Measurement of Reticulated Platelets in Thrombocytopenia — Methodology and Clinical Utility —

HIDEAKI OGATA

The Second Department of Medicine, Kurume University School of Medicine,
Kurume 830-0011, Japan

Summary: In this study, we explored the methodology and clinical utility of reticulated platelets analysis. Reticulated platelets were measured using flow cytometry in peripheral circulation with thiazole orange (TO). We chose fixed platelet-rich plasma as the sample since it has been known to give stable results. The percentage of reticulated platelets in 7 patients with idiopathic thrombocytopenic purpura (ITP) was significantly increased ($8.41 \pm 5.35\%$; mean $\pm$ SD), compared with that in 21 normal subjects ($1.92 \pm 1.27\%$). The value in 16 patients with liver cirrhosis was slightly below normal ($1.86 \pm 0.85\%$). These findings suggested that thrombopoiesis was not promoted under the condition of hypersplenism or of abnormal pooling, and that any elevation in the percentage level of reticulated platelets could be clinically useful in the diagnosis of ITP.

Key words reticulated platelets, thrombocytopenia, liver cirrhosis, ITP

INTRODUCTION

The pathological cause for thrombocytopenia is hypoproduction, excessive destruction, or an abnormal pooling of platelets. Any assessment of these three thrombocytopenic disorders first requires discriminating diagnosis. It is technically difficult to distinguish between the hypoplastic platelet state and any change in turnover. The current methods employed to examine thrombopoiesis are bone marrow aspiration, quantification of platelet-bound immunoglobulin G, and platelets survival studies. However, each of these has remained painful, expensive and troublesome. Accordingly there is a need for a noninvasive simple test to estimate thrombopoiesis in bone marrow.

Some methods for indirectly examining bone marrow platelet production have been recently described [1-3]. Ingram and Coppersmith first reported circulating platelets with an increased amount of RNA (reticulated platelets) in the peripheral blood of dog and demonstrated an increase in the percentage level of reticulated platelets following acute blood loss, using a

microscopy technique to stain them supravivally with new methylene blue [4]. They found that reticulated platelets were probably analogous to reticulocytes. Erythrocytic reticulocytes have been associated with erythropoiesis in bone marrow.

Newly-generated erythrocytes in peripheral blood still contain some amounts of cytoplasmic RNA and lose the RNA as they grow more mature. Thiazole orange (TO), which becomes intensely fluorescent when bound to RNA, is commonly used for flow cytometric measuring of reticulocytes. TO is a very useful dye for reticulocytes analysis [5]. This dye has been applied to reticulated platelets analysis recently.

We have also determined the percentage levels of reticulated platelets in thrombocytopenic patients using flow cytometric analysis with TO in analogy to reticulocyte. Examining fixed platelet-rich plasma as the sample has provided reliable and stable results.

Measuring the percentage level of reticulated platelets could lead to an estimate of bone marrow thrombopoiesis, in stead of other procedures. Using this new technique, we have managed to discriminate between the two thrombocytopenic states of immune thrombocytopenia and of hypersplenism.

MATERIALS AND METHODS

Assay of reticulated platelets

The sample of whole blood sample is collected into a Venoject II tube contained disodium (Terumo, Inc., Tokyo, Japan). Platelet-rich plasma is prepared by centrifugation at 150 g for 15 min, and is pelleted by further centrifugation at 1,500 g for 10 min. The pellet is then washed once in citrate glucose EDTA buffer (sodium chloride 117 mmol/ℓ, sodium citrate 14 mmol/ℓ, dextrose 11 mmol/ℓ, sodium phosphate dibasic 9 mmol/ℓ, potassium phosphate monobasic 2 mmol/ℓ, and disodium EDTA 10 mmol/ℓ in distilled water at pH 7.4) and then twice in Tyrode's BSA EDTA buffer (sodium chloride 140 mmol/ℓ, potassium chloride 2.7 mmol/ℓ, dextrose 5.5 mmol/ℓ, sodium acid phosphate 0.42 mmol/ℓ, sodium bicarbonate 12 mmol/ℓ, disodium EDTA 10 mmol/ℓ, and bovine serum albumin 2 mg/ml in distilled water at pH 7.1). Platelets are resuspended and fixed in 1% formaldehyde at 4 °C for 2 hs. Fixed platelets are

washed twice in Tyrode's buffer, and prepared as an assay sample after staining with TO solution (0.1 μg/ml at final concentration) at room temperature for 1 h in a dark box.

