

Screening for Platelet Antibodies in Adult Idiopathic Thrombocytopenic Purpura: A Comparative Study Using Solid Phase Red Cell Adherence Assay and Flow Cytometry

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Background: Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder caused by antiplatelet autoantibodies. In this study, we compared 2 methods for screening serum platelet antibodies in patients with ITP.

Methods: A total of 44 adult patients were clinically classified with ITP. We used 2 indirect tests to detect human leukocyte antigen antibodies and/or platelet-specific antibodies in their sera. In method I, we used solid phase red cell adherence (SPRCA) assay. In method II, by flow cytometry, platelets from plateletpheresis components were used as target cells, and fluorescein isothiocyanate-conjugated sheep anti-human IgG Fc was used as the staining reagent. Positive results were defined as any test with the percentage of fluorescence exceeding the reference range by 3% or more in method II. Direct tests detecting platelet-associated IgG on platelets of patients with ITP were done by flow cytometry.

Results: Serum specimens from 44 adult patients with ITP (28 female, 16 male) were tested. SPRCA assay could only detect platelet antibodies in 22 patients (50%). By method II, 31 serum specimens (70.5%) yielded positive results. There was a difference between the results of the SPRCA test and method II, with a high degree of significance ($p < 0.001$) by the McNemar test. No significant difference in platelet counts was observed for patients with and without discernible platelet antibodies by SPRCA assay ($p = 0.90$). The direct test was positive in 12 patients (66.7%) out of 18 ITP patients tested.

Conclusion: Flow cytometry is more sensitive than SPRCA assay for detecting platelet antibodies. Detection of platelet antibodies is useful in explaining the immune mechanism and platelet transfusion refractoriness in ITP. [*J Chin Med Assoc* 2006;69(12):569–574]

Key Words: flow cytometry, human leukocyte antigen, idiopathic thrombocytopenic purpura, platelet antibody, solid phase red cell adherence assay

Introduction

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder. Platelet antibody IgG is increased in acute and chronic ITP patients. In children, it is most often an acute disorder, and platelet antibody IgM is significantly higher in acute ITP.¹ In adults, it is most often a chronic condition persisting for more than 6 months.

Platelet refractoriness is caused by human leukocyte antigen (HLA) antibodies and platelet-specific

antibodies.^{2–6} The characteristic of decreased recovery and survival of transfused platelets in nonalloimmunized patients with ITP suggests that platelet-specific antibodies and/or HLA autoantibodies are present in the plasma of almost all cases. In patients with ITP, platelet-specific antibodies that recognize platelet glycoproteins were reported more frequently.^{7–10}

A number of methods have been developed for the detection of platelet-specific antibodies and HLA antibodies, such as solid phase red cell adherence (SPRCA) assay,^{11,12} platelet immunofluorescence assay,¹³ and

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antigen-capture assays such as the monoclonal antibody-specific immobilization of platelet antigen assay.¹⁴ The speed and simplicity of the SPRCA method allow many hospital laboratories to use the SPRCA assay for platelet antibody screening and crossmatching. Drawbacks of these tests are their insensitivity.

Flow cytometry is a sensitive method for the detection of platelet antibodies.¹⁵⁻¹⁷ Many platelet antibody tests have been evaluated in the diagnosis of ITP due to its immune nature.¹⁸ Several publications have described flow cytometric methods for the detection of platelet antibodies in patients with ITP.^{19,20}

In this study, we used 2 indirect methods to detect HLA antibodies and/or platelet-specific antibodies in sera and 1 direct method to detect IgG antibodies on platelets obtained from adult patients with ITP. We evaluated whether or not flow cytometric assays using normal platelets as targets are more informative for the evaluation of ITP.

Methods

Patients and controls

A total of 44 adult patients were clinically classified as having ITP using the following criteria: true thrombocytopenia (reviewed by blood smear); bone marrow aspiration with an increased or normal percentage of megakaryocytes; no other evident clinical cause of the thrombocytopenia other than an immune mechanism.¹⁶

Sera from another 10 patients (6 female, 4 male; median age, 57 years; age range, 17-83 years) with non-immune thrombocytopenia (aplastic anemia, 2; paroxysmal nocturnal hemoglobinuria, 1; myelodysplastic syndrome, 2; hepatitis, 1; disseminated intravascular coagulation, 1; multiple myeloma, 1; lymphoma, 1; familial thrombocytopenia, 1) were also studied.

