

Original Article

Platelet antibody detection by flow cytometry: an effective method to evaluate and give transfusional support in platelet refractoriness

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Background: Immune platelet refractoriness is mainly caused by human leukocyte antigen antibodies (80-90% of cases) and, to a lesser extent, by human platelet antigen antibodies. Refractoriness can be diagnosed by laboratory tests and patients should receive compatible platelet transfusions. A fast, effective and low cost antibody-screening method which detects platelet human leukocyte/platelet antigen antibodies is essential in the management of immune platelet refractoriness.

Objective: The aim of this study was to evaluate the efficiency of the flow cytometry platelet immunofluorescence test to screen for immune platelet refractoriness.

Methods: A group of prospective hematologic patients with clinically suspected platelet refractoriness treated in a referral center in Campinas, SP during July 2006 and July 2011 was enrolled in this study. Platelet antibodies were screened using the flow cytometry platelet immunofluorescence test. Anti-human leukocyte antigen antibodies were detected by commercially available methods. The sensitivity, specificity and predictive values of the immunofluorescence test were determined taking into account that the majority of antiplatelet antibodies presented human leukocyte antigen specificity.

Results: Seventy-six samples from 32 female and 38 male patients with a median age of 43.5 years (range: 5-84 years) were analyzed. The sensitivity of the test was 86.11% and specificity 75.00% with a positive predictive value of 75.61% and a negative predictive value of 85.71%. The accuracy of the method was 80.26%.

Conclusion: This study shows that the flow cytometry platelet immunofluorescence test has a high correlation with the anti-human leukocyte antigen antibodies. Despite a few limitations, the method seems to be efficient, fast and feasible as the initial screening for platelet antibody detection and a useful tool to crossmatch platelets for the transfusional support of patients with immune platelet refractoriness.

Keywords: Blood platelets; Antigens, human leukocyte; Flow cytometry; Histocompatibility; Antigens, human platelet

Introduction

Refractoriness to platelet transfusions - platelet refractoriness (PR) - is defined as inappropriately low platelet count increments following two or more, preferably consecutive, transfusions⁽¹⁾. PR must be determined by objective data which determine platelet transfusion outcomes, such as the corrected count increment (CCI) and the predicted percentage recovery (PPR)⁽²⁾.

This condition may be caused by immune and non-immune factors. Non-immune causes, represent the main etiology (more than 80% of cases) of platelet refractoriness and include splenomegaly, fever/sepsis, antibiotics or disseminated intravascular coagulation⁽³⁾. Immune causes, occurring in less than 20% of the cases, involve alloimmunization against human leukocyte antigens (HLA) and, to a lesser extent, human platelet antigens (HPA) following exposure through transfusion, pregnancy or transplantation. Among immune causes, HLA antibodies are responsible for approximately 80-90% of PR cases and HPA antibodies for approximately 10-20% of cases, associated with HLA antibodies or not. It is important to note that the presence of antiplatelet antibodies does not mean PR, since in approximately 30% of cases, they occur in the absence of clinically detected PR⁽⁴⁾.

The immune causes of PR can be diagnosed by laboratory tests and patients should receive compatible platelet transfusions⁽⁵⁾. Testing for HPA antibodies is technically demanding and few laboratories offer the exam in their routine. The platelet antibody detection tests available include microcytotoxicity using Amos modification that detects both IgG and IgM antibodies, the platelet immunofluorescence test (PIFT) either by microscopy or flow cytometry (Capture-P[®]) and monoclonal antibody immobilization of platelet antigens (MAIPA)^(2,6-8). The latter is the gold standard technique that permits the identification and quantification of platelet specific antibodies however this technique is very laborious and time-consuming. Thus, a fast, effective and low cost antibody-screening method, which could detect both HLA and HPA platelet antibodies is essential for the recognition and for clinical support in immune PR.

The main aim of the study was to evaluate the efficiency of the flow cytometry platelet immunofluorescence test (FC-PIFT) as a screening test to identify immune PR.

Conflict-of-interest disclosure:
The authors declare no competing financial interest

Submitted: 2/17/2013
Accepted: 4/5/2013

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DOI: 10.5581/1516-8484.20130062

Methods

A group of hematologic patients with clinically suspected PR treated at the hospital complex of the Universidade Estadual de Campinas during the period July 2006 to July 2011 was prospectively enrolled in this study. Serum samples were collected before transfusion for direct platelet antibody screening and samples were then stored at -20°C until processing.

