

Attempt to improve the diagnosis of immune thrombocytopenia by combined use of two different platelet autoantibodies assays (PAIgG and MACE)

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Background and Objectives. Despite an extensive search for a definitive diagnostic assay for platelet autoantibodies, the laboratory diagnosis of immune thrombocytopenia (ITP) still remains a clinical challenge. Data in the literature have so far demonstrated that measurement of platelet-associated IgG (PAIgG) is sensitive, especially when flow cytometry is employed, but lacks adequate specificity. Measuring specific autoantibodies by antigen capture techniques increases specificity, but a large part of patients escape autoantibodies detection by such means too. The aim of the present study was to compare the diagnostic value of PAIgG with a modified antigen capture ELISA (MACE) in patients with primary and secondary immune thrombocytopenia and in patients with non-immune thrombocytopenia.

Design and Methods. One hundred and four patients with a platelet count lower than $100 \times 10^9/L$ were studied. Forty-two patients had primary ITP (P-ITP), 23 patients had ITP secondary to other immune diseases (S-ITP) and 39 patients had thrombocytopenia due to decreased platelet production (non-immune; NITP). PAIgG was measured by immunofluorescent flow cytometry, whereas specific platelet-associated autoantibodies (against GP IIb/IIIa, Ib/IX, Ia/Ia) were measured by a commercially available modified antigen capture assay (MACE, GTI, USA).

Results. The sensitivity of the PAIgG assay for ITP was 60%, the specificity was 77%, the positive predictive value was 81% and the negative predictive value was 54%. The sensitivity of MACE was 60%, specificity was 97%, the positive predictive value 97% and the negative predictive value 59%. We found a 73% concordance between PAIgG and MACE assays. Both PAIgG and MACE had significantly greater sensitivity in S-ITP than in P-ITP.

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Interpretation and Conclusions. Forty percent of patients with clinically diagnosed immune thrombocytopenia had no detectable platelet autoantibodies, possibly because of intrinsic methodological detection problems, different stages of disease, or absence of a true immune etiology. ©2002, Ferrata Storti Foundation

Key words: ITP, platelet, autoantibodies, PAIgG, antigen capture assay, MACE.

Various techniques have been developed to detect platelet autoantibodies in order to facilitate the diagnosis of immune thrombocytopenia (ITP). The measure of platelet-associated immunoglobulins (PAIgG) is considered a sensitive tool, especially when flow cytometry is employed for analysis.^{1,2} However, PAIgG suffers from lack of specificity, since elevated levels have been reported in patients with both immune and non-immune thrombocytopenia,^{2,3} being affected by plasma IgG, circulating immune complexes, age and activation of platelets.^{4,5} In fact PAIgG can detect both surface IgG and α -granule IgG following platelet lysis.⁴ Therefore, irrespective of the assay used, PAIgG does not necessarily measure true anti-platelet autoantibody.^{2,6}

Given such pitfalls, the *American Society of Hematology* (ASH) established in 1996 that the PAIgG assay is unnecessary and inappropriate in the diagnostic algorithm of ITP.⁷ Nevertheless, the PAIgG assay continues to be widely used.

The availability of monoclonal antibodies allows the detection in ITP of true platelet autoantibodies that bind to specific platelet surface glycoproteins and platelet antigen capture techniques have been developed.⁸⁻¹¹ Several studies have since shown that pathogenic autoantibodies are mainly IgG directed against epitopes located on platelet glycoproteins IIb/IIIa, Ib/IX and occasionally on Ia/IIa.¹⁰⁻¹⁷ Furthermore, the assay of specific platelet

autoantibodies by means of antigen capture techniques seems to be more useful than PAIgG in the diagnosis of immune thrombocytopenia.^{18,19}

The aim of the present study was to compare the diagnostic value of PAIgG and a modified antigen capture ELISA (MACE) in 42 patients with idiopathic or primary ITP (P-ITP), 23 patients with ITP secondary to other immune diseases (S-ITP) and 39 patients with thrombocytopenia due to decreased platelet production (not-immune; NITP).

Design and Methods

Patients

One hundred and four patients with a platelet count lower than $100 \times 10^9/L$ were assessed for entry into the study between May 1998 and July 2000. All patients were diagnosed and enrolled at our Department.

All patients underwent comprehensive history taking, physical examination and blood sampling for platelet antibody assays, independently of treatment. Results of antiplatelet antibodies testing were not used in clinical diagnosis.

The main clinical features of the patients are summarized in Table 1.