RNase treatment

Fixed platelets are treated with RNase before incubation with TO by the following procedure. Platelets are washed twice in Tyrode's buffer after fixation, then resuspended in 1 ml of buffer. The platelets suspension is incubated with 1 mg of RNase at 37 °C for 1 h.

Flow cytometric assay

The samples are analyzed in a Ortho Cytron flowcytometer equipped with an argon-ion laser. Fluorescence is detected as red fluorescence through a 530/30 nm band-pass filter. The platelets population in forward versus lateral scattering is confirmed as the population of CD41 positive events in the cytogram and is then gated for analysis area. A positive range is set such that less than 1% of the

TABLE 1.
Clinical characteristics and results for 16 patients with liver cirrhosis and for 8 patients with idiopathic thrombocytopenic purpura

Patient	Age	Sex	Diagnosis	Platelet count ($\times 10^9/\ell$)	Reticulated platelet (%)
1	61	M	LC + HCC	22	2.7
2	41	M	LC	23	1.5
3	69	F	LC	167	0.8
4	61	M	LC + HCC	67	2.4
5	78	M	LC	94	1.3
6	54	F	LC	52	1.7
7	61	F	LC	125	3.7
8	65	F	LC	125	1.5
9	55	M	LC	81	0.7
10	70	F	LC	89	1.1
11	76	F	LC	78	1.6
12	55	M	LC	102	2.9
13	67	F	LC	93	1.9
14	75	F	LC	52	1.1
15	42	M	LC	62	3.0
16	61	M	LC	126	1.9
17	37	M	ITP	26	12.2
18	19	F	ITP	29	10.1
19	61	F	ITP	44	2.4
20	47	M	ITP	33	5.0
21	63	M	ITP	36	1.5
22	40	F	ITP	54	13.7
23	61	F	ITP	26	14.0

control: 21 normal volunteers (15 male and 6 female, age range 24-42 years, platelet range $157-325 \times 10^9/\ell$)

population in the RNase-treated sample is incubated as the forward scatter versus red fluorescence on a cytogram.

Subjects

Twenty-one normal volunteers (15 male and 6 female, aged 24-42 years, platelet range $157-325 \times 10^9/\ell$), 7 patients with idiopathic thrombocytopenic purpura (ITP) whose platelet counts were in the range of $26-54 \times 10^9/\ell$, and 16 patients with liver cirrhosis due to HCV infection whose platelet counts

were in the range of $22-167 \times 10^9/\ell$ were examined (Table 1).

Statistics

Statistical analysis was performed using Wilcoxon's signed-ranks test. The results of analysis were expressed as mean $\pm$ SD. $p < 0.05$ was set as the significant level.

RESULTS

Fixation of platelets

The effect of the fixation of the platelets with formaldehyde on the reticulated platelet percentage was examined. Using the intact platelets of a normal control subject, the percentage of reticulated platelet was found to increase with increasing incubation time with TO (Fig. 1).

In the case of the platelets fixed by formaldehyde, the percentage did not vary over a wide range of incubation time from 20 min to 120 min. The positivity rate was found to remain within the narrow range of 2.2% to 2.4%. Consequently, we chose fixed platelets for the following experiments.