Platelet antibodies were screened by the FC-PIFT. Sera from 24 regular male blood donors with no history of previous transfusions were analyzed and a negative fluorescence standard curve was defined. Pooled platelets from two O blood group male donors with no history of previous sensitization were buffer washed and re-suspended in 0.1% phosphate buffered saline (PBS)/ ethylenediaminetetraacetic acid (EDTA) (final concentration 100,000 platelets/mL) and then incubated with patient serum (5 mL) for 30 minutes at 37°C. Negative and positive controls were added to each test batch. After three consecutive washes, cells were incubated for 50 minutes with fluorescein isothiocyanate (FITC) goat anti-human immunoglobulin G [AffiniPure F(ab') Fragment Goat Anti-Human 2 IgG, Fc Fragment Specific - Jackson Immuno Research – Baltimore, USA] at 1:50 dilution. Samples were read after a second buffer wash in a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the CellQuestH software (Becton Dickinson). For data acquisition and analysis, 10,000 events were analyzed. The test was considered positive if the median fluorescence (MF) obtained was greater than or equal to two standard deviations (SD) above the negative MF control and inconclusive if MF was between one and two SD above the negative MF control.

Anti-human leukocyte antigen antibody analysis

The detection of anti-HLA antibodies (PRA) was determined by commercially available methods: enzyme-linked immunosorbent assay (ELISA - LAT® One Lambda Inc, Canoga Park, CA, USA) and Luminex technology (LabScreen® and LabSingle Antigen® One Lambda Inc, Canoga Park, CA, USA) according to the manufacturer's instructions.

Flow cytometry platelet immunofluorescence test versus anti-human leukocyte antigen antibody analysis

The results of FC-PIFT and PRA obtained from the samples collected were compared. The sensitivity and specificity as well as predictive values of FC-PIFT were determined taking into account that the majority of antiplatelet antibodies present HLA specificity.

Statistical analysis

The R software version 2.13.1 (2011-07-08) was used for statistical analysis. The Fisher exact test was applied for count data with the level of significance set at 0.5% (p -value ≤ 0.05). Since the great majority of antibodies involved in PR present HLA specificity⁽³⁾, PRA was used as a reference test to calculate sensitivity, specificity, and positive and negative predictive values of FC-PIFT^(9,10).

Results

Population characteristics

This prospective study analyzed 76 blood samples from 32 female and 38 male patients with a median age 43.5 years (range: 5-84 years). The group characteristics are described in Table 1. Of the patients studied, 55.7% (n = 39) presented myeloproliferative oncologic disorders and 27.1% (n = 19) lymphoproliferative disorders. Non-oncologic disorders were detected in 17.1% (n = 12) (Bernard Soulier Syndrome, Glanzmann's thrombasthenia and aplastic anemia).

Table 1 - Clinical data of patients enrolled in the study

Disorder	Patients		Non-oncologic (N = 12 - 17%)
	Oncologic (N = 58 - 83%)		
	Myeloproliferative	Lymphoproliferative	
Gender - n (%)			7 (58)
Male	20 (34)	11 (19)	5 (42)
Female	19 (33)	8 (14)	
Age (years)			28.5
Median	49.7	40.5	7-79
Range	15-84	5-60	0.013

Correlation Flow cytometry platelet immunofluorescence test versus anti-human leukocyte antigen antibody analysis

There were correlations between positive FC-PIFT and positive PRA in 38.15% (n = 29) and negative FC-PIFT and negative PRA in 39.47% (n = 30) of the samples. On the other hand, the FC-PIFT was positive when the PRA was negative in 13.16% (n = 10) and the FC-PIFT was negative when the PRA was positive in 6.58% (n = 5) of the cases. Finally the FC-PIFT was inconclusive when the PRA was positive in 2.6% of the total samples (n = 2). Data are shown in Table 2.

