RESEARCH ARTICLE

Correlation between the cell population in the automated hematology analyzer high-fluorescence region and atypical lymphocyte flags

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Introduction: During routine blood measurements using an automated hematology analyzer, two easily confused types of suspect flags related to lymphocytes often appear: atypical and immature lymphocytes. The aim of this study was to investigate the correlation of high fluorescence cell (HFC) parameter and lymphocyte flags determined from an automated hematology analyzer.

Methods: A total of 93 patients affected by various pathologic conditions (viral infection, immunological disease, oncological disease and tumor) were divided into an "atypical lymphocytes" group ("atypical" for short), an "immature lymphocytes/blasts" flag group (abnormal), a mixed-flag group that includes "atypical lymphocytes" (mixed), and a non-flag group (non-flag).

Results: The numbers of HFCs in the atypical, abnormal, mixed, and non-flag groups were 1.8% (0.9%-5.5%), 0.7% (0.1%-5.0%), 2.3% (1.2%-5.0%), and 0.8% (0.7%-1.2%), respectively. The HFCs of "atypical" appeared as a separate cluster with clear boundaries. The HFCs of "abnormal" as an unclear boundaries, and it was difficult to accurately distinguish between the HFCs from the immature lymphocytes and the normal lymphocytes. The lower limit of HFC when the atypical lymphocyte flag appeared was $0.04 \times 10^9/L$. The number of HFCs was similar to atypical lymphocytes detected by microscopy and $CD19^+CD20^-CD27^{++}$ cells by flow cytometry at 78% and 76%, respectively. The number of HFCs detected in "atypical" and $CD19^+CD20^-CD27^{++}$ cells showed good consistency ($r = .715$), whereas the consistency was poorest for "abnormal" ($r = .176$).

Conclusion: It demonstrates that HFCs reflects atypical lymphocytes better than immature lymphocytes/blasts.

KEYWORDS

atypical lymphocytes, automated hematology analyzer, high fluorescence cell, immature lymphocytes/blasts

1 | INTRODUCTION

During routine blood measurements using an automated hematology analyzer, two easily confused types of suspect flags related

to lymphocytes often appear: atypical and immature lymphocytes. Atypical lymphocytes are lymphocytes with an abnormal morphology that appear in the peripheral blood during viral diseases as well as in patients with immune disease.¹ Immature lymphocytes appear among the precursor/immature lymphocytes in the peripheral blood, and are often found in hematological malignancies or the use of bone marrow-stimulating drugs after the radiation and chemotherapy of tumors. In the white blood cell (WBC) differential scatter plot (DIFF)

Abbreviations: APC, allophycocyanin; DIFF, differential scatter plot; FITC, fluorescein isothiocyanate; HFC, high-fluorescence cell; IMG, immature granulocyte; PE, phycoerythrin; WBC, white blood cell.

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of an automated hematology analyzer, the high-fluorescence region is located in the upper region of lymphocytes, and cells that fall in this region are known as high-fluorescence lymphocytes, referred to as high-fluorescence cells (HFCs). The increase of HFC may be related to the increase of pathological lymphocytes. However, the correlation between HFC increase and “atypical lymphocyte” flag, or “abnormal lymphocyte” flag remains unknown.

2 | EXPERIMENTAL

2.1 | Sample sources

A total of 93 anticoagulant (ethylenediaminetetraacetic acid salt)-treated whole blood samples were collected from outpatients and hospitalized patients at Peking Union Medical College Hospital between July 2014 and September 2015. According to the flag information of the hematology analyzer and clinical diagnoses, the samples were divided into an “atypical lymphocytes” flag group (38 samples, including 25 cases of viral infection, 8 cases of immunological disease, and 5 cases of oncological disease), an “immature lymphocytes/blasts” flag group (21 samples, including 15 cases of viral infection and 7 cases of tumors), a mixed-flag group that included “atypical lymphocytes” (23 samples, including 15 cases of viral infection, 3 cases of immunological disease, and 5 cases of oncological disease), and a non-flag group (11 samples, including 5 cases of viral infection and 6 cases of immunological disease). Three of these samples were used to determine the source of the high-fluorescence region of cells: one from a patient with infectious mononucleosis diagnosed according to an “atypical lymphocytes” flag along with 4% atypical lymphocytes visible by microscopy (hereafter referred to as “atypical”), one from a patient with lymphoma diagnosed according to an “immature lymphocytes/blasts” flag (hereafter “abnormal”), and one from an apparently healthy individual with the addition of cultured plasma leukemia cells (hereafter “plasma cells”). Human plasma leukemia cells (NCI-H929) were purchased from the Peking Union Cell Resource Center (IBMS, CAMS/PUMC).

