Original Article

Platelet Transfusion Outcome and Flow Cytometric Monocyte Phagocytic Assay (FMPA)

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Abstract

Background: This study was designed to evaluate platelet transfusion outcome via flow cytometric monocyte phagocytic assay (FMPA).

Method: Fifteen patients with a history of multiple platelet transfusions and fifteen controls were enrolled in this study. CMFDA-labeled platelets were incubated with patients' sera and were finally incubated with monocytes in a tube and analyzed by flow cytometry. Monocytes that phagocytosed platelets were detected as a CMFDA-positive platelet population via monocyte gate. The FMPA results were compared with CCI results for the patients.

Result: The FMPA result correlated with 1-hour (r = -0.885, *P* = 0.001) and 24-hour (r = -0.884, *P* = 0.001) CCI. There is a significant difference in means of FMPA results between the patients with immune platelet refractoriness (68.46 ± 10.4%), non-refractory group (37.73 ± 15.21%) and the control group (18.27 ± 2.86%).

Conclusion: Our data showed that FMPA has good results in evaluation of platelet transfusion outcome and may be useful as an indicator of platelet transfusion response.

Keywords: CMFDA, flow cytometry, FMPA, platelet transfusion,

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Introduction

Patients with platelet refractoriness may develop alloantibodies against platelet antigens, e.g. Human Platelet Antigens (HPAs) and Human Leukocyte Antigens (HLAs).[1,2] Platelet refractoriness occurs in 30%–70% of transfused patients.[3,4] Corrected count increment (CCI) is used as an indicator of platelet transfusion response. A CCI more than 7.5×10^9/L during 1 hr after transfusion and more than 4.5×10^9/L[3] after 20–24 hr after transfusion shows a good response to platelet transfusion. Platelet count less than 7.5×10^9/L or 4.5×10^9/L reflects platelet refractoriness.[5–7]

Antibodies (auto-or allo-antibodies) destroy antibody-coated platelets by phagocytosis. Immune and non-immune causes are involved in platelet refractoriness.[2,8,9] HLA-matched platelet transfusion and platelet cross matching are suggested to decrease the immune refractory state, but they are not always useful.[10,11] Therefore, developing a screening test may be useful in order to predict the platelet transfusion outcome. In this study, we used flow cytometric monocyte phagocytic assay (FMPA) using CMFDA (5-choloro methyl flourescein) to evaluate platelet transfusion outcomes in patients with AML (acute myeloid leukemia), according to instructions explained by Lim *et al.*[10]

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Patients and Methods

Fifteen patients (4 females and 11 males) with AML (age range 37 to 68 year, mean = 38.6 y) and 15 apparently healthy subjects (7 females and 8 males in the same age range) and without AML were enrolled in this study. The patients received multiple, random and non-HLA/non-HPA typed platelet products (ranged 2–8 units). We did not have access to the previous CCI results but in this study, CCI was calculated for the patients after the last transfusion (CCI results are shown in Table 1). Several days after the last platelet transfusion in the hospital, blood samples were taken and sera were separated and kept at -70°C until testing.

FMPA was performed and 1- and 24-hour CCIs (corrected increment count) were calculated according to the formula[12]:

$$\text{CCI} = \frac{[\text{Pt count(post)} \times 10\ (11) - \text{pt count (pre)} \times 10(11)] \times \text{body surface area (m2)}}{\text{Transfused Pt count} \times 10\ (11)}$$

In brief, CMFDA-labeled platelets were prepared, then treated with patient's serum and mixed with monocytes to perform phagocytosis.

Platelet rich plasma (PRP) preparation

Platelet rich plasma (PRP) was obtained from whole blood, with EDTA anticoagulant from six random donors with blood group O. Blood groups were determined using Anti-A and Anti-B reagents

(LORNE). Six whole blood specimens were mixed and centrifuged (200 g/15 min), then the upper layer was separated as PRP. Pooled PRP was washed three times with 0.3% EDTA-PBS buffer and resuspended at a final concentration of 5×10^8 platelet per mL without aggregation.[13]

CMFDA-labelled platelets preparation

After preparing PRP and 3 times washing steps, platelets were incubated with 5 µm CMFDA in the dark for 45 minutes at room temperature (RT). Then, the cells were centrifuged at 2000 g for 10 min and washed twice and suspended in 0.3% EDTA-PBS to a final concentration of 5×10^8 cells/mL.

To sensitize the cells, CMFDA-labeled platelets were mixed with patient's serum (volume adjusted to a ratio 1:9), and were incubated at 37°C for 30 minutes. Then the cells were centrifuged at 2000 g for 10 min and washed twice and suspended in 0.3% EDTA-PBS.

