

Short communication

Flowcytometric analysis of basophil counts in human blood and inaccuracy of hematology analyzers

Background: Differential leukocyte counts are of proven clinical value, but information about basophil counts in normal and disease conditions is scarce although basophils are regarded as key effector cells in allergy.

Aims: To establish and validate flowcytometric methods for counting basophils in peripheral human blood, to determine reference values, and to examine the accuracy of two widely used hematology analyzers.

Methods: Basophils were measured in whole blood by flowcytometry after staining with antibodies against the IL-3-receptor (CD123) or the eotaxin-receptor (CCR3) combined with other markers used for gating or validation purposes.

Results: The basophil percentages in 95 healthy adults showed an excellent correlation between the two independent flowcytometric methods, demonstrating that both are accurate and precise. The most robust marker is CCR3, which seems to be sufficient to specifically identify basophils. Normal values of relative and absolute blood basophil counts were 0.22–1.28% and 0.014–0.087 G/L (95% reference intervals), respectively. Basophil counts measured with two hematology analyzers Coulter GEN-S and ADVIA-120 showed no correlation between these instruments. Comparing the data obtained by flowcytometry and the analyzers demonstrate that basophil counts of the GEN-S are erratic, while the ADVIA-120 gives at least an estimation of true basophil numbers.

Conclusions: We provide a solid description and validation of a novel and rapid method for the flowcytometric enumeration of basophil in whole blood. The fact that the most heavily used Hematology autoanalyzer gives completely erroneous results could explain why basophil counts have not yet received recognition as a clinically useful diagnostic marker.

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Differential leukocyte counts have a long tradition as diagnostic tools in Medicine. Basophils represent a morphologically distinct granulocyte population in human blood. They are a rich source of histamine, leukotrienes, IL-4 and IL-13 (1, 2), major mediators of allergic inflammation (1). Although basophils cannot be missed in stained blood smears, their low frequency leads to an insufficient precision of visual basophil counts (3). A method based on manual counts of cells stained with toluidine blue has been described many years ago, but this technique is too laborious for routine clinical use (4). Thus, little is known about basophil counts in normal and disease conditions. Automated leukocyte differentiation analyzes several thousands of leukocytes thereby enabling in principal accurate determinations of basophil numbers (5). However, because morphological leukocyte differentiation is used as gold standard in Clinical Hematology, the validation of basophil counts has not attracted sufficient interest (6).

Using defined basophil markers and exclusions of other leukocyte populations we describe two independent

methods for measuring the basophil percentage by flowcytometry in whole blood. The results are compared with data from two hematology analyzers.

Methods

Blood donors

In this study, approved by the local ethics committee, venous blood anticoagulated with EDTA from an unselected population of 95 healthy adult volunteers (mean age 33.4, range 17–60 years, 30 males and 65 females) was collected between 7 and 9 a.m. and analyzed within the same day in parallel by two unrelated flowcytometry methods and by the hematology analyzers Coulter[®] GEN-S (Beckman Coulter, Fullerton, CA, USA) and Bayer ADVIA[®]-120 (Bayer Diagnostics, Tarrytown, NY, USA).

Leukocyte staining

Staining was performed in whole blood. A 100- μ L blood was incubated with the antibodies for 15 min at room temperature in the dark. After lysis of erythrocytes with LysingSolution[®], cells were

washed in CellWASH® and fixed with CellFIX® (BD Bioscience, San Jose, CA, USA).

Two combinations of Abs were used:

- (1) Staining based on CD123: 5 µl CD123-PE, 20 µl anti-lin1-FITC (CD3/14/16/19/20/56), 10 µl HLA-DR-PerCP, 5 µl CD11c-APC [(BD), Franklin Lakes, NJ, USA].
- (2) Staining based on CCR3: 10 µl anti-immunoglobulin E (IgE)-FITC (Caltag, Burlingame, CA); 10 µl CD3-PerCP (BD); 10 µl anti-CCR3-PE (R&D, Minneapolis, MN, USA).

