples from the CONT group had values ≥1.2; while, in the GC group, all samples but one had values < 1.2 (Fig. 4).

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