Flow Cytometric Study of Neutrophilic Granulopoiesis in Normal Bone Marrow Using an Expanded Panel of Antibodies: Correlation With Morphologic Assessments

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Flow cytometry studies of surface markers of neutrophils have been performed mostly on peripheral blood, and for a limited spectrum of diseases. Studying maturation defects on developing neutrophils in the bone marrow (BM) may be helpful in BM diseases, such as myelodysplastic syndromes and Shwachman-Diamond syndrome. We applied an expanded panel of antibodies to examine normal maturation patterns in 26 control samples of BM together with microscopic correlation. Promyelocytes correlated well with the CD24⁺ and CD11b⁺ populations, and metamyelocytes correlated well with the CD16⁺ population (intermediate positivity). An excellent correlation was also identified

between the sum of bands and segmented neutrophils and each of the following: CD16⁺⁺ (strong positivity), CD35⁺, CD87⁺, and CD64⁺. Although visually identified segmented neutrophils paralleled CD10 positivity, there was an appreciable difference between both methods. We conclude that neutrophilic granulocyte maturation in the BM is accompanied by a change in surface antigens that reflects certain stages of development. A successful strategy for detecting maturation defects is to include several antibodies that are known to be expressed or absent at the same stage of maturation, such as CD16, CD35, CD64, and CD87. *J. Clin. Lab. Anal.* 18:36–41, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Surface antigens of granulocytes are studied much less than other hematopoietic cells. Early studies of granulocytic surface antigens focused on the diagnosis of certain inherited disorders, such as leukocyte adhesion molecule deficiency, in which a specific antigen is known to be missing (1). Studies of peripheral blood (PB) granulocytes have expanded to include other antigens, such as CD55 and CD59, for the diagnosis of paroxysmal nocturnal hemoglobinuria (2). Since these studies are performed on PB, they may not pose a great challenge because the vast majority of PB granulocytes are of the same maturation stage (i.e., segmented neutrophils). Recently, there has been increasing interest in the use of surface antigen studies on bone marrow (BM) granulocytes to support the diagnosis of other inherited and acquired BM disorders, such as myelodysplastic syndromes and Shwachman-Diamond syndrome (3–6). In these conditions, granulocytic abnormalities can manifest themselves as a deficiency of a certain antigen, persistent expression of an early antigen, or the expression of an anomalous antigen

(5,7). Obviously, diagnosing maturation defects of BM neutrophilic granulocytes is a more challenging approach, which relies on an appropriate understanding of the normal maturation pattern of granulocytic surface antigens (8). Previous studies of normal granulocytic maturation involved a small number of surface antigens, with no expanded comprehensive panel that could be used to study granulocytic maturation in the BM (9–14). The study of granulocytes has well-known pitfalls and limitations. Granulocytes have intracytoplasmic storage pools of numerous surface antigens that can be translocated to the surface with granulocytic activation or excessive manipulation (15). For example, earlier studies routinely used density gradient separation of granulocytes, which has since been associated with

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significant changes in granulocytic surface antigen expression (16,17). Moreover, when gates are set using forward and side scatter properties, eosinophils and monocytes may be included in the gate. Both types of cells are known to have a different surface antigen expression, which could interfere with data interpretation. Antigens, such as CD10 and CD16, are known to be expressed on mature and maturing neutrophils, but not on normal eosinophils and monocytes. In this study we examined neutrophilic granulocyte maturation in BM from control individuals, using an expanded panel of antibodies, and correlated the results with those from a microscopic examination.