Table 2 - Distribution of the results of the flow cytometry platelet immunofluorescence test (FC-PIFT) versus anti-human leukocyte antigen antibody (PRA)

	FC-PIFT		PRA	
	n	%	n	%
Positive	39	51	36	47
Negative	35	46	40	53
Inconclusive	2	3		

Flow cytometry platelet immunofluorescence test - sensitivity and specificity

The sensitivity FC-PIFT was 86.11% and the specificity was 75.00% with a positive predictive value of 75.61% and a negative predictive value of 85.71%. The accuracy of the method was 80.26%. Data are shown in Table 3.

Table 3 - Comparison of the results of the flow cytometry platelet immunofluorescence test (FC-PIFT) versus anti-human leukocyte antigen antibody (PRA)

	Positive PRA		Negative PRA	
	n	%	n	%
Positive FC-PIFT *	29	38.2	10	13.2
Negative FC-PIFT*	5	6.6	30	39.4
Inconclusive FC-PIFT	2	2.6	0	0

*p-value \leq 0.001; Odds Ratio = 16.53; 95% confidence interval = 4.72-70.34

Discussion

The present study demonstrates that FC-PIFT is an interesting tool to identify patients with immune PR. The test is fast, relatively simple and allows the selection of compatible platelet donors by crossmatching to support thrombocytopenic patients or those with platelet dysfunction bleeding.

The identification of antibodies against antigens present on the platelet surface strongly suggests immune PR, nevertheless the majority of PR cases have non-immune causes. When immune factors are present, the identification of the antibodies linked to the platelets, as well as the availability of compatible platelet components, may significantly enhance the response to platelet transfusion and improve patient outcome.

There is a significant association between platelet transfusion failure and patient survival; this increases the clinical impact of platelet refractoriness⁽¹¹⁾. Providing an adequate post-transfusion platelet count increment in refractory patients is not an easy task and so transfusion of compatible platelets is crucial, particularly in immune PR⁽¹²⁾. HLA is the most frequent cause of immune PR and finding multiple HLA-compatible related donors for one individual is very difficult.

Successful transfusion of patients with platelet-refractory thrombocytopenia is extremely important^(13,14). However, several potential donors are needed to sustain HLA platelet matched transfusion programs considering not only HLA diversity but also the transfusional demand of these patients. Pool size calculations may provide essential data for a rational planning of platelet transfusion support programs and help guide different institutions that aim to build a platelet donor registry. Feasibility and costs should be taken into account when considering the donor pool size required⁽¹⁵⁾.

The use of HLA platelet matching is not the only approach to manage alloimmune platelet transfusion refractoriness. Crossmatching and support with antigen negative platelet units allow rapid selection of donors, mainly for those patients with uncommon HLA types to whom it might be virtually impossible to find HLA-compatible donors⁽¹⁶⁻¹⁸⁾. Recently, the use of the HLA-Matchmaker algorithm has been reported as an emerging concept for the management of refractory patients⁽¹⁹⁾. The combination of matching compatible antigens and the application of mismatch acceptability determined by serum screening for HLA antibodies has offered an effective approach for HLA-based platelet transfusion support of refractory patients.

This study showed that the FC-PIFT has a high correlation with PRA, and also demonstrated good sensitivity, accuracy and a high positive predictive value. A larger number of patients

analyzed could improve the statistics of the study, reducing the confidence interval found (Table 3). However, due to the low frequency of hematological patients with suspected PR, even with cooperative studies, the ideal number of patients enrolled probably could not be achieved. FC-PIFT does not discriminate the specificity of antiplatelet antibodies (anti-HLA antibodies or anti-HPA antibodies) and some limitations of the method should be discussed. The frequency of positive FC-PIFT with negative PRA (13%) suggests the presence of HPA antibodies (in agreement with the literature 10-20%). Moreover, the great polymorphic diversity of the HLA system imposes another limitation for FC-PIFT; the use of pooled platelets from two random donors may not ensure the ideal range of antigens. However, the use of more platelet donors could cause a 'dilution' of the HLA antigens.

Conclusions

Even with limitations, the FC-PIFT seems to be efficient, fast and feasible as an initial screening to detect platelet antibodies and a useful tool to crossmatch platelets for the transfusional support of patients with refractoriness. The use of additional techniques that identify anti-HPA antibodies, such as MAIPA, is essential for the appropriate clinical management of these cases.

Acknowledgements

The authors wish to thank Roberto Zulli for his assistance with the statistical analysis and Raquel Susana Foglio for corrections in the English version of the manuscript.

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