The negative serum control was from 1 of the normal blood donors (25 male; median age, 23 years; age range, 19-26 years) who had never received a transfusion. A pool of 5 highly sensitized patient sera was used as positive control.

Indirect methods

We used 2 indirect methods (method I, SPRCA assay; method II, flow cytometry using donor platelets as target cells) to detect HLA and/or platelet-specific antibodies in the sera.

Method I: SPRCA test

We used the SPRCA test (Capture-P Solid Phase System; Immucor, Norcross, GA, USA). We employed

the platelets from 7 donors as the target cells. The platelets from each donor's apheresis platelets were layered in different wells, and the patient's serum and negative and positive control sera were added in individual wells. The wells were washed and anti-IgG-coated indicator red cells added. If antibodies to the platelets are present, the indicator red cells would form a thin film in the well; but if no platelet antibodies are present, the indicator red cells would pellet to the bottom of the well, forming a red button.

Method II: flow cytometry using donor platelets as target cells

Platelet preparation: We utilized platelets from 10 fresh plateletpheresis components as target cells to screen platelet antibodies (HLA antibodies and/or platelet-specific antibodies) in the sera. The platelets were washed twice with a mixture of phosphate-buffered saline (PBS) and anticoagulant citrate dextrose solution (ACD) formula A (Baxter Healthcare Co., Fenwal Division, Deerfield, IL, USA) (PBS/ACD=9/1) with the pH adjusted to 7.0, by centrifugation at 1,400g for 5 minutes. Washed platelets were pooled. We adjusted the platelet count to $200 \times 10^6/L$ with PBS/ACD. The reagent platelets were prepared on the day of analysis and were not stored for future use.

Antibody test: Equal volumes (50 μ L) of patients' sera and pooled platelets were incubated at 37°C for 30 minutes, respectively. The cells were then washed twice with PBS/ACD mixture at 1,400g for 5 minutes. The sensitized platelets were resuspended in 100 μ L PBS/ACD and incubated for 30 minutes in the dark at room temperature with 10 μ L fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgG Fc (The Binding Site Ltd., Birmingham, UK). They were then washed twice in PBS/ACD. We resuspended the pellet in 200 μ L PBS/ACD by shaking the tubes on a vortex for 15 seconds and promptly analyzed them on a flow cytometer (Epics XL; Coulter Electronics, Hialeah, FL, USA). We analyzed approximately 5,000 platelets per test.

A negative reference range was determined by using sera from never-transfused males. We set the analysis region with the negative reference range in the FL1 histogram. Positive results were defined as any test with the percentage of fluorescence exceeding the reference range by 3% or more.²¹ Negative and positive control sera were included in each test.

Direct method using flow cytometry

For detecting platelet-associated IgG (PAIgG) on the platelet surface, platelets from patients with ITP were washed twice. The platelets were incubated with

FITC-conjugated sheep anti-human IgG Fc. They were then washed twice and analyzed on the flow cytometer. The negative platelet control was from normal males who had never received a transfusion. The platelets that were sensitized with a pool of 5 highly sensitized patients' sera were used as positive control. We analyzed approximately 5,000 platelets per test. Positive results were defined as any test with the percentage of fluorescence exceeding the negative control by 3% or more.²²

Statistical analysis

Statistical analysis of the data was performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). The platelet counts of patients with positive and negative tests were compared using the Mann-Whitney test. The difference between the results of SPRCA and method II was compared by the McNemar test. The level of agreement of the results of SPRCA assay and method II was assessed by the κ coefficient. The figure of distribution of percent fluorescence was done using SigmaPlot 8.02 (Systat Software, Point Richmond, CA, USA). A p value < 0.05 was considered to be significant.

Results

A total of 44 patients with ITP (28 female, 16 male) were studied. The median age of the patients with ITP was 53 years (range, 23–90 years). The median interval between initial onset of thrombocytopenia and blood sampling for platelet antibody testing was 35 days (range, 0–2,555 days), and 21 sera were sampled within 7 days.