Analysis by flowcytometry and hematology analyzers

The data of 50 000 events were acquired using a FACSCalibur® and analyzed using CellQuest® Pro software (BD Bioscience). Calibrations and color compensations were performed daily. Differential leukocyte counts were performed with two hematology analyzers: Coulter® GEN-S and Bayer ADVIA®-120. All measurements were done under quality-management guidelines of our accredited laboratories.

Statistical analysis

To check the accuracy of the flowcytometric methods and compare them with hematology analyzers we calculated the linear regressions of the percentages of basophils using Excel software,

Microsoft Corp., Redmond, WA. The frequency distribution of the percentages of basophils was performed using Prism software, GraphPad Software Inc., San Diego, CA. For the calculation of the reference intervals, expressed as median values with 95% double-sided reference intervals, we employed a nonparametric percentile method of reference interval calculation using the MedCalc (MedCalc Software, Mariakerke, Belgium) statistics program (7).

Results and discussion

Staining based on CD123

Previous studies demonstrated that IL-3 strongly enhances the functions of mature basophils (2, 8). Although other leukocytes also express IL-3 receptors, we found that basophils express particularly high densities of the IL-3Rα (CD123) (8). We used an Ab-combination marketed by BD for analyzing plasmacytoid and myeloid dendritic cells (DC). The lineage cocktail is used to gate out lymphocytes, monocytes and neutrophils (9). Apart from basophils, plasmacytoid DC are the only CD123-high lin-neg/dim population, which can be separated from basophils by their high HLA-DR expression. Apart from the DC and basophils, eosinophils (CD123-med,

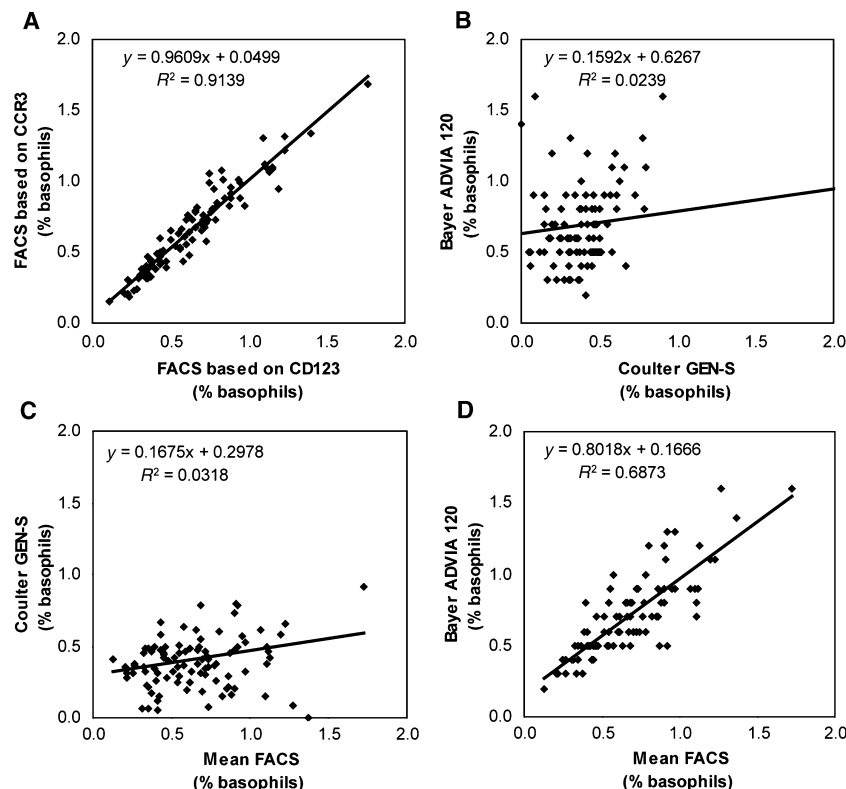


Figure 1. Accuracy of basophil measurements by flowcytometry and hematology analyzers. Panel A shows the linear regression analysis of the basophil percentages obtained by the two independent measurements obtained by flowcytometry. Panel B compares the data obtained by the two hematology analyzers. The mean of the percentages of basophils from the two flowcytometry measurements were compared with the data from Gen-S (C) and with ADVIA 120 (D).

lin-med, HLA-DR-med, CD11c-med) are also easily identified (see Supplement Fig. 1).

