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Detection of platelet-associated immunoglobulins by flow cytometry for the diagnosis of immune thrombocytopenia: a prospective study and critical review

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ABSTRACT

Background and Objectives. **Flow cytometry (FC) to identify platelet-associated (PA) immunoglobulin (Ig) is a potentially useful diagnostic test for idiopathic thrombocytopenic purpura (ITP). However, the restricted application of PAIg measurement to thrombocytopenic populations primarily comprised of ITP patients will artificially enhance the test's diagnostic specificity. For this reason, we performed a prospective study in which the results of a sensitive technique for detecting PAIg, as is FC, were correlated to the cause of the thrombocytopenia.**

Design and Methods**. A total of 118 patients with platelet counts <100×10⁹/L and 30 normal donors with a platelet count >200×10⁹/L were studied for PAIg employing a flow cytometer. Forty-two children and 20 adults were diagnosed as having immune thrombocytopenia and 27 children and 29 adults had nonimmune thrombocytopenia of different etiology.**

Results. **Raised levels of PAIg were found in 56/62 patients with immune thrombocytopenia and in 34/56 patients with non-immune thrombocytopenia. Diagnostic values of PAIg for the detection of immune thrombocytopenia were: sensitivity 90.3% and specificity 39.3%. An enzyme-linked immunoabsorbant assay (ELISA) for the detection of autoantibodies to platelet glycoprotein (GP) complexes was used in adults, 9 with immune-related thrombocytopenia and 16 with non-immune thrombocytopenia, in order to determine the true non-specific nature of the positive PAIg test. By ELISA, 8/9 patients with immune thrombocytopenia and 7/16 with non-immune thrombocytopenic disorders showed autoantibodies to platelet GP complexes. Conclusions: PAIg detection by FC constitutes a sensitive but non-specific assay thus making it unnecessary and inappropriate for establishing the diagnosis of ITP.**
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Key words: immune thrombocytopenia; flow-cytometry; ELISA; platelet-associated immunoglobulins

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Immune-mediated thrombocytopenia, whether idiopathic, drug induced or resulting from some underlying disease, is a clinical condition characterized by increased platelet destruction due to sensitization of platelets by autoantibodies. It is now well recognized that these autoantibodies are mainly IgG, often associated with IgM, directed against glycoprotein (GP) complexes IIb/IIIa, GPIb/IX, and GPIa/IIa.[1]

The demonstration in 1975 by Dixon *et al.*[2] of abnormally high concentrations of platelet-associated (PA) IgG in idiopathic thrombocytopenic purpura (ITP) led to the assumption that PAIgG was responsible for platelet damage and since then a large number of methods for detection of PAIg have been described.[3-8] In the mid 1980s, flow cytometry (FC) emerged as a method to identify PAIg in normal and ITP platelets.[9-13] Several studies have shown that FC methods are diagnostically useful in the setting of immune-mediated thrombocytopenia.[9,11,14,15] However, as often happens with a new laboratory probe, the patient population chosen to examine the sensitivity and specificity was primarily restricted to the condition that provided the rationale for the development of the test. This process can potentially lead to artificially favorable results and when the test is applied to a wider spectrum of clinically and biologically related conditions, it may be shown to be less specific.[16] Taking this into account and in order to establish the sensitivity and specificity of PAIg detection by FC, we performed a prospective study which included patients with immune thrombocytopenias and other thrombocytopenic disorders.

Design and Methods

Study design

For a 1-year period, clinicians from the Instituto Nacional de Pediatría and Instituto Nacional de la Nutrición Salvador Zubirán were invited to submit patient's blood samples for analysis. The participating physicians were requested to include as wide a spectrum of thrombocytopenic disorders as possible. Two samples were requested; one for the whole blood platelet count (Coulter Counter, model STKS, Hialeah, FL, USA) and the other for measuring PAIg. After the samples had been analyzed, the physicians caring for the patients were contacted and solicited to indicate their patient's diagnosis. The result of the

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PAIg assay was not used in designating the cause of the thrombocytopenia.