Patients' sera were tested for platelet antibodies by methods I and II. The test results are presented in Table 1. The results of the SPRCA assay and method II were in moderate agreement (κ coefficient = 0.555). All assays yielded negative results for negative control sera.

Table 1. Results of solid phase red cell adherence (SPRCA) assay (method I) and flow cytometric assay* (method II) in 44 patients with idiopathic thrombocytopenic purpura

SPRCA (method I)	Flow cytometric analysis (method II)	Patients (n)
Positive	Positive	15
Positive	Negative	7
Negative	Positive	16
Negative	Negative	6

*Using donor platelets as target cells.

The SPRCA assay could only detect platelet antibodies in 22 patients (50%). The mean platelet counts of patients with positive ($40.0 \pm 32.7 \times 10^9/L$) and negative ($41.7 \pm 40.4 \times 10^9/L$) SPRCA tests did not differ significantly ($p = 0.90$).

Using method II, 31 serum specimens (70.5%) yielded positive results. There was a difference between the results of SPRCA testing and method II, with a high degree of significance ($p < 0.001$) according to the McNemar test.

Using method II, the mean percentage of fluorescence of negative serum control and non-immune thrombocytopenia were $3.03 \pm 1.68\%$ and $2.45 \pm 0.95\%$, respectively, and they were similar ($p = 0.33$). The mean percentage of fluorescence in ITP patients was $12.01 \pm 12.01\%$, which was significantly higher than those of the negative control ($p < 0.001$) and non-immune thrombocytopenia ($p < 0.001$) (Figure 1).

Of the 10 patients with non-immune thrombocytopenia, neither of these 2 different assays for platelet antibodies had any positive results.

The platelets obtained from 19 patients with ITP were available for direct testing. Twelve (66.7%) of these 18 specimens produced positive results.

Discussion

Circulating platelet autoantibodies are involved in the pathogenesis of ITP.^{7–10} The primary mechanism of thrombocytopenia in ITP is that platelets opsonized by platelet autoantibodies are destroyed by the reticuloendothelial system. Activation of complements on the platelet surface may occur in some patients with ITP. Tsubakio et al demonstrated that ITP autoantibodies, anti-HLA and platelet-specific antibodies (e.g. anti-HPA-1a) activate the complement pathway to a similar degree, and platelet lysis by autoantibodies and complement may occur in some chronic ITP patients.²³ McMillan et al found that autoantibodies in plasma obtained from some adult patients with ITP inhibit *in vitro* megakaryocyte production, suggesting that a similar effect may occur *in vivo*.²⁴

Some patients with ITP were associated with autoimmune disorders such as autoimmune hemolytic anemia and idiopathic neutropenia.^{25,26} The autoantibodies are directed against antigens specific for the various peripheral blood cells, i.e. platelets, erythrocytes, and granulocytes, and they are not cross-reactive. Polyclonal autoantibodies suggested generalized immune dysregulation in patients with multiple autoimmune manifestations.²⁷