If basophils only are of interest, a two-color measurement based on light scatter and CD123-high/HLA-DR-neg/dim expression may be sufficient. It should be noted, however, that a proportion of basophils express HLA-DR at variable, albeit lower, levels even in a normal population. Thus, one cannot exclude that in certain diseases HLA-DR is up regulated on basophils to a degree making a distinction from CD123-high DC difficult.

Staining based on CCR3

Our previous studies have shown that basophils and eosinophils, in contrast to other blood leukocytes, constitutively express the eotaxin-receptor CCR3 (10), thereby mediating their attraction to several CC-chemokines (10, 11).

High densities of CCR3 were detected on basophils of all 95 donors, thus representing a most discriminatory basophil marker: CCR3-high basophils and eosinophils are easily separated by their light scatter characteristics (in the upper lymphocyte or upper granulocyte gates, respectively). Since activated Th2-lymphocytes can also express CCR3 (12), we included anti-CD3 to identify CCR3-positive Th2-cells. However, the number of CCR3-positive T-cells was negligible, at least in normal individuals, indicating that measurements based on light scatter and CCR3-high alone may be sufficiently precise.

The anti-IgE label was included in the analysis as a further basophil marker, showing that all basophils (based on light scatter and CCR3-high) stained also for IgE and that there was no IgE-positive CCR3-negative population. Since the density of the FcεRI on basophils was variable (consistent with previous findings [13]), the discriminatory power of anti-IgE is, however, less consistent (see Supplement Fig. 2). A flowcytometric analysis of basophil counts based on staining of IgE and IgE-receptor together with manual counting of toluidine stained cells has been used in a study of chronic urticaria patients (14). With both methods a subgroup of patients with basopenia could be identified which related to serum histamine releasing activity, indicating that these methods are of clinical value. Although marked basopenia could be detected, the correlation of basophil counts between the manual and their flowcytometric method was not strong enough to be directly comparable in clinical practice.

Absolute and relative basophil counts determined by flowcytometry

Linear regression of basophil percentages of the independent flowcytometry measurements shows a very strong correlation ($R^2 = 0.9139$) between these methods based on entirely different phenotypic characteristics