Methods

Samples of 5mL and 10 mL of peripheral blood from 30 presumably healthy blood donors and patients, were collected into vacutainer tubes containing 1.5 mg/mL of ethylenediaminetetraacetic acid (EDTA). All specimens were processed within 5 hours of collection. The blood samples were centrifuged at 20°C and 200 × g for 10 min and the platelet-rich plasma obtained was removed and centrifuged at 20°C and 800 × g for 10 min to prepare the platelet pellet. The platelet pellet was washed three times with phosphate buffer saline (PBS) containing EDTA (0.009 mol/L Na_2 EDTA; 0.01 mol/ L Na_2 HPO_4; 0.0018 mol/L KH_2PO_4; 0.17 mol/L NaCl and 0.0033 mol/L KCl) and then fixed by incubation for 10 min at room temperature with 2% paraformaldehyde in PBS--EDTA. The fixed platelets were washed twice with PBS-EDTA and adjusted to a concentration of 5×10^9/L. Aliquots (200 µL) of the platelet suspension were added to 12×75 mm polystyrene tubes previously coated with 30 µL of 5% bovine albumin. Fluorescein isothiocyanate (FITC) conjugated $F(ab)_2$ fragments of affinity–isolated rabbit antihuman IgG and IgM (Dako, Glostrup, Denmark) were used to detect PAIg. CD41 monoclonal antibody (Dako, Glostrup, Denmark) was employed to identify the platelet population and the non-specific fluorescence was established by FITC conjugated goat anti-mouse monoclonal antibody. Platelets were analyzed for surface immunoglobulins on an Cytoron Absolute flow cytometer (Ortho Diagnostic Systems. Raritan, NY, USA). Platelet analysis was performed on log forward scatter light versus log right angle scatter light on approximately 20,000 platelets.

A commercial solid phase enzyme-linked immunosorbant assay (ELISA; PakAuto, GTI, Brookfield, WI, USA) for the detection of autoantibodies to glycoprotein complexes IIb/IIIa, Ib/IX and Ia/IIa in plasma and platelet eluate was tested in 25 adult patients with thrombocytopenia from the Instituto Nacional de la Nutrición Salvador Zubirán. Briefly, plasma or platelet eluate samples were added to microwell strips coated with platelet glycoproteins and unbound immmunoglobulins were removed by washing. An alkaline phosphatase labeled anti-human globulin reagent was added to the wells and incubated. The unbound antiglobulin was washed away and enzyme substrate was added. The reaction was stopped by adding sodium hydroxide solution and the optical density of produced color measured at 405 nm or 490 nm. Positive and negative control plasmas and platelet eluates were assayed in each run.

The sensitivity and specificity, as well as, the positive and negative predictive values for FC PAIg test were calculated as described elsewhere.[17]

Results

The reference values, obtained from normal blood donors with platelet counts >200×10^9/L, for PAIgG ranged between 1.5% and 18.3% (mean = 8.5%; SD = 4.1) and for PAIgM between 0.3% and 14.6% (mean = 6.5; SD = 3.6). The cut-off positive value (mean±2SD) for IgG was 16.8% and 13.8% for IgM.

A total of 118 patients with platelet counts <100×10^9/L were studied. Sixty-nine were children from the Instituto Nacional de Pediatría, aged 2 months to 16 years (median 5.9 years). The adults were from the Instituto Nacional de la Nutrición Salvador Zubirán ranging in age from 17 to 64 years (median 45 years). Forty-two children and 20 adults were classified as having a classical immunologic disorder and hence immune thrombocytopenia and thrombocytopenia of different etiology was identified in 27 children and 29 adults.

The results of the PAIg measurements are shown in Table 1. Ninety out of 118 (76.3%) thrombocytopenic patients showed increased PAIg values. PAIgM was elevated in 7 patients, PAIgG in 18, and both PAIgM/PAIgG in 65 patients. Fifty-one out of 57 patients (89.5%) with ITP and all patients suffering from either systemic lupus erythematosus or primary antiphospholipid syndrome showed increased PAIg values. Sixty-one percent (34/56) of the patients with other thrombocytopenic disorders had elevated levels of PAIg: 50% with malignant disorders, 78% with hematologic diseases, 62.5% with hepatic disorders, 83% with infective diseases, and 55.5% with miscellaneous disorders. The results of the diagnostic values of PAIg for the detection of immune thrombocytopenia were: sensitivity 90.3% and specificity

Table 1. Flow cytometry detection of platelet-associated (PA) IgG and/or IgM in patients with immune thrombocytopenia and other thrombocytopenic disorders.