2.2 | Confirmation of the HFC source in the WBC DIFF scatter plot

Conventional measurement of the whole blood was performed using a BC-6800 automated hematology analyzer (Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). The absolute number and percentage of HFCs were recorded. The sources of HFCs were determined by blood smear preparation dyeing and microscopic observation of cell morphology. The SP1000i automated slide preparer/stainer was purchased from Sysmex (Kobe, Japan), and slides were observed under the 80i optimal microscope (Nikon, Tokyo, Japan).

2.3 | Confirmation of HFCs and minimum detection limit for the atypical lymphocytes flag

A sample with an absolute number of $0.21 \times 10^9/L$ HFCs and confirmed to contain atypical lymphocytes by microscopic morphological

inspection was selected for determination of the limit of detection. After dilution through a concentration gradient of 0.9, 0.5, 0.4, 0.3, 0.2, and 0.1 with 0.9% normal saline, the results were detected and recorded with the automated hematology analyzer.

2.4 | Pre-treatment and gating strategy for flow cytometry analysis

Fifty microliters of peripheral whole blood was collected and placed in a flow cytometry test tube. Fluorescein isothiocyanate (FITC)-CD27 (5 μ L), phycoerythrin (PE)-CD20 (5 μ L), PE-Cy5-CD19 (5 μ L), and allophycocyanin (APC)-CD138 (10 μ L) (BD Biosciences, Franklin Lakes, NJ, USA) were added in this order, mixed, and placed in the dark for 15 minutes. Red blood cell lysis buffer (700 mL) was added, and after 15 minutes the mixture was centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, 2 mL of phosphate buffered saline was added, and the sample was mixed and centrifuged at 300 g for an additional 5 minutes. The supernatant was removed, and 0.5 mL of fixative was added, mixed, and placed into the flow cytometer (FACSaria II, BD Biosciences). Figure 1 shows the flow cytometry gating strategy. The FSC/SSC gate was used first to isolate WBCs, and was then used to isolate lymphocytes within the WBC gate. Within the lymphocyte gate, the PE-Cy5 CD19/FITC CD27 gate was used to isolate CD27⁺CD19⁺ cells. Within the CD27⁺CD19⁺ gate, the PE CD20/FITC CD27 gate was used to isolate CD27⁺⁺CD19⁺CD20⁻ cells. After detection was complete, FlowJo 7.6 software was used to analyze the results.

2.5 | Data analysis

The consistency between the numbers of HFCs determined automatically and atypical lymphocytes identified microscopically was evaluated by checking whether the deviation between the numbers falls within the acceptable range for determining the coincidence rate based on the H20A standards of the American Clinical and Laboratory Standards Institute.

The consistency between the percentage of HFCs detected from the automated hematology analyzer and the percentage of CD19⁺CD20⁻CD27⁺⁺ cells in flow cytometry was determined by evaluation of the coincidence rate. Moreover, line charts of the HFC% and CD19⁺CD20⁻CD27⁺⁺% values of the “atypical lymphocytes” flag group, “immature lymphocytes/blasts” flag group, mixed-flag group containing “atypical lymphocytes”, and non-flag group were constructed to analyze the consistency between the values.

3 | RESULTS

3.1 | Expression of HFCs and CD19⁺CD20⁻CD27⁺⁺ between different flag groups

The HFC% and CD27⁺⁺CD19⁺CD20⁻% values of the mixed flag group containing “atypical lymphocytes” and the “atypical lymphocytes” flag group were substantially higher than those of the

FIGURE 1 Flow cytometric gating strategy for CD19⁺CD20⁻CD27⁺⁺ cells

non-flag group and the “immature lymphocytes/blasts” flag group. However, the HFC% and CD27⁺⁺CD19⁺CD20⁻% values of the “immature lymphocytes/blasts” and the non-flag groups were similar (Table 1).

3.2 | WBC differential distribution characteristics of pathological lymphocytes in different flag groups

For the “atypical” sample, 4% atypical lymphocytes were observed by microscopy. In the routine WBC DIFF scatter plot, there was a significant increase in the proportion of cells in the high-fluorescence region, with an increase in HFC% but no abnormality in the immature granulocyte percentage (IMG%) and no “immature granulocyte” flag. For the “plasma cell” sample, there was an increase in the number of cells in the right part of the high-fluorescence region and a significant increase in IMG% with the appearance of the “immature granulocyte” flag, but no abnormality in HFC%. For the “abnormal” sample, HFCs, lymphocytes, and monocytes were all found in the same region without clear boundaries. HFC% could not be

determined, and there was no abnormality in IMG%; At the same time, a “WBC differential scatter plot abnormality” flag appeared (Figure 2 and Table 2).

