Modification of Solid Phase Red Cell Adherence Assay for the Detection of Platelet Antibodies in Patients With Thrombocytopenia

Preyanat Vongchan, PhD,1 Weerasak Nawarawong, MD,2 and Robert J. Linhardt, PhD3

Key Words: Solid phase red cell adherence; Thrombocytopenia; Platelet alloantibodies; Platelet transfusion; Platelet refractoriness

Abstract

Platelet refractoriness is caused by HLA antibodies and platelet-specific antibodies. Current methods used to detect antiplatelet antibodies have limitations. Solid phase red cell adherence (SPRCA) lacks sensitivity and requires a second assay using chloroquine-treated intact platelets to specify the response due to anti-HLA. We modified SPRCA by using 2 types of antihuman platelet antibodies with different specificities toward platelet lysate and tested samples from 361 patients (69 with unexplained thrombocytopenia and 292 with poor response to platelet transfusions not explainable by alloimmunization or the clinical situation) and 50 from healthy volunteers. Our method compared favorably with platelet suspension direct immunofluorescence. All samples from healthy volunteers were negative; of the samples from the patient population, 240 were positive (147 samples had only antiplatelet and 3 samples had only anti-HLA antibodies). This modified technique had a sensitivity of 98% and a specificity of 91%.

Three clinical conditions may result from alloimmunization against human platelet antigens: neonatal alloimmune thrombocytopenia, posttransfusion purpura, and platelet transfusion refractoriness. Refractoriness to platelet transfusion is a difficult clinical problem that can complicate the supportive care of patients with multiple transfusions. Rates of refractoriness of 40% to 70% have been reported in patients with a prolonged requirement for platelet transfusion during chemotherapy-induced marrow aplasia or marrow transplantation for malignancy.1,2 Patients receiving cellular blood components may form HLA antibodies and platelet-specific alloantibodies,3 and patients who receive multiple transfusions are at increased risk of developing red cell antibodies.4,5 There were reports that platelet antibody specificity in transfusion recipients differed significantly from that observed in patients with neonatal alloimmune thrombocytopenia or posttransfusion purpura.5,7 Immunologic platelet destruction, mediated by alloantibodies directed against antigens on platelets, is frequently the principal or an important contributing factor in the platelet refractory state.7,8 There is a general correlation between alloimmunization to HLA and platelet antigens and clinical platelet refractoriness.4,9-13 This correlation provides the basis for blood product selection strategies in the management of refractory conditions.2

Because detection of alloantibodies is important for diagnosis and treatment, the diagnosis has to be done quickly. A variety of methods have been used in the detection of antiplatelet alloantibodies. These techniques include antigen capture enzyme-linked immunosorbent assay (ELISA),13,14 flow cytometry,14,15 platelet aggregometry, serotonin release assays, platelet factor 4 assays,16,17 and solid phase red cell...
adherence (SPRCA) assay. Each of these techniques has important limitations. Immunobead assay and the monoclonal antibody–specific immobilization of platelet antigens assay have been considered as possible reference methods. However, these assays are time-consuming and not easily adapted for routine use. In recent years, other sensitive and specific tests to detect platelet antibodies have been developed, but they are still rather tedious. The SPRCA assay has been used for the detection of drug-dependent platelet antibodies. The speed and simplicity of SPRCA allow many hospital laboratories to use it for platelet antibody screening and cross-matching. A major drawback of SPRCA is its insensitivity, the requirement for intact pooled platelets, and the need to perform it twice in the case of a positive result to rule out the presence of anti-HLA antibodies.

In this study, we modified standard SPRCA using 2 types of rabbit antihuman platelet polyclonal antibodies, anti-HLA adsorbed and nonadsorbed, to coat onto microtiter plates in parallel. Thus, platelet lysate with and without HLA antigens was selectively immobilized on wells, and anti–HLA-specific antibodies in serum samples were then specified in 1 step. Requirements for intact platelets and chloroquine treatment were eliminated by using this modified SPRCA assay. We adapted this assay so that it did not use specialized or expensive equipment and could be performed in transfusion service laboratories. Our modified assay was used to screen antiplatelet antibodies in patient serum samples and compared with the platelet suspension immunofluorescent technique (PSIFT) as a reference method. We then evaluated our modified SPRCA (M-SPRCA) for screening and identification of anti-HLA and platelet-specific antibodies in the serum samples of patients with thrombocytopenia.