Flowcytometry analysis

To confirm that the reticulated platelet population measured was included among the platelet population, a normal control subject sample was labeled with CD41 MoAb directed against GPIIb/IIIa. The CD41 MoAb-positive population area recognized to

Fig. 1. Effect of fixation on positive rate of reticulated platelet. The effect of fixation on the positive rate of the reticulated platelet was investigated. The positive rate was increased depending on the incubation time of thiazole orange with intact platelets, and was stable with fixed platelet.

A: CD 41 positive cells

B: Fw vs Rt scattering

Fig. 2. Population of platelets in the cytogram. CD 41 positive cells were recognized as the platelet population in forward versus right scattering, and gated as an assay area for quantifying the percentage level of reticulated platelet.

be platelets comprehended the population gated to assay the number of reticulated platelets (Fig. 2).

RNase treatment

The effect of treatment of platelet-rich plasma with RNase on reticulated platelet measurement was investigated. Fig. 3 demonstrates the percentage level of reticulated platelets in a patient with ITP with (right), and without (left), RNase treatment of the sample. It was found that treatment with RNase resulted in a clear distinct decrease in TO positivity percentage in the patient. Fig. 3 also shows that the TO positive platelets were larger than the negative platelets.

Values of reticulated platelet percentage in subjects

The percentage of reticulated platelets was $1.92 \pm 1.27\%$ (mean $\pm$ SD) in the normal volunteers, $1.86 \pm 0.85\%$ in the cirrhotic patients, and $8.41 \pm 5.35\%$ in the patients with ITP. All the patients with liver cirrhosis showed no elevation in the percentage of reticulated platelets. In those with ITP 4 of the 7 patients showed an increased percentage of reticulated platelets, while the other 3 had normal values (Fig. 4). The percentage level of reticulated platelets in the patients with liver cirrhosis was similar to that in the normal volunteers. On the other

hand, a significantly higher level was found in the patients with ITP, than in the normal volunteers ($p < 0.02$) and than in the patients with liver cirrhosis ($p < 0.09$).

Fig. 4. Distribution of reticulated platelets in different disease groups.

Fig. 3. Effect of RNase on reticulated platelet. The populations of RNase-untreated (A) and RNase-treated (B) platelets of a patient with idiopathic thrombocytopenic purpura stained with thiazole orange are shown in forward versus red fluorescence cytograms. The positive cell population was decreased in the RNase-treated platelets.

DISCUSSION

Similar to the value of determining the level of reticulocytes in erythropoiesis, reticulated platelets could be useful to estimate bone marrow platelet production in various thrombocytopenic disorder. To use a sample of the peripheral blood from a patient has made the technique of analysis less invasive and more simple, in contrast to bone marrow aspiration, or biopsy each of which is biopsy, painful. However, there remains the technical difficulty in determining platelet life span when exposed to radioisotope during examination.

The aim of this study was to establish a method to analyze TO-positive platelets using flow cytometry to discriminate the pathogenesis from that of immune thrombocytopenia, and that of abnormal pooling of platelets.

Some authors have recently reported analysis of reticulated platelets [2]. Kienast and Schmitz have applied TO staining evaluated by fluorescence-activated flow cytometry to human platelets in reticulated platelets analysis [6]. They found that the average percentage of reticulated platelets in normal subjects was $8.6 \pm 2.8\%$ (mean $\pm$ SD). However, the other investigators have reported different values [1-3,7].

The various findings might have resulted from differences in the sample-being either whole blood or platelet-rich plasma and differences in fixation of the platelets. In the present study, we used fixed platelet-rich plasma. The rate of percentage of reticulated platelets gradually increased with increasing incubation time with TO when the platelets were not fixed with formaldehyde (Fig. 1). Meanwhile, it was not changed over 2 hs when the platelets were fixed. The reason for elevation may be due to a characteristic property of TO that permeates the live platelet membrane and binds to the nucleotide of the granule [5]. Therefore we chose to utilize fixed samples. The percentage of reticulated platelets in patients with ITP was increased compared to that in patients with liver cirrhosis or normal subjects.