3.3 | Determination of the limit of detection for the atypical lymphocytes flag from HFC values

The results above indicated that an increase in the number of HFCs is primarily due to the presence of atypical lymphocytes from viral infection. Thus, we further determined the limit of detection of the “atypical lymphocytes” flag in HFCs using the automated hematology analyzer. A sample with a known absolute count of $0.21 \times 10^9/L$ HFCs was diluted through a concentration gradient of normal saline. When the absolute count of HFCs was $0.04 \times 10^9/L$, the automated hematology analyzer could still produce the “atypical lymphocytes” flag, but when the absolute count was $0.03 \times 10^9/L$, no such flag was produced (Table 3). Therefore, an absolute count of $0.04 \times 10^9/L$ HFCs was as the lower limit of detection for the automated hematology analyzer “atypical lymphocytes” flag.

TABLE 1 HFC% and CD27⁺⁺CD19⁺CD20⁻% in different flag groups (median and range)

Cells	“Atypical lymphocytes” flag group	“Immature lymphocytes/blasts” flag group	Mixed-flag group containing “atypical lymphocytes”	Non-flag group
HFC%	1.8 (0.9-5.5)	0.7 (0.1-5.0)	2.3 (1.2-5.0)	0.8 (0.7-1.2)
CD27 ⁺⁺ CD19 ⁺ CD20 ⁻ %	0.28 (0.01-2.34)	0.13 (0.01-1.02)	0.30 (0.04-1.02)	0.14 (0.09-0.34)

HFC, high-fluorescent cells.

FIGURE 2 Distribution of pathological lymphocytes in the white blood cell differential scatter plots of different flag groups (“atypical lymphocytes” sample, “plasma cell” sample, “abnormal lymphocytes” sample). SS, side scatter, related to intracellular granular density; FL, fluorescence; DIFF, differential scatter plot; HFC, high-fluorescent cells

Parameters	“Atypical lymphocytes” sample	“Plasma cell” sample	“Immature lymphocytes” sample
HFC (×10 ⁹ /L)	0.27	0.01	0
HFC (%)	3	0.1	0
IMG (×10 ⁹ /L)	0.02	3.11	0.82
IMG (%)	0.2	35.5	0.5
Flags	Atypical lymphocytes Lymphocytes increased	WBC differential abnormality Differential abnormality Immature granulocytes? Nuclear left shift? Basophils increased	WBC differential abnormality

TABLE 2 Comparison of pathological lymphocytes in different diseases with relevant routine blood parameters

HFC, high-fluorescent cell; IMG, immature granulocyte; WBC, white blood cell.

TABLE 3 Changes in “atypical lymphocyte” flags at different high-fluorescent cell (HFC) concentrations

Sample	Dilution factor	HFC (×10 ⁹ /L)	“Atypical lymphocyte” flag
1	1	0.21	Yes
2	0.9	0.19	Yes
3	0.5	0.10	Yes
4	0.4	0.09	Yes
5	0.3	0.05	Yes
6	0.2	0.04	Yes
7	0.1	0.03	No

3.4 | Comparison of automated HFC counts and results of atypical lymphocytes detected by microscopy and flow cytometry

The number of HFCs determined by the hematology analyzer and the number of atypical lymphocytes found by microscopy, as a reference method, showed a coincidence rate of 78%. Similarly, HFCs and the percentage of CD19⁺CD20[−]CD27⁺⁺ cells detected by flow cytometry

showed a coincidence rate of 76%. Moreover, the microscopic results is 0%-10%, the results of flow cytometry are 0.1%-17.8%. The median of two methods are 1% and 1.5%.

Next, the correlation between HFCs and the percentage of CD19⁺CD20[−]CD27⁺⁺ cells was analyzed according to the hematology analyzer lymphocyte flags. The correlation coefficients (*r*) of the percentages of HFCs and CD19⁺CD20[−]CD27⁺⁺ cells in the “atypical lymphocytes” flag group, “immature lymphocytes/blasts” flag group, mixed-flag group containing “atypical lymphocytes”, and the non-flag group were 0.715, 0.176, 0.516, and 0.206, respectively. Thus, the consistency between the percentages of HFCs and CD19⁺CD20[−]CD27⁺⁺ cells was highest in the “atypical lymphocytes” flag group and was lowest in the “immature lymphocytes/blasts” flag group. Figure 3 shows the percentages of HFCs and CD19⁺CD20[−]CD27⁺⁺ cells for each group.

