Flow cytometry quantification of granulocytic alkaline phosphatase activity in unlysed whole blood

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BACKGROUND

The determination of Granulocyte Alkaline Phosphatase (GAP) activity is a simple and useful test in several hematological diseases and malignancies, such as Chronic Myeloid Leukemia, Polycythemia Vera and leukemoid reactions. The cytochemical GAP assay score is based on microscopy visualization of a cell-bound brown precipitate, 100 neutrophils are assessed on a blood smear, and categorized into 3 to 4 different color-intensity categories. Although this method is the most common assessment method in modern medicine, it is observer-dependent. Reference values or reference intervals are established for each laboratory, and experienced personnel is required. We have developed a new flow cytometry (FCM) no-lyse no-wash (NLNW) assay to detect cellular alkaline phosphatase in intact neutrophilic cells, using a fluorogenic live cell permeant substrate.

MATERIALS AND METHODS

Freshly drawn whole blood specimens anticoagulated with EDTA were used in this study (n=29).

Cytochemical Method. Based on the hydrolysis of phosphates esters from a naphthol phosphate (substrate) at alkaline pH values. The liberated naphthol is coupled to a diazonium salt (4'amino-4'-methoxy-diphenylamine) to form a brown azo-dye, which is precipitated according to the enzyme location/activity. The intensity of staining is dependent on the activity of the enzyme.

GAP Index:

One hundred neutrophils were classified as follows: Level 0, without enzymatic activity; Level 1, enzymatic activity; Level 2, cytoplasm with highest enzymatic activity (Figure 1). The formula used to calculate the GAP index was the following:

[(L0 granulocytes x 0) + (L1 granulocytes x 1) + (L2 granulocyes x 2)]

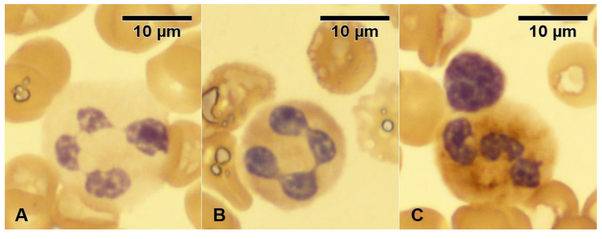

Figure 1. Representative optical microscopy observations of GAP activity by cytochemistry. Representative example of color-intensity categories of cell-bound brown precipitate in neutrophils and optical microscopy: Level 1 (A); Level 2 (B) and Level 3 (C).

Digital image analysis:

Digital images of blood cells from digitized blood smears were obtained at 100x immersion oil objective with a Olympus BX53 optical microscope equipped with a Olympus DP21 digital camera. Images were taken with a resolution of 1600x1200 pixels in TIFF format and analyzed using ImageJ v1.50i software.

Digitized pictures were converted to black and white 32-bit visualizations (Figure 2 A, B). The grayscale channels were used to obtain multivariate data which provides 256 levels of intensity between black (level 0) and white (level 255). Background and inter-cell spaces were discriminated using the "Threshold adjustment tool" (Figure 2 C, D). The darkest peak in histogram refers to cells, and the whitest refers to inter-cell space (Figure 2 C). Neutrophils were then selected using the "Freehand selection tool" (Figure 2E, F). Once both areas were located, the nuclei were discriminated using the "Threshold adjustment tool" (Figure 2 G, H). The darkest peak in the histogram refers to nucleus, and the whitest refers to cytoplasm. After processing (Figure 2 H), multivariate data can be obtained using the "Analyze-measure tool".

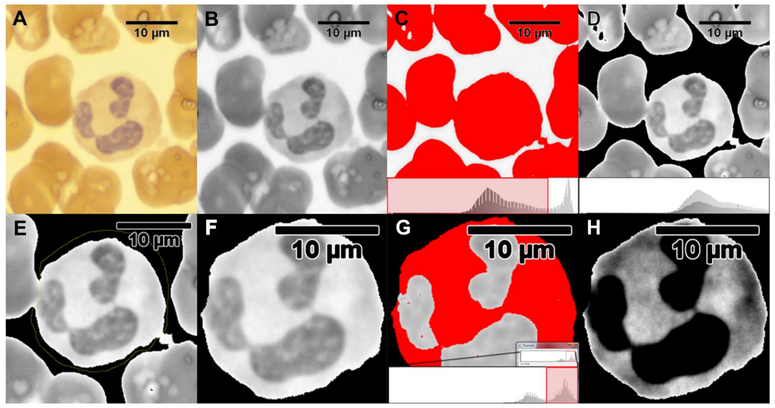

Figure 2. Image processing and analysis provides a means to extract and quantify microscopy images obtained on digital CCD cameras. A. Blood microscopic image with one photography obtained at 100x magnification. B. Conversion to 32-bit image (grayscale). C, D. Discrimination of background/inter-cell space using threshold adjustment tool and selecting the peak of cells. E, F. Isolation of neutrophil with the freehand tool. G, H. Discrimination of nuclei using threshold adjustment tool and selecting the cytoplasmic peak.