Materials and Methods

Patients and Control Subjects

Samples from 361 patients with various diagnoses and in thrombocytopenic crisis and 50 negative control subjects (healthy donors) were studied. The serum samples were from Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, and permission was granted to collect samples that were scheduled to undergo destruction.

Production of Polyclonal Rabbit Antihuman Platelet Polyclonal Antibodies

The production of nonadsorbed antiplatelet antibody (NAP) is described in this section. Platelet-rich plasma (PRP) was isolated from 5 healthy volunteers with blood group O, Rh+ by standard methods recommended by the American Association of Blood Banks. PRP was centrifuged (2,800g for 30 minutes) at room temperature to yield platelet concentrate (PC). The PC was then washed 3 times with 8.9 mmol/L of EDTA–phosphate-buffered saline (PBS), pH 7.2, and adjusted to 1 × 10⁸ platelets/mL with sterile PBS. A rabbit was immunized weekly with human platelets (1 × 10⁹ platelets/500 µL) via an intramuscular route for a period of 2 months. Rabbit blood was collected before each immunization, and serum was separated and used to investigate the rising titer of anti-platelet antibodies by PSIFT. PC pooled from 5 healthy volunteers was prepared, washed twice with EDTA-PBS, pH 7.2, and fixed with 3 mL of 1% paraformaldehyde in PBS for 5 minutes at room temperature. Paraformaldehyde-fixed PC was then washed twice with EDTA-PBS, suspended, and adjusted to 150 × 10⁹ platelets/mL with the same buffer. Rabbit serum (50 µL) at various dilutions was incubated with 5 µL of

Table 1

<table>
<thead>
<tr>
<th>History</th>
<th>M-SPRCA Pattern</th>
<th>Negative</th>
<th>Interpretation of Antibodies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic thrombocytopenia (n = 69)</td>
<td>28</td>
<td>3</td>
<td>45</td>
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<tr>
<td>Hematologic malignancy (n = 61)</td>
<td>45</td>
<td>0</td>
<td>74</td>
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<tr>
<td>Various organ malignancy (n = 42)</td>
<td>28</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>Uncontrolled bleeding (n = 72)</td>
<td>56</td>
<td>0</td>
<td>78</td>
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<tr>
<td>Infection (n = 109)</td>
<td>77</td>
<td>0</td>
<td>70.6</td>
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<tr>
<td>Autoimmune disease (n = 8)</td>
<td>3</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>Total (n = 361)</td>
<td>237</td>
<td>3</td>
<td>66.5</td>
</tr>
</tbody>
</table>

M-SPRCA, modified solid phase red cell adherence; P-N pattern, positive only in the nonadsorbed antiplatelet antibody well; P-P pattern, positive in both wells.

* Interpretation proved by platelet suspension immunofluorescent technique for antiplatelet antibodies and indirect immunofluorescence technique using pooled normal leukocytes for anti-HLA antibodies.
paraformaldehyde-fixed platelets at 37°C for 30 minutes and washed 3 times with EDTA-PBS. Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit immunoglobulins (Igs; 50 µL diluted 1:30 in EDTA-PBS, DakoCytomation, Glostrup, Denmark) was added, and the reaction was incubated at room temperature for another 30 minutes. Finally, the reaction was washed twice with EDTA-PBS and suspended with 300 µL of 0.5% paraformaldehyde and analyzed by flow cytometry.