Sixty-five patients were classified as having immune thrombocytopenia (ITP), while 39 had thrombocytopenia secondary to decreased bone marrow production (non-immune; NITP). Groups were comparable for age and degree of thrombocytopenia, while a higher male prevalence was observed among those with NITP.

Forty-two patients were classified as having primary *idiopathic* immune thrombocytopenia (P-ITP), based on the occurrence of isolated thrombocytopenia, normal or increased number of bone marrow megakaryocytes, normal spleen size and exclusion of secondary causes of thrombocytopenia.

Twenty-three patients had ITP secondary to other immune diseases (S-ITP): 5 patients had systemic lupus erythematosus, 2 had other disorders of connective tissue, 5 had lymphoproliferative disorders, 1 had graft-versus-host disease, 3 Evans' syndrome, 3 antiphospholipid syndrome, 2 Crohn's disease, 1 hepatitis C virus infection and 1 Mycoplasma-related infection.

At the time of sampling, 22 patients (12 P-ITP and 10 S-ITP) were on steroids, i.v. Ig, danazol, and azathioprine, each alone or in combination; one patient had undergone a splenectomy 1 year before.

Thirty-nine patients had NITP: 5 had thrombocytopenia due to chemotherapy for lymphoma or acute leukemia; 6 had had a bone marrow transplant; 7 had lympho- or myeloproliferative disease

Table 1. Main features of the patients with immune (ITP) and non-immune (NITP) thrombocytopenia.

Patients	Immune thrombocytopenia		Non-Immune thrombocytopenia	
	Primary ITP	Secondary ITP	Tot.	
N°	42	23	65	39
Males/Females	11/31	8/15	19/46	26/13
Age (years)*	45±19	41±23	43±21	50±24
Platelets ($\times 10^9/L$)*	48±28	39±27	45±28	48±22

*Mean \pm 1 standard deviation.

(2 chronic myeloid leukemia, 1 myelofibrosis, 2 chronic lymphocytic leukemia, 2 non-Hodgkin's lymphoma); 2 had myelodysplastic syndrome; 3 had myelophthisis secondary to solid cancer; 3 had plasma cell dyscrasia (1 Waldenström's syndrome and 2 multiple myeloma); 1 had thrombotic thrombocytopenic purpura; 3 had liver disease with splenomegaly; 1 had megaloblastic anemia; 6 had pseudo-thrombocytopenia; 1 had familial macrothrombocytopenia and 1 had a platelet function defect of uncertain etiology.

Five patients (2 NITP patients and 3 S-ITP patients) received platelet concentrates for hemorrhagic and/or severe thrombocytopenia. In these patients, to avoid concomitant platelet reactive alloantibodies, we checked the corrected count increment after transfusion as previously described.²⁰ No patient was refractory to platelet transfusions.

Thirty-four healthy age- and sex-matched donors were studied as normal controls.

Flow cytometric analysis of platelet-associated IgG (PAIgG)

Platelet-associated IgG (PAIgG) was assayed by a modification of direct immunofluorescence analysis.²¹ Platelets were washed twice by differential centrifugation using phosphate-buffered saline pH 7.4 containing 2% EDTA, and 0.1% bovine serum albumin, (PBS/EDTA/BSA). One hundred microliters of washed platelets were centrifuged through a 20% sucrose cushion (5 min in a Beckman microfuge); the pellet was resuspended with 100 mL of FITC-conjugated goat anti-human IgG Fab₂ diluted 1:20 (Sigma, St. Louis, USA) and incubated in the dark for 15 min at 25°C. Free antibody was eliminated by centrifuging the sample through the sucrose cushion and the pellet was resuspended in

Table 2. Prevalence of platelet-associated IgG (PAIgG) and specific platelet-associated autoantibodies (MACE) in patients with immune (ITP) and non-immune (NITP) thrombocytopenia.

	PAIgG positive	MACE positive
Primary ITP	21/42 (50%)	20/42 (48%)
Secondary ITP	18/23 (78%)	19/23 (83%) ^o
All ITP	39/65 (60%)	39/65 (60%)
NITP	9/39 (23%)*	1/39 (3%)*

* $p < 0.03$ vs P-ITP and S-ITP; ^o $p = 0.013$ vs P-ITP.

PBS/EDTA/BSA and fixed with paraformaldehyde 1% f.c. Platelet-bound immunofluorescence was then directly measured using a flow cytometer analyzer (FACScan, Becton-Dickinson, USA). Logarithmic amplification was used for both light scatter and fluorescence signals. The fluorescence signal was collected from 10,000 events. Non-specific fluorescence was set using FITC-conjugated mouse IgG (Becton-Dickinson); the threshold was positioned such that 1% of fluorescence was obtained. Positivity was considered as the percentage of fluorescent cells greater than 2 standard deviations above the mean of normal controls.