Monocyte-enriched mononuclear cells (MNCs) preparation

Monocytes were prepared from heparinized whole blood from a healthy adult (group O). In brief, the platelets were separated from plasma by centrifugation (2000 g/10 min) of the suspension, then supernatant was transferred to another tube and centrifuged (1000 – 1500 g /10 min). Plasma without the platelets was added to the pellet in the first tube, then one volume of the suspension was added to one volume of 6% dextran and allowed to stand for 60–70 min. The supernatant was transferred to another tube and centrifuged (1000×g/10 min). The supernatant was transferred to a new tube and one volume of Nycoprep was added, then centrifuged (400–500×g/15 min). The middle layer consists of monocytes. The cells were separated in another tube, then washed and resuspended in PBS in final concentration of 1×10^7 cell/mL.

The prepared monocytes were incubated with mouse monoclonal PE-labeled anti-human CD14 to detect monocytes by flow cytometry (as a control tube) in each run.

Phagocytosis

One volume of sensitized and CMFDA-labeled platelets (part 2) was mixed with 5 volumes of monocyte-enriched mononuclear cells (MNCs). PGE2 was added to final concentration of 40 ng/mL to prevent platelet aggregation and non-specific adherence of platelets to monocytes. The sensitized platelets and monocytes were incubated in a 5% CO_2 incubator at 37°C for 2 hours then centrifuged (1000–1500 g/10 min) and washed with 0.3% EDTA-PBS.

FMPA

The mixture was incubated with PE-labeled anti-human CD14 in the dark for 15 minutes at RT, then washed with 0.3% EDTA-PBS and analyzed by flow cytometry. CFMDA spectral properties are similar to FITC and detectable in channel 1 and monocytes were detectable in channel 2. Monocytes that had phagocytosed the sensitized and labeled platelets were detectable in CMFDA positive platelet population by CD14+ monocyte gating.

As a control tube, 100µL of the labeled platelet suspension was added to a tube (with no sera), incubated at 4°C for 30 minutes and then washed with PBS and analyzed by flow cytometry. A serum with positive result for Panel Reactive antibody (PRA) test was used as positive control and FMPA was also performed without platelet sensitization to evaluate the random adhesion of CMFDA-labeled platelets to monocytes. Post transfusion 1- and 24-hour CCIs were calculated to determine platelet transfusion evaluation.

The results of FMPA test in patients and controls were compared by Mann-Whitney test. Correlation between FMPA and CCI results was analyzed by Pearson coefficient test (significant level, $P < 0.5$). All tests were performed by SPSS 16 software.

Results

No patient had fever, splenomegaly, or DIC, and no antibiotic was used. FMPA was significantly higher ($P < 0.001$) in patients (47.31 ± 19.98) than controls (18.27 ± 2.86) (Table 1). Figure 1 shows the percentage of monocytes that phagocytosed CMFDA-labeled platelets in two samples (as FMPA percent). FMPA result

Table 1. Results of FMPA, 1- and 24- hour CCIs; (Mean ± SD).

State	Patients code	1-hour CCI	24-hour CCI	FMPA %	FMPA Control group N = 15
Group1: patients with platelet refractoriness	1	6500	5800	68.25	14.58
	2	4200	3800	86.47	21.62
	3	7200	6300	60.98	24.61
	4	6900	5800	63.14	15.29
	5	6700	6000	63.51	21.32
Mean ± SD		**6300 ± 1202**	**5540 ± 993**	**68.46 ± 10.4**	
Group 2: patients without refractoriness	6	10500	7800	40.61	18.98
	7	16300	12000	26.51	20.09
	8	7600	6800	56.37	19.29
	9	8000	7700	50.08	17.98
	10	11800	8600	14.35	18.71
	11	13000	10200	19.74	15.27
	12	8500	7100	52.21	14.76
	13	7500	6700	41.81	16.29
	14	12400	9300	28.88	18.37
	15	9700	8500	36.81	17.01
Mean ± SD		**10530 ± 2875**	**8470 ± 2857**	**37.73 ± 15.21**	18.27±2.86