MATERIALS AND METHODS

BM neutrophilic granulocytes from 26 control subjects were studied. The subjects all underwent a BM examination for the staging of various neoplastic conditions. All of the subjects had normal complete blood counts (CBCs) and no evidence of BM involvement by the neoplastic process. Air-dried BM aspirate smears were stained with the Wright stain. Morphologic assessments of the BM aspirate slides were performed by two independent observers (M.T.E. and Y.G.). Each observer performed a 500 differential cell count, and the average percentages of cell types were recorded. The percentage of neutrophilic cell subtypes was corrected to reflect their percentage within the neutrophilic granulocyte population only (morphologic gating) to allow for comparison with the flow cytometry results. Flow cytometry was performed using a lysed whole-blood technique on EDTA-anticoagulated BM within 3 hr of collection. The following panel of antibodies was used (clones in parenthesis): CD10 (HI10a), CD11b (D12), CD11c (S-HCL-3), CD14 (MøP9), CD24 (ML5), CD33 (P67.6), CD34 (8G12), CD35 (E11), CD36 (CB38 (NL107)), CD43 (1G10), CD45 (2D1), CD45RA (L48), CD45RO (UCHL-1), CD49d (9F10), CD64 (10.1), CD66b (G10F5), CD87 (VIM5) (Becton Dickinson Biosciences, San Jose, CA); CD15 (80H5) (Immunotech, Miami, FL); and CD3 (S4.1), CD16 (3G8), and HLA-DR (TU36) (Caltag Laboratories, Burlingame, CA). A three-color analysis was performed using FACScan (Becton Dickinson Biosciences). The granulocyte gate was set using CD45-side scatter properties (Fig. 1A). The following antibody combinations were used: CD24 and CD14, CD3 and HLA-DR, CD34 and CD87, CD11b and CD16, CD15 and CD35, CD11c and CD45RA, CD43 and CD45RO, CD49d and CD64, CD33 and CD10, and CD36 and CD66b. Gates were adjusted to minimize eosinophils and monocytes as previously described (7). Eosinophils (CD49d⁺ CD64⁻) and monocytes (CD14⁺ CD24⁻) accounted for <2%

of events within the gate (Fig. 1B and C). In most antibodies, the scattergraph cursors were set using a visually identified negative population. Results were expressed as percentages of positive cells. CD16 expression was divided into high (16⁺⁺), low-intermediate (16⁺), and negative (16⁻) based on the high expression of CD16 seen on PB neutrophils, as previously described (Fig. 1D) (3). We attempted to correlate the morphologic BM differential count with antibody reactivity. In addition, we examined how the antibodies correlated with each other relative to their proposed reactivity during neutrophilic maturation. Statistical differences between results were confirmed using Student's *t*-test. This study was reviewed and approved by the institutional review board (IRB).

RESULTS

A total of 26 control subjects (13 males and 13 females) were studied. The median age was 20.5 years (range 1–71 years). The pertinent CBC results were as follows (median and ranges): hemoglobin 12.3 gm/dl (11.1–14.9), total white cell count $5.6 \times 10^9/l$ (4.2–10), absolute neutrophil count $2.6 \times 10^9/l$ (1.8–7.8), and platelet count $235 \times 10^9/l$ (167–412).

All of the pan granulocytic markers (CD33, CD15, CD43, and CD66b) were expressed in virtually all cells in the gate (99% $\pm$ 1%), although the intensity of CD33 expression was less compared to the other markers (12). As previously described (18), CD36 was virtually absent on the gated cells. HLA-DR and CD45RA stained <3% of BM neutrophils for all patients.

Summary of Morphologic and Flow Cytometric Correlations (Table 1)

Promyelocytes

A microscopic examination of BM showed the percentage of promyelocytes within neutrophilic granulocytes at 7% $\pm$ 4% (mean $\pm$ standard deviation). This

TABLE 1. Summary of neutrophilic granulocyte cell types and the best surface marker correlates

Cell type	Flow cytometric profile
Promyelocytes	CD24 ⁻ , CD11b ⁻
Myelocytes	CD11b ⁺ , CD16 ⁻
Metamyelocytes	CD16 ⁺ (intermediate intensity)
Promyelocytes and myelocytes	CD49d ⁺
Myelocytes, metamyelos, bands, and segs	CD24 ⁺ , CD11b ⁺
Metamyelos, bands, and segs	CD16 ⁺ , CD49d ⁻
Bands and segs	CD35 ⁺ , CD87 ⁺ , CD16 ⁺⁺ (high intensity), CD64 ⁻

Metamyelos: metamyelocytes; segs: segmented neutrophils.

percentage did not significantly differ from the percentages of CD24⁻ cells ($5.6\% \pm 3.9\%$) or CD11b⁻ cells ($7.5\% \pm 3.4\%$; $P > 0.1$) (Fig. 1B and D). There was also no statistical difference between the percentages of the CD24⁻ and CD11b⁻ cells.