Modified antigen capture ELISA (MACE)

A solid-phase modified antigen capture ELISA was used for the detection of specific autoantibodies against platelet glycoproteins IIb/IIIa, Ib/IX and Ia/IIa (MACE) (MACE Auto, GTI, Brookfield, WI, USA).

Platelets were washed as for PAIgG analysis and stored at -80°C in the preservative solution (GTI). Next, 3×10^7 platelets were lysed by 0.1% Triton-X 100 and then centrifuged in a Beckman microfuge for 10 minutes. Fifty microliters of the supernatant were added to the wells coated with monoclonal antibody to GP IIb/IIIa (AP2 clone), GP Ib/IX (142.1 clone) and GP Ia/IIa (143.1 clone). Alkaline phosphatase-conjugated anti-human IgG identified bound autoantibodies. Samples showing optical density (OD) values greater than 3 standard deviations above the mean of normal controls were regarded as positive.

Statistical analysis

Statistical analysis was performed with commercially available software for personal computers (STATISTICA for Windows 98).

Variables were expressed as mean values and SD. The differences between mean values were evaluated by the two-tailed Student's t-test. Differences in relative frequency of antiplatelet antibodies were compared by the χ^2 test. Statistical correlation was calculated with Pearson correlation coefficient. A p value < 0.05 was considered statistically significant.

The sensitivity of the PAIgG and MACE assays was calculated as the ratio between the positive ITP samples and the total number of ITP; the specificity was calculated as the ratio between the number of negative NITP samples and the total number of NITP samples. Positive predictive value was determined by the ratio between positive ITP samples and the total number of positive samples (ITP plus NITP) in the assay; negative predictive value was determined by dividing the number of negative NITP samples by the total number of negative samples (NITP plus ITP) in the assay.

Results

Results of the PAIgG and MACE assays are summarized in Table 2.

PAIgG levels were increased in 21/42 (50%) of patients with P-ITP, in 18/23 (78%) of patients with S-ITP and in 9/39 (23%) of patients with NITP.

Specific platelet autoantibodies were demonstrated by MACE in 20/42 (48%) P-ITP patients, in 19/23 (83%) S-ITP patients and 1/39 (3%) patients with NITP; the percentage of MACE positivity was higher in S-ITP than in P-ITP ($p < 0.03$). The majority (54%) of ITP patients had antibodies against multiple glycoproteins (Figure 1). No statistical difference in PAIgG and MACE positivity was observed when comparing treated and untreated patients. The sensitivity and the specificity of the PAIgG and MACE assays are summarized in Figure 2.

The sensitivity of the PAIgG assay for ITP was 60%, the specificity was 77%, the positive predictive value was 81% and the negative predictive value was 54%. The sensitivity of the MACE assay was 60%; the specificity was 97%, the positive predictive value 97.5% and the negative predictive value 59%.

There was a significant degree of concordance (73%) between results obtained with the PAIgG and MACE assays (χ^2 19.9, $p < 0.001$) (Table 3). On the other hand, in the 39 patients with demonstrable platelet anti-glycoprotein autoantibodies, the fluorescence intensities of PAIgG and optical density of antigen capture ELISA were significantly ($r = 0.42$, $p = 0.007$) correlated (Figure 3).