4 | DISCUSSION

Some atypical lymphocytes are formed from the differentiation of lymphocytes toward plasma cells following antigen stimulation.

FIGURE 3 Analysis of correlation trends between HFC% and CD19⁺CD20[−]CD27⁺⁺% according to lymphocyte-related flags

Atypical lymphocytes are often observed in pathological conditions such as in viral infection, antigen stimulation, drug use, blood transfusion, cardiopulmonary bypass, hemodialysis, and after surgery. These lymphocytes play an important role in the development and progression of diseases, including inflammation, autoimmune disease, and multiple myeloma.^{2,3} By contrast, immature lymphocytes are more closely related to hematological malignancies and drug treatment.

As we know, most of atypical lymphocytes express T-cells. However, the research from Linssen et al showed that there are close association between HFC and CD19⁺CD20[−]CD27⁺⁺ cells. In our studies, we divided all samples into four groups, the median of “atypical lymphocytes” flag group and a mixed-flag group that included “atypical lymphocytes” are higher than other groups. Therefore, the root cause of mixed-flag group that included “atypical lymphocytes” has the highest median is the combined action of “atypical lymphocytes” and other cells.

During routine complete blood count and white blood cell differential (CBC+Diff), an automated hematology analyzer can provide parameters related to lymphocytes such as the “atypical lymphocytes” flag, “immature lymphocytes/blasts” flag or HFCs. The results of the present study showed that when the number of HFCs reached a threshold value, the hematology analyzer produced the “atypical lymphocytes” flag. Determining whether or not there is a correlation between a clear detection of HFCs and the lymphocyte-related flags originating from different diseases will have reference value for conducting a targeted morphological review during routine blood analysis and for assisting with clinical diagnosis and treatment.

Atypical lymphocytes arising from viral infection are located in the HFC region of the analyzer output, accompanied by an increase in the HFC% value. Although plasma cells arising from tumors that are frequently detected as lymphoplasmacytic lymphoma and B-cell lymphoma are located near the HFC region, we found that the HFC%

does not increase in such cases, whereas IMG%, which is related to immature/blast cells, increases instead. Immature and blast lymphocytes arising from tumors are distributed in the lymphocyte, monocyte, and granulocyte regions with no HFC% increase, suggesting abnormality in the WBC DIFF scatter plot. CD138 is a transmembrane proteoglycan expressed on the surface of many tumor cells.^{4,5} In the bone marrow, CD138 is only present in B lymphocytes, and changes in its expression are related to the specific stage of differentiation; it is expressed in pre-B cells and plasma cells, but not in mature B cells. Oncological disease can stimulate lymphocyte differentiation to plasma cells, during which immature lymphocytes show a stronger tendency to differentiate to abnormal lymphocytes. However, changes in atypical lymphocytes in infectious disease do not significantly affect CD138 expression.

Therefore, to further analyze the correlation between HFC parameters and atypical lymphocytes, we analyzed the consistency between the morphologically immature lymphocytes determined by microscopy and the number of CD19⁺CD20[−]CD27⁺⁺ cells determined by flow cytometry. CD19⁺CD27⁺⁺ and CD19⁺CD138⁺ are markers of activated B cells and plasma cells. Because a subset of plasma cells in the peripheral blood express CD27 but not CD138, CD27 was selected as the marker for plasma cells in this study.^{6–8} The results revealed good coincidence rates between the numbers of HFCs and CD19⁺CD20[−]CD27⁺⁺ cells and between the numbers of HFCs and atypical lymphocytes identified by microscopy. This indicates that there is a high concordance between cells expressing CD19⁺CD20[−]CD27⁺⁺ and atypical lymphocytes. In contrast, the number of CD19⁺CD20[−]CD27⁺⁺ cells was poorly correlated with the number of IMGs. In the “atypical lymphocytes” flag group, arising from viral infection and immunological disease, the consistency between the changes in the number of HFCs and CD19⁺CD20[−]CD27⁺⁺ cells was significantly greater than that of the “immature lymphocytes/blasts” flag group arising from oncological disease. This indicates that HFC detection in the DIFF scatter plot can

reflect atypical lymphocytes related to viral infection or immunological disease relatively well, but is not ideal for indicating immature lymphocytes related to tumors.

In summary, an increased number of HFCs in the WBC DIFF scatter plot from the hematology analyzer and the "atypical lymphocyte" flag has certain clinical significance in monitoring viral infection and immunological disease. At the same time, the high number of cases of viral infection and immunological disease included in the "atypical lymphocytes" flag group could indicate that HFCs are more strongly correlated with atypical lymphocytes than with immature lymphocytes/blasts.

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