Comparison of Methodologies for Detecting Reticulated Platelets and Establishment of Normal Reference Range

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Aims: To compare the detection methodologies of reticulated platelets and establish the normal reference range. Methods: The percentage of reticulated platelets in a healthy population was measured by two methods; the Sysmex XE-5000 blood cell analyzer using polymethyl oxazine staining, and a flow cytometer using thiazole orange staining. The correlation between the two methods was analyzed. The advantages and disadvantages of the two methods were clarified. Statistical analysis for the reference ranges in the two methods was conducted. Result: The correlation between the two methods was r = 0.67. The reference range for polymethyl oxazine staining was 1.0%-7.5%, and the reference range for thiazole orange staining was 3.0%-10.5%. Conclusion: Polymethyl oxazine staining was simpler and more stable than thiazole orange staining, and is therefore a preferable method for detecting reticulated platelets.

Key Words Reticulated Platelets (RPs), Thiazole Orange (TO), Polymethyl Oxazine (PO), Immature Platelet Fraction (IPF)

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INTRODUCTION

When compared to the mature platelets, neo-platelets that are newly released from megakaryocytes into the blood circulation are larger, have more granular cytoplasm, and can be stained by methylene blue. Their residual RNA in cytoplasm is stained with the dye. Such "juvenile" platelets are referred to as reticulated platelets (RPs) or immature platelets. Similar to reticulated erythrocytes, they are in an immature phase in the megakaryocyte-to-platelet transition [1,2]. The RNA content in the peripheral platelets is closely related to the activity of megakaryocytes. When the megakaryocyte activity is increased, RNA content is increased accordingly. That is, the RPs and the immature platelet fraction (IPF) are increased. Megakaryocyte activity can be extrapolated from RPs. Moreover, the percentage of RPs reflects the severity of damage of platelets and the generation of platelets in bone marrow [2,3]. It also allows differentiation between thrombocytopenia related to bone marrow disorder and thrombocytopenia related to peripheral platelet consumption. Clinically, measurement of RPs has important implications in the differentiation, diagnosis, and treatment of autoimmune thrombocytopenic purpura (AITP), thrombotic thrombocytopenic purpura (TTP), and abnormalities after chemotherapy or bone marrow transplantation [3-6]. Previously, RPs had been detected by flow cytometry with thiazole orange (TO). Now, a new method employs polymethyl oxazine (PO), followed by detection with an automated blood cell analyzer.

In this study, we compared the two methods to make clear their advantages and shortcomings and established the reference ranges.

MATERIALS AND METHODS

1. Instruments and reagents

The XE-5000 blood cell analyzer was used along with imported genuine reagent kits and genuine whole blood

quality control material (manufactured by Sysmex Corporation), under participation in the global online quality control system of Sysmex. The FACSCalibur flow cytometer (manufactured by Becton Dickson, BD) was used with CD41a-PE antibody and TO dye purchased from BD. Venous blood vacuum collection tubes with EDTA-K2 were manufactured by Guangzhou Improve Medical Instruments Co., Ltd.

2. Data of sample providers

Samples were obtained from a total of 22 groups of healthy volunteers who received health examinations in the clinic of our hospital between February and May 2010. Among these 336 healthy volunteers, 160 were male and 176 were female; their ages ranged from 23 to 82 years. Inclusion criteria were that various biochemical, immunological, and hematological parameters were within normal ranges and that the result of health examination showed to be in good health.

3. Analyses with XE-5000

As a fully automated analyzer, it was used to analyze the samples after preheating for 15 minutes following start-up. Automated measurement of IPF was completed within one minute after placing an EDTA-K2 anticoagulated whole blood sample into the instrument. A total of 30 samples were analyzed at 30 minutes and at 2 hours after blood sampling, to check the influence of sample storage times on the results. For statistical analysis of reference range, a total of 22 batches of the samples from the 336 individuals were analyzed within three months.

4. Analyses with flow cytometer

Through the pre-experiments conducted using the above-described 30 samples, method No. 5 was identified as the best method (***Table 1***). A total of three batches of samples from 57 individuals for statistical analyses of reference range were analyzed within one week.

5. Statistical processing

SPSS 10.0 was used for statistical analysis. Since the data were in a non-normal distribution, reference ranges were obtained by percentile method. Correlation between the two methods was analyzed and made into a scatter plot.

RESULTS

Analysis diagrams of RP with the two methods are shown in ***Fig. 1***.

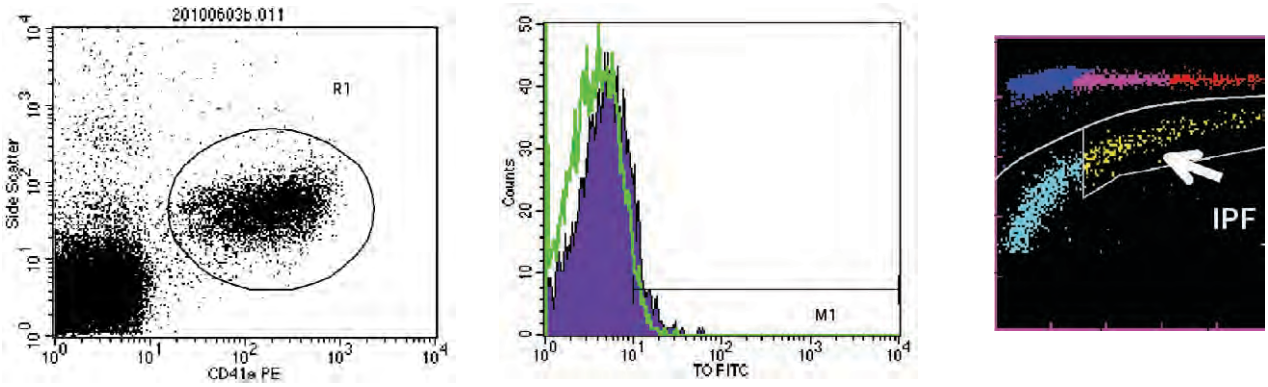
As being shown in ***Table 2***, when samples with different storage times were tested with the XE-5000, the differences of result were statistically insignificant ($P > 0.05$). In contrast, the differences of result obtained by the flow cytometer were statistically significant ($P < 0.0001$).