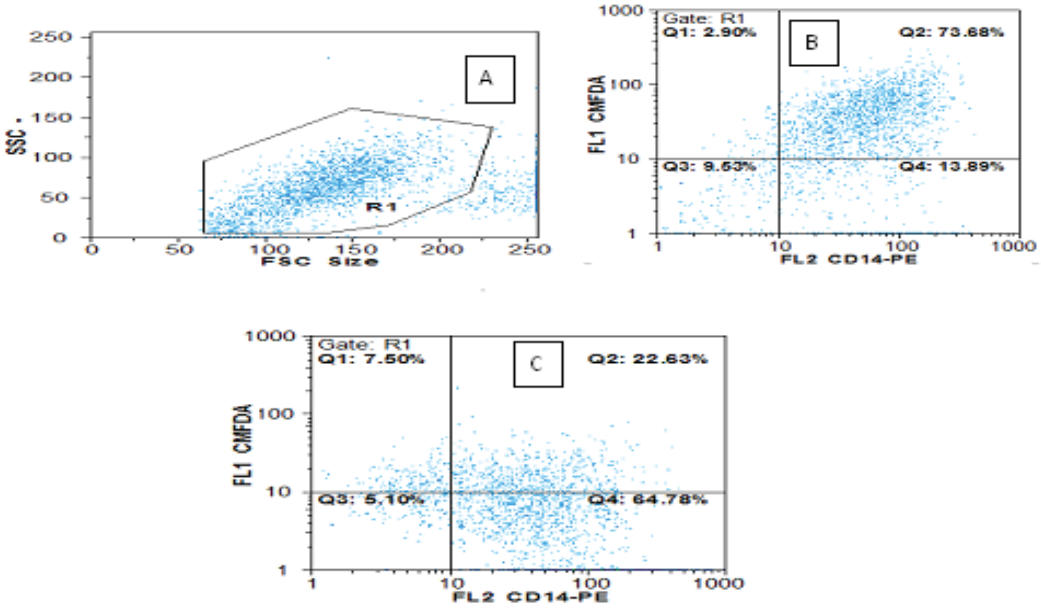

Figure 1. Dot Scaterogram FMPA: **A)** CD14 monocyte population, **B)** CMFDA positive population (monocytes that phagocytized CMFDA-labeled platelets) with PRA (panel reactive antibody) positive serum (containing HLA-Abs) as positive control; **C)** CMFDA positive population with low MFDA (22.83%).

using CMFDA-labeled platelet without sensitization was 9.7 ± 4.1%.

The patients were subdivided into two groups according to CCI results; the first 5 patients had 1 hour CCI results less than 7500 that reflected immune refractory state ($P < 0.001$). The results of FMPA and CCI during 1 and 24 hours after transfusion in patients (Mean ± SD) are shown in Table 1.

There is a significant difference between patients with platelet refractoriness and patients without refractoriness ($P = 0.005$). The FMPA results strongly correlated with 1-hour (r = - 0.885, $P = 0.001$) and 24-hour (r = -0.884, $P = 0.001$) CCIs.

Discussion

Our study showed FMPA result had a negative correlation with 1-hour and 24-hour CCI, which means that in our patients, lower CCI was correlated with higher FMPA and vice versa.

Lim *et al.* previously suggested that FMPA was more predictable than crossmatching to predict platelet refractoriness because four out of 12 patients in their study showed high FMPA with low CCIs in whom crossmatching was not positive.[5] They reported that 1- and 24-hour CCIs were correlated to FMPA results. [(10)]

Platelet refractoriness (PR) is defined as failure to respond to two consecutive platelet transfusions.[14] CCI is used as an index of platelet transfusion response. PR rate is reported to be 30% – 70% in patients with malignant hematopoietic disorders. Platelet refractoriness may have non-immunologic (related to products factor or patients factor like: fever, splenomegaly, sepsis) and immunologic causes.[15] Immunologic PR in mediated by alloantibodies against platelet antigens; anti-HLA (human leukocyte antigens) and HPA (human platelet antigens) antibodies in the patients' serum.[3,16] It is reported that significant reduction in CCI is not always caused by platelet-specific antibodies.[11,17]

Transfusion of HLA-identical or cross matched platelets or providing platelets that are negative for related antigens are the main strategies for PR management.[8] HLA-matching is a time-consuming and expensive solution and nearly 40% HLA-matched platelet transfusions are unsuccessful.[11–18]

Lim *et al.*[10] previously suggested FMPA as a reliable test to predict platelet outcome because CMFDA in not radioisotope, the technique is flow cytometry that is quantitative, simple and very close to *in vitro* conditions and measures the immune response to transfused platelets. As our results are similar to their report, we can conclude that although the technique is not very fast, it can direct us to perform platelet crossmatching just for positive result of FMPA and not for all patients.

Conflict of interest

There is no conflict of interest. The manuscript has been seen and approved by all authors and it is neither being published nor being considered for publication elsewhere.

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