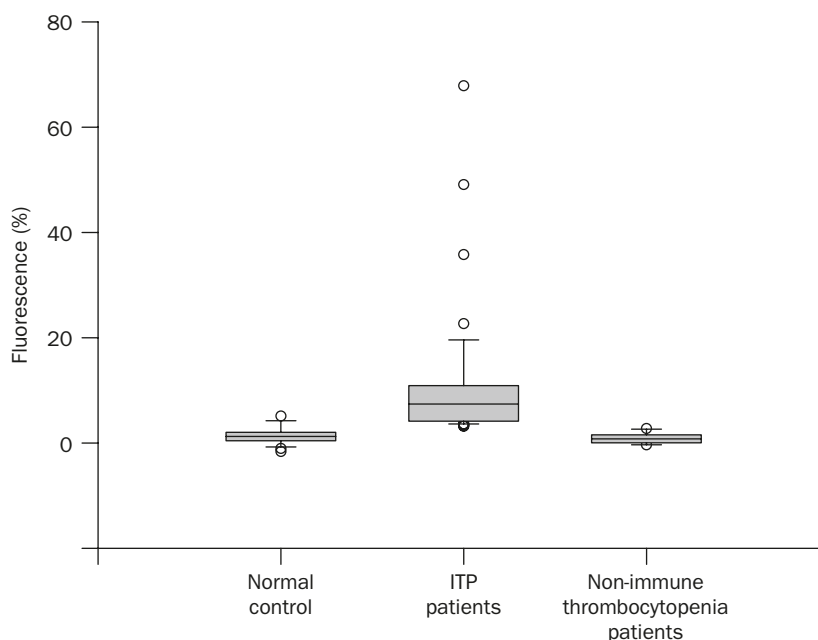


Figure 1. Box plot for percent fluorescence by method II in 25 normal controls, 44 idiopathic thrombocytopenic purpura (ITP) patients and 10 non-immune thrombocytopenia patients. The box represents the interquartile range, and the line within the box represents the median. The bar represents the standard deviation. The open circles are outliers.

Both the SPRCA test and flow cytometric assay that use platelets as the target cells will detect platelet-specific antibodies and antibodies to HLA class I antigens. These tests will detect both alloantibodies and autoantibodies, but will not distinguish between them. The SPRCA test is a common laboratory test to evaluate the presence of platelet antibodies. The interpretation of the SPRCA test is straightforward, and the kit provides good control material to ensure that the results are valid. But it has been reported that antibodies are not always detected with this assay. Jones et al reported that the SPRCA method showed 65% sensitivity and 100% specificity for detecting PAIgG in ITP.²⁸ Raife et al reported that the sensitivity of the SPRCA test for the clinical diagnosis of ITP was 43% for the indirect method.²⁹ In our study, the SPRCA method showed 50% sensitivity and 100% specificity for detecting plasma antiplatelet antibodies.

Flow cytometry is sensitive, but it lacks the ability to differentiate between pathologic and non-pathologic PAIgG. Many patients with non-immune thrombocytopenia also have high PAIgG.³⁰ Thrombocytopenia in some patients with septicemia may be related to the binding of IgG to platelets.³¹ Total PAIgG measurements in thrombocytopenic patients may reflect platelet size, which is known to be increased in response to thrombopoietic stress.³² One exception to this interpretation is that increased total PAIgG content merely

reflects their increased plasma IgG in patients who have disorders with increased plasma IgG concentrations.³³

Latorraca et al studied 14 patients with chronic ITP using flow cytometry; the direct test (anti-platelet antibodies on the platelet surface) was positive in 7 patients (50%), and the indirect test (detecting platelet antibodies in sera) was positive in 8 patients (57%).¹⁹ In our study, the direct test was positive in 66.7% of patients tested, while the indirect test using method II was positive in a higher number of patients (70.5%).

Although the clinical diagnosis of ITP is generally satisfactory in “typical” cases, demonstration of platelet autoantibodies supports an immune-mediated pathologic process in patients with ITP. Sensitive methods for platelet antibodies are not readily adaptable to routine clinical laboratories. In this study, the 70.5% sensitivity by flow cytometry is still not very sensitive. The diagnosis of ITP needs both clinical evidence and laboratory data to exclude other causes of thrombocytopenia. According to the guidelines of the American Society of Hematology, no serologic tests are necessary to diagnose clear ITP.²¹

Possible factors influencing the detection rate may include the following: the selection of patients in different stages of disease; presence of both alloantibodies and autoantibodies in some patients with ITP; the methodologic difference in flow cytometry; the use of fresh donor platelets to decrease background fluorescence;

and the number of donors from whom the target platelets were obtained. In this study, a pooled platelet suspension from 10 donors was used for flow cytometry; nevertheless, in the SPRCA assay, platelets from 7 single donors were used as target cells. The target platelets used in flow cytometry and the SPRCA assay were obtained from different donors, because these 2 tests were performed on different dates and fresh platelets were chosen as targets.

We conclude that flow cytometry assay using donor platelets as target cells allows detection of autoantibodies in 70% of patients with ITP. Flow cytometry is more sensitive than SPRCA assay for detecting platelet antibodies. Definite proof regarding the presence of platelet antibodies is useful in explaining the immune mechanism and platelet transfusion refractoriness.

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