Myelocytes

The percentage of visually identified myelocytes within the neutrophilic granulocyte population was $27\% \pm 9.2\%$. The percentage of CD11b⁺ CD16⁻ cells was $31.9\% \pm 10.8\%$ (Fig. 1D). Although these numbers overlap, the difference was borderline statistically significant ($P = 0.06$).

Metamyelocytes

The percentage of metamyelocytes was $24\% \pm 6.1\%$. A statistically nonsignificant difference was observed for

the granulocytes with low to intermediate expression of CD16 (CD16⁺) of $22.6\% \pm 6\%$ ($P = 0.4$). This population was easily defined by flow cytometry, and the number was reproducible for individual cases (Fig. 1D). The percentage of cells with the phenotype CD64⁺ 49d⁻ of $25.6\% \pm 9\%$ was also not statistically different from the percentage of metamyelocytes. However, this population was difficult to identify by visual observation of the flow cytometry scattergraphs, and the percentage was mostly determined by calculation (Fig. 1C).

Bands and segmented neutrophils

The microscopic percentage of this population was $42.2\% \pm 12.8\%$. Several markers correlated well with this population, including CD16⁺⁺ ($38\% \pm 11.6\%$),

Fig. 1. Flow cytometry scattergraphs of (A) CD45-side scatter gate showing granulocytic gating, (B) CD24-CD14 showing the CD24⁻ tail of promyelocytes and the location of monocytes, (C) CD49d-CD64 showing the location of eosinophils, and (D) CD11b-CD16 showing areas of low-intermediate positivity for CD16 (CD16⁺) and high positivity (CD16⁺⁺).

CD35⁺ ($43.6\% \pm 12\%$), CD87⁺ ($39.6\% \pm 10.8\%$), and CD64⁺ cells ($40\% \pm 11.1\%$) (Figs. 1C and D, and 2A and B). These results confirm our previous observation (in a smaller study) that CD87 is expressed at the band stage of development (14).

Segmented neutrophils

The percentage of segmented neutrophils by visual examination was $24.8\% \pm 14.3\%$. As expected, we encountered the problem of significant interobserver variation when we attempted to identify segmented neutrophils. The percentage of CD10⁺ cells was the lowest of all examined markers in the BM ($18\% \pm 8\%$) (Fig. 2C). CD10 is known to be expressed at the segmented stage of neutrophilic maturation (19,20).

There was a borderline statistical difference between CD10⁺ cells and microscopically identified segmented neutrophils ($P = 0.04$).

Promyelocytes and myelocytes

The percentage of cells in this group, by visual evaluation, was $35.6\% \pm 11.9\%$. This percentage correlated very well with the percentage of CD49d⁺ cells ($35.2\% \pm 9.8\%$; $P > 0.8$) (Fig. 1C). On the other hand, CD11c⁺ cells did not provide a good correlation for this subset of cells, with the percentage of CD11c⁺ cells being $48.6\% \pm 10.6\%$ (Fig. 2D). It has been reported that CD11c is expressed at the myelocyte stage and maintained throughout neutrophilic maturation (21).

Fig. 2. Flow cytometry scattergraphs of (A) CD87⁺, (B) CD35⁺, (C) CD10⁺, and (D) CD11c⁺ populations.

Myelocytes, metamyelocytes, bands, and segmented neutrophils

The percentage of the sum of these cells was $92.7\% \pm 4\%$, which correlated with the percentage of $CD24^+$ cells ($94.7\% \pm 3.3\%$) and $CD11b^+$ cells ($92.7\% \pm 3.5\%$). This study expands our previous observation that CD24 is expressed at the myelocyte stage of maturation (13).