Flow Cytometry Method. Leukocytes were stained with 10µM Vybrant™ DyeCycle™ Violet Stain (DCV, Thermo Fisher) protected from light in a dedicated water bath at 37ºC. DCV was excited at 405 nm and its emission was collected using the following filter combination: 413 LP, 495 DLP, and 440/50 BP. DCV fluorescence was displayed in logarithmic scale. DCV threshold levels were set empirically using a SSC vs. DCV-H dual parameter plot to eliminate from detection the large amounts of red blood cells that are found in unlysed whole blood. Samples were incubated with Alkaline Phosphatase Live Stain (APLS) for 20 minutes, in a dedicated water bath at 37ºC (in the dark). APLS controls were incubated at 4ºC to prevent enzyme activity. Acquisition was stopped when 50,000 events were collected in the leukocyte gate, on the Attune Acoustic Focusing Cytometer (Thermo Fisher), with APLS collected using 488 nm excitation and 530/30 nm emission. APLS fluorescence intensities were classified as follows: no intensity for no enzyme activity (L0), and increased levels from dim to bright accordingly (L1, L2, and L3) (Figure 3). The formula used to calculate the APLS index was the following:

$$\left(\frac{(Mdn_{L1}-Mdn_{L0})}{rSD_{L0}\times 2}\right)\times \%L1_{events}+\left(\frac{(Mdn_{L2}-Mdn_{L0})}{rSD_{L0}\times 2}\right)\times \%L2_{events}+\left(\frac{(Mdn_{L3}-Mdn_{L0})}{rSD_{L0}\times 2}\right)\times \%L3_{events}$$

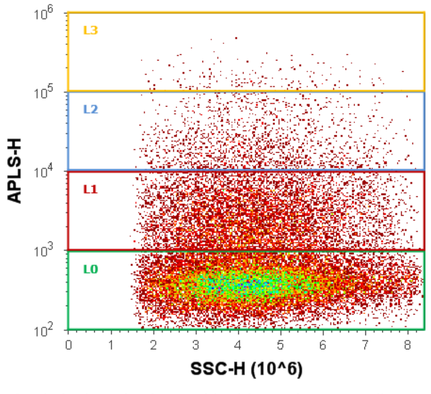

Figure 3. Bivariate density-plot in which the X-axis represents side light scatter and the Y-axis represents alkaline phosphatase activity. Region L0 contains autofluorescent cells. Regions L1 to L3 contain cells of low to high fluorescence intensity, used to identify and quantify changes in the median intensity and number of granulocytes. Neutrophils were gated based on nucleic acid dye viable staining, scatter properties, and single cell events.

Statistical methods used in this study were Wilcoxon test, Bland-Altman test and Kruskal-Wallis test using GraphPad Prism Software®.

RESULTS AND DISCUSSION I

Comparison of cytochemistry and FCM index results suggest that FCM has a higher sensitivity than the cytochemistry method, allowing the detection of higher values which can not be detected in a color-intensity classification (Figure 4). Cytochemical and FCM GAP index values normalized to 100, show differences between FCM and microscopy visualization (Cytochemistry) indexes were statistically significant (95% CI, 17.38-45.95, P-value <0.0001).

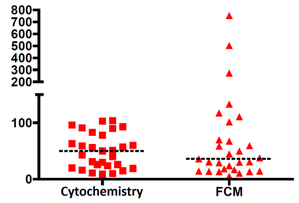

Figure 4. Comparison of GAP activity scores with cytochemistry and FCM index. GAP scores obtained from n=29 samples, with cytochemistry method (left) and FC method (right).

RESULTS AND DISCUSSION II

Bland–Altman plots for method comparisons used 29 freshly drawn whole blood samples. For each sample, the average GAP index score was represented against the difference between the GAP index score obtained with the compared methods. The average ± 1.96 standard deviation of the difference (s.d.d.) value describes the 95% confidence interval for the difference between two methods. Bland-Altman analysis showed good agreement, and only two of the specimens tested were not in agreement (±1.96 rSD; +274.8, -359.5) (Figure 5).

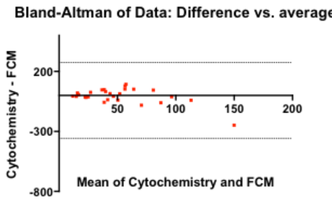

Figure 5. Bland-Altman plots for GAP index comparisons using freshly drawn whole blood. Bland-Altman comparison of cytochemistry vs. FCM GAP index. Bias (-42.3) and limits of agreement (±1.96 rSD; +274.8, -359.5).

RESULTS AND DISCUSSION III

The image analysis software ImageJ was used to quantify the intensity of cell-bound precipitates. These results were compared with the classical cytochemical method. Homogeneous immersion objectives are necessary to better discriminate background from cells and. Without these, neutrophils with increased enzymatic activity will not be completely discriminated from background. The median of the pixel intensity showed statistically significant differences in the color intensity (Kruskal-Wallis statistic = 57.86; p-value < 0.0001) (Figure 6).

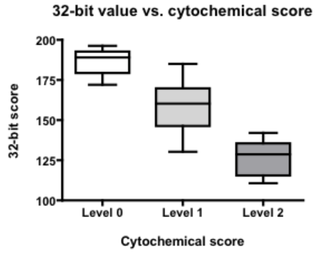

Figure 6. Intensity of cell-bound precipitates of neutrophils cytoplasm measured with image software analysis. X-axis represents the GAP level classification as showed in figure 1 and the Y-axis represents the median value of color intensity obtained with image software analysis.

CONCLUSIONS

Comparison of FCM and enzyme cytochemistry techniques showed that scores were not similar in terms of range**.** Moreover, FCM data resulted in increased variability of fluorescence intensity assignment, whereas image analysis software reported GAP values comparable with observer-dependent analysis. The staining and analytical flow cytometric methods reported here facilitate the detection, and quantification of subpopulations of leukocytes with different alkaline phosphatase activity. These experiments demonstrate the value of FCM as an adjunct to conventional cytochemical methods to quantify the GAP activity in unlysed whole blood. However, clinical data obtained with FC should be interpreted with caution due to the potential existence of different GAP activities.

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