As a result of our findings, clinical measurement of reticulated platelets may become a useful method for the diagnosis of ITP, since the assaying PA-IgG was non-specific to this disease. In this study, one half the patients with ITP showed no increase in the percentage of reticulated platelets. Accordingly, we have postulated that ITP may be an heterogeneous thrombocytopenic disorder and consist of some different subpopulations with autoimmune states.

The reticulated platelet count corresponded well with the bone marrow biopsy result [7].

The patients with liver cirrhosis showed no elevated percentage in reticulated platelets. Generally, the pathological mechanism of thrombocytopenia in cirrhotic patients has been found to be mainly due to abnormal pooling and partially-increased peripheral platelet destruction through some immunological mechanism [8-12]. The mechanism has been demonstrated by platelet life-span measurements, and platelet distribution with radioisotope, and by PA-IgG.

In this study, we have demonstrated that the percentage of reticulated platelet in cirrhotic patient was not increased. This finding suggested that thrombopoiesis was not promoted in liver cirrhosis.

Previous studies have reported that the spleen played a central role in the distribution and destruction of platelets in liver cirrhosis and that the platelet survival time was reduced in this state.

The reason for the platelet production not being promoted under the condition, of increased destruction of platelets in peripheral blood is not yet clear. However, the present study has demonstrated that measurement of the reticulated platelets can provide us a technique to discriminate between ITP and liver cirrhosis easily.

ACKNOWLEDGMENTS: The author thanks Professor K. Tanikawa (The Second Department of Medicine, Kurume University School of Medicine) for critical reading of the manuscript. Thanks are also extended to Dr. Yoshimoto and Mrs. Kusaba for their technical assistance in flow cytometry measurement.

REFERENCES

1. Ault KA, Rinder HM, Mitchell J, Darmody MB, Vary CPH et al. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. *Am J Clin Pathol* 1992; 98:637-646.
2. Richards EM, and Baglin TP. Quantitation of reticulated platelets: methodology and clinical application. *Br J Haematol* 1995; 91:445-451.
3. Rinder HM, Munz UJ, Ault KA, Boan JL, and Smith BR. Reticulated platelet in the evaluation of thrombocytopenic disorder. *Arch Pathol Lab Med* 1993; 117:606-610.
4. Ingram M, and Coppersmith A. Reticulated platelets following acute blood loss. *Br J Haematol* 1969; 17:225-229.
5. Lee LG, Chen C-H, and Chiu LA. Thiazole orange: A new dye for reticulocyte analysis. *Cytometry* 1986; 7:508-517.

6. Kienast J, and Schmitz G. Flow cytometric analysis of thiazole oragen uptake by platelets: A diagnostic aid in the evaluation of thrombocytopenic disorders. *Blood* 1990; 75:116-151.
7. Romp KG, Peters WP, and Hoffman M. Reticulated platelet counts in patients undergoing autologous bone marrow transplantation: an aid in assessing marrow recovery. *Am J Hematol* 1994; 46:319-324.
8. Toghill PJ, Geern S, and Ferguson R. Platelet dynamics in chronic liver disease with special reference to the role of the spleen. *J Clin Pathol* 1977; 30:367-371.
9. Aoki Y, Hirai K, and Tanikawa K. Mechanism of thrombocytopenia in liver cirrhosis: kinetics indium-111 tropolone labelled platelets. *Eur J Nucl Med* 1993; 20:123-129.
10. Aster RH. Pooling of platelets in the spleen: Role in the pathogenesis of "Hypersplenic" thrombocytopenia. *J Clin Invest* 1966; 45:645-657.
11. Marongiu F, Mamusa AM, Mameli G, Mulas G, Cambuli AB et al. Thrombocytopenia and liver cirrhosis evidence for relationship between platelet count, spleen size and hepatic synthetic activity. *Thromb Res* 1987; 45:275-278.
12. Wadenvik H, Denfors I, and Kutti J. Splenic blood flow and intrasplenic platelet kinetics in relation to spleen volume. *Br J Haematol* 1987; 67:181-185.