Metamyelocytes, bands, and segmented neutrophils

The percentage of this population was $64.5\% \pm 11.5\%$. This was not statistically different from the percentage of $CD49d^-$ cells ($64.8\% \pm 9.8\%$), $CD45RO^+$ ($61.1\% \pm 16\%$), and CD16 positive cells ($CD16^+$ and $CD16^{++}$) ($60.6\% \pm 11.9\%$). We found that it was most difficult to reproduce the results of CD45RO, compared to the other markers, for individual patients. The percentage of $CD11c^+$ cells ($51.4\% \pm 11.6\%$) did not correspond to the other markers or to the morphologic assessment of this group. Our study suggests that CD11c is expressed at the late stages of metamyelocyte development, after the surface expression of CD16.

DISCUSSION

The study of granulocytic surface markers is gradually attracting more interest. However, the question of how to interpret flow cytometry results on granulocytes (22) is still a matter of debate. Moreover, some notable references contain debatable schemes of granulocytic surface marker maturation (23). Previous studies have provided isolated views of a small number of markers. In this study, we applied an expanded panel of antibodies to examine neutrophilic granulocyte maturation in the BM together with morphologic assessments. Although this study was based on statistical analysis and involved no sorting experiments, our previous sorting studies showed a strong correlation between morphologic types and statistical analysis (13,14). For individual maturational stages in the BM, promyelocytes and metamyelocytes showed the best correlation with specific markers. Promyelocytes corresponded to the $CD24^-$ and $CD11b^-$ flow cytometry populations. In a previous, smaller experiment that included cell sorting, CD24 was shown to be expressed at the myelocytic stage of development (13). Metamyelocytes correlated with the cell population with intermediate expression of CD16. Previous studies have indicated that CD16 begins to be expressed at the metamyelocyte stage and reaches its peak at the band and segmented stages of neutrophilic development (9,10). The expression of CD16 should not be studied in the presence of infection

because it is decreased or even completely absent under such circumstances (24,25). Although the percentage of $CD10^+$ cells in the marrow tended to parallel that of segmented neutrophils, there was an observable difference between both percentages, which may be related to the limitation of morphologic identification of neutrophils. The percentage of $CD10^+$ was less than that of morphologically identified neutrophils in most cases, which indicates that morphologic classification has a tendency to overestimate segmented neutrophils. Several subsets of maturational stages correlated well with certain markers. Promyelocytes and myelocytes correlated well with $CD49d^+$ cells. Myelocytes, metamyelocytes, bands, and segmented neutrophils closely matched the percentages of $CD24^+$ and $CD11b^+$ cells. Metamyelocytes, bands, and segmented neutrophils corresponded to the percentages of $CD49d^-$ cells, CD16-positive cells (both $CD16^+$ and $CD16^{++}$), and $CD45RO^+$ cells, although the latter were not as reproducible as the previous two markers. The best correlation with a group was obtained for bands and segmented neutrophils with $CD16^{++}$, $CD35^+$, $CD87^+$, and $CD64^-$. Although it was previously reported that CD11c is expressed at the myelocytic stage (26), in the current study it did not correlate well with a specific stage of development, and apparently was expressed at the late stages of metamyelocytes.

The current results suggest that granulocytic maturation in BM can be studied with the use of a judicious panel of antibodies to follow the normal scheme of maturation, as demonstrated in this work. Other antibodies, which were not available to us during this study, may be useful in future experiments. These include CD54, which is strongly expressed at the promyelocytic stage and is weakly or not at all expressed with further maturation, and CD66a, which begins to be expressed at the myelocytic stage of development and is maintained with further maturation (27,28). The application of several antibodies to surface markers known to be expressed or absent at a certain stage of maturation, such as CD16, CD35, CD87, and CD64, may provide an acceptable means of assessing maturational abnormalities.

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