		# of patients with elevated		
	Total of pts tested	PAIgM	PAIgG	PAIgM/ PAIgG
Classical Immunologic				
Idiopathic thrombocytopenic purpura	57	4	12	35
Systemic lupus erythematosus	4	0	1	3
Primary antiphospholipid syndrome	1	0	0	1
Malignant				
Lymphoproliferative	6	0	1	2
Carcinoma	4	0	0	3
Acute leukemia	14	1	0	5
Hematologic				
Aplastic anemia	4	1	0	3
Myelodysplastic syndrome	3	0	1	0
Megaloblastic anemia	2	0	0	2
Hepatic				
Cirrhosis	8	0	1	4
Infective				
Hepatitis C virus	4	0	0	4
Tuberculosis	2	0	1	0
Miscellaneous				
Hyper and hypothyroidism	5	0	1	2
Diabetes mellitus	3	1	0	1
Chronic renal failure	1	0	0	0

Table 2. Autoantibodies to platelet (Plts) and plasma glycoprotein (GP) complexes IIb/IIIa, Ib/IX, and Ia/IIa in patients with immune thrombocytopenia and other thrombocytopenic disorders.

		# of patients with GP antibodies in:		
	Total of pts tested	Plts	Plasma	Plts/ plasma
Classical Immunologic				
Idiopathic thrombocytopenic purpura	8	7	1	0
Primary antiphospholipid syndrome	1	0	0	0
Malignant				
Leukemias	4	0	0	0
Hematologic				
Aplastic anemia	2	0	0	0
Myelodysplastic syndrome	2	0	1	0
Cyclic thrombocytopenia	1	0	0	0
Hepatic				
Cirrhosis	2	0	1	0
Infective				
Hepatitis C virus	5	2	1	2

39.3%. The positive predictive value for a positive PAIg test for immune thrombocytopenia in a thrombocytopenic patient was 62.2%. In contrast, the negative predictive value was 78.6% indicating that a negative PAIg test in a thrombocytopenic patient makes immune thrombocytopenia diagnosis unlikely.

In an attempt to determine the true non-specific nature of the positive PAIg result in non-immune thrombocytopenic cases, platelet and plasma samples from 25 adult patients with platelet counts $<100\times10^9$/L, 9 with classical immune-mediated thrombocytopenia and 16 with purportedly non-immune disorders, were assayed with a commercial ELISA in search of autoantibodies directed to plasma and platelet GP complexes (Table 2). Eight out of 9 patients with immune-mediated thrombocytopenia had GP antibodies, 7 in platelet eluate and 1 in plasma. In contrast, GP antibodies were detected in 7/16 patients with non-immune thrombocytopenic disorders and, interestingly, 5 of them had hepatitis C virus infection.

Discussion

Herein we describe our experience in flow cytometric detection of PAIg in patients with platelet counts $<100\times10^9$/L who were classified on clinical grounds as suffering from immune or non-immune thrombocytopenia. This prospective study confirms that the measurement of PAIgG and PAIgM is highly sensitive (90.3%) for the diagnosis of patients with classical immune-mediated thrombocytopenia. Indeed, as shown in Table 3, Courash and Rheinschmidt[9] in 171 patients with thrombocytopenia or a clinical suspicion of a qualitative platelet defect reported that flow-cytometric measurement of PAIgG had a 93.8% sensitivity in detecting patients with a clinical diagnosis of immune-mediated thrombocytopenia. Similarly, Rosenfeld *et al.*,[11] evaluating PAIgG, PAIgM, and PAIgA in 50 patients with immune thrombocytopenia and in 44 with non-immune thrombocytopenia, found a sensitivity of 92%. In contrast, Christopoulos *et al.*[14] in a smaller number of patients, 16 with immune thrombocytopenia and 9 with non-immune thrombocytopenia, reported that the measurement of PAIgG had a 75% sensitivity and Tazzari *et al*.[15] found an 85% sensitivity in 67 patients with thrombocytopenia, 54 of them with immune thrombocytopenia. The reasons for the apparent discrepancy in specificity between studies is uncertain (Table 3), but could in part reflect our inclusion of various thrombocytopenic disorders. The sensitivity and specificity of a test is proportional to the prevalence of disorder in the series examined. Ideally, our study should have evaluated every thrombocytopenic patient present in both hospitals. This would have included a significantly higher proportion of patients with hematologic malignancies and chemotherapy-induced thrombocytopenia. The inclusion of more of these patients would have led to a further lowering of specificity. However, in clinical practice, the diagnostic performance of the test is influenced by appropriate patient selection and hence the group studied in our series is an attempt to give such a perspective.

Our study shows that a positive PAIg test is a rela-

Table 3. Main technical features, sensitivity and specificity of some published flow-cytometric methods for platelet-associated Ig detection.