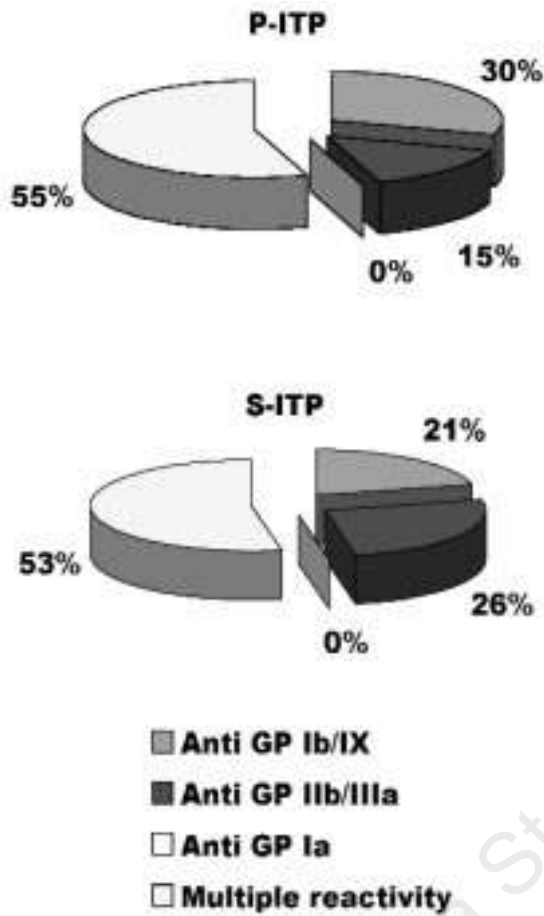


Figure 1. Target antigens of specific platelet autoantibodies obtained by antigen capture ELISA (MACE) in 20/42 patients with primary (P-ITP) and in 19/23 patients with secondary (S-ITP) immune thrombocytopenia.

Table 3. Correlation of the results obtained with platelet-associated IgG (PAIgG) and platelet-associated autoantibodies (MACE) assays.

PAIgG	MACE		Total
	Positive	Negative	
Positive	30	18	48
Negative	10	46	56
Total	40	64	104

$\chi^2 = 19.4 p < 0.001$.

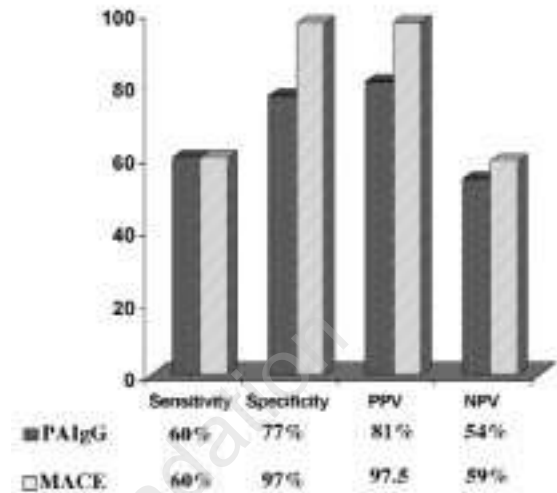


Figure 2. The sensitivity, specificity, positive and negative predictive values (PPV, NPV) for the diagnosis of immune thrombocytopenia obtained with the immunofluorescent assay of platelet-associated IgG and capture method for specific platelet autoantibodies against GPIIb/IIIa, Ib/IX and Ia/IIa in 104 patients with thrombocytopenia (platelet count below $100 \times 10^9/L$).

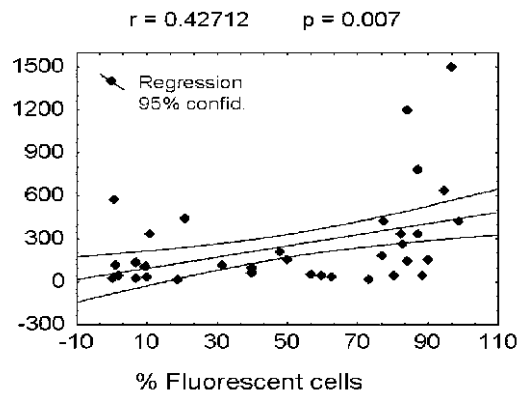


Figure 3. Correlation between fluorescence intensities of platelet-associated IgG (% fluorescent cells) and optical density of antigen capture ELISA (MACE optical density) in 39 patients with demonstrable platelet anti-glycoprotein autoantibodies. In patients with multiple reactivity of platelet autoantibody, the highest optical density was considered.

Discussion

There is a lack of a sensitive and specific single immunoassay for platelet autoantibodies that may support a clinical diagnosis of immune thrombocytopenia.

The quantitative and/or qualitative measure of platelet-associated immunoglobulins (PAIg) is the most widely used phase II method to detect platelet autoantibodies. This assay measures the presence of immunoglobulins on whole or lysed platelets.^{6,22} However, platelet immunoglobulins may represent not only platelet autoantibodies, but also releasable immunoglobulins stored by endocytosis in the α -granules, which are related to platelet size and to immune complexes bound to platelet Fc receptor.⁴ Besides initial studies showing that 60-90% of ITP patients had elevated PAIg levels,^{2,23-25} it soon became clear that the specificity of the test for platelet autoantibodies was low,^{1,2,26,27} since elevated levels of PAIgG are found in thrombocytopenic conditions other than ITP.^{26,28} In our hands, the measure of PAIgG by flow cytometry in 104 thrombocytopenic patients showed a sensitivity and specificity of 60% and 77%, respectively, for the diagnosis of ITP. It is remarkable that the sensitivity of the PAIgG was lower than expected; in fact the sensitivity was 50% in patients with primary ITP and 78% in those with secondary ITP. A higher positivity of PAIg in S-ITP was also reported by other authors^{23,29} and may be explained by increased levels of autoantibodies and/or immune complexes in autoimmune systemic diseases, with or without thrombocytopenia.¹⁴ Surprisingly, the specificity of 77% in our series was higher than the 30-50% reported by most authors.^{27,28,30,31} Such discrepancies could be explained by technical reasons. In fact, the density gradient separation of platelets to remove cell fragments, as used by us, may decrease the positivity and increase the specificity of the PAIgG test.^{2,32}