The correlation coefficient between the two methods was 0.67 (***Fig. 2***).

The reference range for the XE-5000 by statistical analysis of 22 batches of samples from 336 individuals in three months was 1.0 - 7.5%. On the other hand, the reference range for the flow cytometry was 3.0 - 10.5% (***Table 3***).

Table 1 *Six methods used for pre-experimental flow cytometric analyses*

Method	Step 1	Step 2	Step 3	Step 4
1	CD41a+whole blood+TO	incubation 1h	detection	
2	CD41a+whole blood	incubation 15min+TO	incubation 1h	detection
3	CD41a+plasma+TO	incubation 1h	detection	
4	CD41a+plasma	incubation 15min+TO	incubation 30min	detection
5	CD41a+plasma	incubation 15min+TO	incubation 1h	detection
6	CD41a+plasma	incubation 15min+TO	incubation 2h	detection



A B C

Fig. 1 *Analysis diagram of RPs with the two methods*

A: CD41a-PE labeled platelet cluster
B: Analysis diagram of RPs with flow cytometer after TO staining
C: Scattergram of IPF on XE-5000 with PO staining

Table 2 *Results obtained with two methods after different sample storage times*

Method	Sample storage time	Number of samples	Average	Median	95% CI	
XE-5000	30 min	30	2.84	2.86	1.0 - 7.5%	$P > 0.05$
	2 h	30	2.82	2.85	1.0 - 7.5%	
Flow cytometer	30 min	30	2.13	2.58	0.7 - 10.0%	$P < 0.0001$
	1 h	30	6.25	6.70	3.0 - 10.5%	
	2 h	30	11.2	12.12	6.2 - 18.6%	

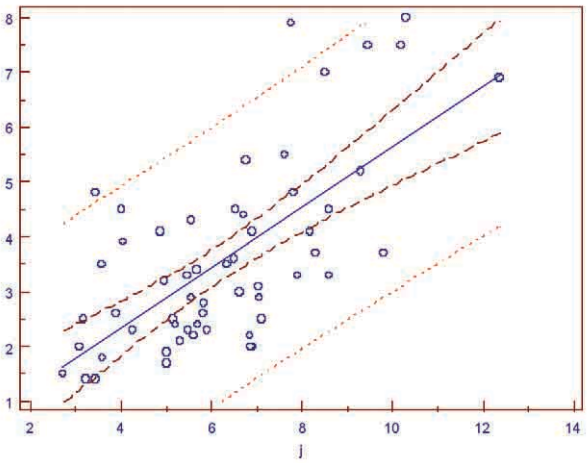


Fig. 2 *Scatter diagram of correlation between two methods*
(n = 57, r = 0.67)

Table 3 *Reference ranges of two methods*

Method	Total number of sumples	Average	Median	Reference range (95% CI)
XE-5000	336	2.83	2.88	1.0 - 7.5%
Flow cytometer	57	6.27	6.71	3.0 - 10.5%

DISCUSSION

The measurement of RPs is conducted on the use of specific fluorescent stains. Such stains can penetrate through the cell membrane and go into the cytoplasm and bind to the residual RNAs. Semiconductor laser irradiation to the stained cells causes them to emit different scattered light and fluorescence intensities. Thus, the RPs can be measured by detecting these scattered light and fluorescence.

Flow cytometric detection of RPs is susceptible to the influence of numerous factors, and the process has been hardly standardized[7,8]. In this study, we detected RPs in the same sample incubated with TO stain for 30 minutes, 1 hour, and 2 hours using flow cytometer. The results were significantly different, and were poorly correlated. The percentage of RPs rose over the staining incubation time. Different incubation times led to significantly different results, which are consistent with previously-reported data[7,8]. Similarly, we evaluated the effect of sample storage time to RPs detection with the XE-5000. In the case, the entire process from sample aspiration to analysis was conducted in a preset fully automated mode. The only difference in detection conditions was the storage time between the samples collection from the patients and the measurement. In this experiment, the results from the same sample showed no significant difference after sample storage times of 30 minutes and 2 hours ($P > 0.05$) and were well correlated ($r = 0.998$).

The reference range was established as 1.0 - 7.5%, which is consistent with a published data[9]. It was determined by the XE-5000 because the instrument produced more consistent results than the flow cytometer for detection of RPs.

Normally, with flow cytometric detection of RPs, the process starts with reagent preparation followed by a number of steps which are sample and reagent (CD41a-PE and TO) addition, incubation, cell collection, and analysis. Such a complicated operation impedes standardization. On the other hand, fully automated processing with the XE-5000 is simple and convenient and can be easily standardized.

Without a reference material and a quality control substance, flow cytometry can not ensure its traceability and reliability. In contrast, blood cell analysis with the XE-5000 is reliable because there is a global online quality control system.

In conclusion, flow cytometric detection of RPs involves multiple influencing factors, poor stability, a complicated process, lack of traceability, and higher cost. In contrast, the XE-5000 detection is stable, involves simple and convenient operation, and has lower cost. In addition, it is highly sensitive and specific in the screening of thrombocytopenia [9-12]. Therefore, it can replace flow cytometry[12] and provides a good basis for the widespread use of RPs detection in clinical settings.

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