Author	Platelet fixation	Antibody used	Irrelevant control	Sensitivity %	Specificity %
Corash[9]	PFH 1% x 5 min	Biotin $F(ab)_2$ goat anti-human IgG, FITC avidin	None	93.8	94.5
Rosenfeld[11]	No	FITC goat anti-human IgG, IgA, IgM (whole molecule)	Sheep anti-mouse	92*	81.8*
Christopoulos[14]	No	FITC $F(ab)_2$ goat anti-human IgG	Non-immune goat IgG	75*	77.8*
Tazzari[15]	PFH 1% x 5 min	FITC goat anti-human IgG (whole molecule)	None	85*	100*
Present study	PFH 2% x 10 min	FITC $F(ab)_2$ rabbit anti-human IgG, IgM	Goat anti-mouse	90.3	39.3

*PFH = paraformaldehide. FITC = fluorescein isothiocyanate. *Calculated using raw data available.*

tively non-specific finding since there are increased amounts of PAIg in a wide spectrum of purportedly non-immune thrombocytopenic disorders. Indeed, 34/56 (60.7%) of the patients included in the non-immune thrombocytopenic group had a positive PAIg test. Since our data demonstrated that the measurement of PAIg has a low specificity (39.3%) for the diagnosis of immune-mediated thrombocytopenia, we thought it important to establish the true non-specific nature of the positive flow-cytometric PAIg test by searching for the presence of autoantibodies to platelet and plasma GP complexes with an ELISA in a selected group of 25 patients with thrombocytopenia of different etiology. The presence of GP autoantibodies was recorded in 8/9 patients with immune-mediated thrombocytopenia and in 7/16 patients (43.7%) with non-immune thrombocytopenic disorders, 5 of them with hepatitis C virus infection. When patients with hepatitis C virus infection were excluded from the analysis, the proportion of false-positive cases in ELISA decreased to 18% whereas in flow-cytometric PAIg assay it remained almost unchanged (58%).

Our study does not provide information concerning the biological or clinical implications of the finding of elevated platelet-bound immunoglobulin. It is possible that some of the non-immune thrombocytopenic disorders evaluated could be mediated by immune mechanisms. For example, there is evidence that hepatitis C virus infection may produce a significant autoimmune reaction to platelets leading to thrombocytopenia[18,19] as occurred in all our patients with this infection. However, the causes of increased PAIg in patients with thrombocytopenia due to an apparently non-immune etiology are unknown. Circulating immune complexes[20] may bind to platelet Fc receptors,[21,22] or may bind to circulating fragments of erythrocytes or leukocytes that are co-isolated with platelets. The platelet surface may be altered during systemic illnesses, by membrane fragmentation or by secretion[23] and these changes may expose new antigens[23,24] that could react with naturally occurring antibodies.[25] A diminished platelet surface sialic acid concentration may also cause an increased non-specific association of IgG with platelets.[26] There are currently no data to support any of these possibilities.

We used a flow-cytometric technique for identifying PAIg in paraformaldehyde-treated platelets as has been recommended.[27] Also, as other investigators (Table 3), we used $F(ab')_2$ fragment of rabbit antihuman IgG and IgM to reduce interference from Fc receptor binding. Our data showed that FC is sensitive enough to detect PAIg in immune thrombocytopenia. However, our study also indicates that a positive PAIg test is a relatively non-specific finding since there are increased amounts of PAIg in a wide spectrum of purportedly non-immune thrombocytopenic disorders.

In summary, the overall data are interpreted as indicating that PAIg test has an extremely low specificity thus making it unnecessary and inappropriate for establishing the diagnosis of ITP.[28]

Potential implications for clinical practice

Platelet associated Ig identification by flow cytometry is inappropiate to establish the diagnosis of ITP since it detects increased amounts of PAIg in a wide spectrum of non-immune thrombocytopenic disorders.

Contributions and Acknowledgments

LTR-G: design of the study, analysis of patient samples and collection of data. XL-K: conception and design of the study, critical revision and final approval of the manuscript. RP: conception of the study. OB-B: analysis of patient samples. JP: design of the study, analysis and interpretation of the data, and drafting the manuscript. Doctors Raul Puente and Martin Flores for selection of the patients.

Our criteria is that first and last authorship are given to those with the major participation. LTR-G appears as the first author because this work was her post-graduated thesis, therefore JP appears as the last author. Middle authoships were assigned according to their participation.

Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

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