In 1987 Kiefel and McMillan proposed a phase III method for the assay of platelet autoantibodies based on the immobilization of the autoantibody bound to platelet surface glycoproteins.^{9,10} In spite of the use of different strategies to capture immune complexes,³³ all phase III methods showed a specificity higher than phase II assays;^{28,30,31} nevertheless, in about 40% of patients with a clinical diagnosis of immune thrombocytopenia, platelet autoantibodies are undetectable.

In this study we used a modified antigen capture assay (MACE) to verify the results obtained by flow cytometric determination of platelet-associated IgG and to compare sensitivity, specificity and predic-

tive values of the two assays. In agreement with previous reports,^{15,28,30,31} platelet-specific autoantibodies were demonstrated by MACE in 60% of patients with a clinical diagnosis of immune thrombocytopenia and in only 3% of those with a non-immune form; therefore, the assay achieved a very high positive predictive value (97.5%) for ITP.

Detection of specific platelet antibodies was significantly more frequent in secondary immune thrombocytopenia than in the primary form (83% and 48%, respectively).

While it is well known that P-ITP and S-ITP cannot be differentiated by platelet autoantibodies sharing the same specificity,^{14,34,35} to our knowledge this is the first report showing a higher prevalence of specific platelet autoantibodies in the secondary form of ITP. Based on this finding, further clinical and laboratory investigations should be recommended in patients with thrombocytopenia and demonstrable specific platelet autoantibodies. Consistent with previous data, we found that autoantibodies against GPIIb/IIIa were as common as antibodies against GPIb/IX;^{11,25,31,36} moreover, we found a multiple specificity of autoantibodies in a notable percentage of patients.

Recently, Joutsu *et al.*³⁷ demonstrated a significant correlation between fluorescence intensities for PAIgG obtained by the direct platelet immunofluorescence test and the relative absorbance of a monoclonal antibody immobilization of platelet antigens. Our data confirm a significant degree of concordance between the results obtained with flow cytometric assay of PAIgG and the modified antigen capture assay in patients with specific platelet autoantibody.

In conclusion, we recommend combining PAIgG assay as a screening test (since it is an easy-to-use, rapid and cheap tool), followed by an antigen capture assay to enhance the specificity of the assay of platelet autoantibodies in the diagnosis of immune thrombocytopenia.^{38,39}

Even so, it is disappointing that 40% of patients with clinically diagnosed immune thrombocytopenia have no detectable autoantibodies. This result could depend on different glycoprotein targets,⁴⁰ different stages of disease or the absence of a real immune etiology.

Contributions and Acknowledgments

FF planned the study and was responsible for writing the paper. MLR and GL were responsible for the clinical diagnosis of the patients. RS collected and analyzed the laboratory data. GC was responsible for the laboratory assays. AG critically reviewed the

manuscript. All the authors gave their critical contribution and approved the final version of the paper. The authors are listed in an order reflecting their contribution to the article. The authors are indebted to Dr. R. Ramon for his precious contribution to the statistical analysis and to the realization of the figures. RS: responsible for each Tables and Figures.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlap with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

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What is already known on this topic

Although it is well known that autoantibodies against platelet antigens are responsible for ITP, the role of the tests for detection of these autoantibodies in clinical practice is still a matter of debate.

What this study adds

The study compares the diagnostic value of flow cytometry analysis of platelet-associated IgG with an antigen capture ELISA in a large case series of patients studied at a single institution, and shows that the two methods have a comparable sensitivity. Moreover, the study demonstrates that specific platelet antibodies are more frequent in secondary than in primary forms of immune thrombocytopenia.

Potential implications for clinical practice

In suspected ITP, flow cytometry should be used as a screening test and antigen capture assay as a confirmatory test. The possibility of a secondary form of ITP should be considered carefully in patients with demonstrable platelet autoantibodies.

Carlo Balduini, Associate Editor (Pavia, Italy)