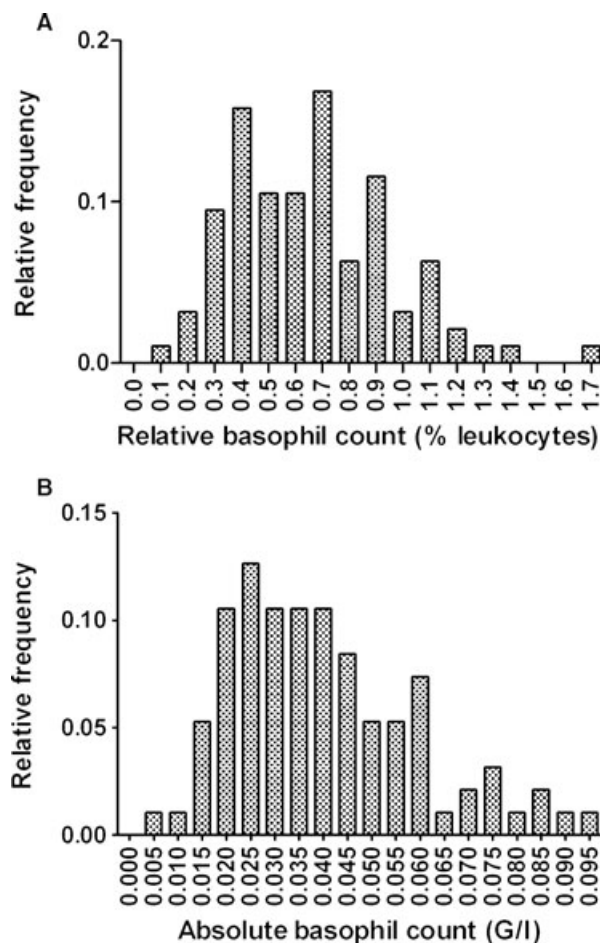


Figure 2. Frequency distribution of peripheral blood basophils. The frequency distribution of the relative (A) and absolute basophil (B) counts, as determined by the mean of the two flowcytometry measurements, in the blood of 95 healthy blood donors is shown. Normal values of relative and absolute blood basophils counts were 0.63% median, interval 0.22–1.28% and 0.037 G/L median, interval 0.014–0.087 G/L, respectively.

(Fig. 1A). Furthermore, both are equally accurate at low and high basophil values.

The frequency distributions and normal basophil values of 95 donors are shown in Fig. 2. Normal values of relative and absolute blood basophils counts were 0.63% median, interval 0.22–1.28% and 0.037 G/L median, interval 0.014–0.087 G/L, respectively. No statistically significant ($P = 0.273$) difference of basophil counts were observed between males and females. Note that in this unselected population asymptomatic atopy was not excluded. It will now be interesting to examine whether basophil numbers differ among nonatopic and atopic populations and whether basophil counts are a useful parameter in patients with symptomatic allergy or in some other diseases. Possibly due to the insufficient accuracy of routine leukocyte differentiation, information on basophil counts in different diseases is scarce and most

studies are rather old and based on blood histamine, an indirect parameter of basophil numbers (15–17).

Comparison with basophil counts determined by two hematology analyzers

A comparison of the basophil percentages obtained by the ADVIA-120 and the GEN-S by linear regression (Fig. 1B) demonstrates that at least one of the machine counts must be erroneous ($R^2 = 0.0239$). Because of the excellent correlation between independent flowcytometry measurements, the means of flowcytometric basophil percentages were taken as gold standard and compared to the data of the hematology analyzers. Figure 1C demonstrates that the basophil counts of the GEN-S, a widely used instrument in Clinical Hematology (5), are completely erratic ($R^2 = 0.0318$). It is, indeed, rather disturbing that so many false results are handed daily to physicians in centers throughout the world, even if these values are neglected for the care of patients. Basophil percentages obtained by flowcytometry fit much better the ADVIA-120 values (Fig. 1D), although variations of more than 100% are not consistently discriminated ($R^2 = 0.6873$). Thus, the ADVIA-120 is clearly more accurate giving at least an estimation of true basophil counts.

Both flowcytometry methods also allow an enumeration of eosinophils. Excellent correlations were found for the eosinophil percentages between flowcytometric methods and the data of the GEN-S or ADVIA-120, demonstrating that all methods are equally accurate for this parameter (data not shown).

This study describes two independent methods for measuring blood basophils, their normal values and distribution, and the pitfalls of hematology analyzers. The simple staining procedure is performed in whole

blood and the measurements could be easily automated. The best and most robust marker is CCR3 and ongoing clinical studies indicate that single staining with anti-CCR3 is sufficiently discriminatory also under pathological conditions. Our study also underscores the poor performance of major hematology autoanalyzers for differentiating basophils, obviously because these have not been sufficiently validated (6). Even if basophil counts are neglected by most physicians, we find it scandalous that completely erroneous results are included in the routine laboratory reports. That automated counts with the Coulter® GEN-S are not suitable for assessing basophil numbers in patients has also been noted by Grattan et al. (18) in a more recent study on chronic urticaria where basophils were counted manually. Although the Bayer ADVIA®-120 (and possibly other analyzers using a technique based on the resistance of basophils to acid stripping) normally gives at least an approximation of basophils counts, they may be subject to other errors in certain conditions. For example it has been shown that myeloma cells in the blood of patients are counted as basophils by this analyzer (19). Since lower numbers of plasma cells are observed in the blood, e.g. during a strong immune response and in auto-immune diseases, false basophil numbers must also be expected with the ADVIA®-120 in these circumstances.

The methods described here now provide the opportunity to determine whether basophil counts are a useful diagnostic parameter in certain diseases.

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