Saturated ammonium sulfate was added dropwise into rabbit serum with stirring to a final concentration of 35% saturation. The mixture was then moved to 4°C for overnight precipitation, centrifuged at 2,500 rpm for 30 minutes at room temperature, and the supernatant containing Igs was collected. Saturated ammonium sulfate was again added dropwise to a final concentration of 50% saturation, moved to 4°C overnight, and centrifuged, and the precipitate containing Igs was collected. This precipitate was suspended with PBS, pH 7.2 (half the volume of the serum sample) and dialyzed against PBS with 4 to 5 changes of buffer. The protein concentration was determined by the Lowry method. Partially purified Ig was further purified by using Melon Gel (Pierce, Rockford, IL). Briefly, ammonium sulfate–purified antibodies in PBS were dialyzed against 1× Melon Gel purification buffer before mixing with 20% slurry gel (1:1 ratio), incubated with shaking for 5 minutes, and then filtered through 45-µm filter paper under reduced pressure; nonspecific proteins are adsorbed to the gel, and only IgG passes through without an elution step. Fractions (1 mL) were collected and observed, their optical density was measured at 280 nm, and fractions containing protein were combined. The resulting purified antiplatelet polyclonal antibodies were dialyzed against 3 changes of PBS, pH 7.2, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After dialysis, the purified antiplatelet polyclonal antibody, NAP, was frozen and stored at –20°C.

Preparation of Anti-HLA/Antiplatelet Associated IgG–Depleted Antiplatelet Antibody

Immune-mediated thrombocytopenia may be caused by antiplatelet specific antigen or antibody to common antigens such as HLA. Transfusion of platelets to such patients needs specific platelets and crossmatching. Since positive results in patient sera using conventional SPRCA are not specific, the assay needs to be performed a second time using chloroquine-treated platelets in which the HLA molecule has been destroyed. A second positive result then indicates that the antibody in serum is specific for the platelet-specific antigen. This current technique was adapted to demonstrate the presence of the antiplatelet surface antigen in a single step. Anti-HLA antibodies in rabbit serum (NAP) must be eliminated. Moreover, since the principle of the test is based on cross-linking between antihuman IgG antibody, antigen– antibody complex immobilized on the microtiter plate, and indicator cells, the antiplatelet associated IgG (PAIG) in NAP needs to be eliminated to prevent nonspecific reactions. Thus, rabbit antiplatelet antibodies containing both anti-HLA and anti-PAIG (or NAP) must be treated to remove anti-HLA and anti-PAIG, as follows.

Depletion of Anti-HLA From Antiplatelet Antibody

Whole blood (10 mL) collected over EDTA was obtained from 5 healthy volunteers who had donated blood for PC preparation. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using the Ficoll-Hypaque gradient centrifugation technique, and granulocytes were obtained by using 6% dextran sedimentation. The PBMCs and granulocytes were pooled, washed 4 times with cold 1% bovine serum albumin (BSA) in PBS containing 0.02% sodium azide (PBS-azide) and counted using an automatic cell counter (Sysmex KX-21N, Mundelein, IL). The cell suspension was adjusted to 1 × 10^7 cells/mL with cold 1% BSA in PBS-azide. Heat-inactivated normal AB serum was added to this cell suspension to give a final concentration of 10% for blocking of the Fc receptor. The reaction was performed on ice for 30 minutes and washed 4 times with PBS, pH 7.2, and a part of the cell suspension was collected. The NAP antibody (4 mL) was added to the cell pellet, mixed, and incubated on ice for another 45 minutes with occasional agitation. Finally, anti-HLA–depleted antiplatelet antibody was obtained as the supernatant by centrifugation.

Determination of Anti-HLA Activity by the Indirect Immunofluorescence Technique

Anti-HLA–depleted antiplatelet antibodies were investigated for reactivity toward HLA on leukocytes by indirect immunofluorescence and analyzed by flow cytometry. Heparinized whole blood was drawn from 5 healthy donors. PBMCs and granulocytes were prepared, washed, and pooled by the method mentioned previously. Cells were adjusted to 1 × 10^7 cells/mL and incubated with heat-inactivated AB serum at a final concentration of 10% for 30 minutes on ice. After the Fc receptors were blocked, a 50-µL aliquot was added along with 50 µL of anti-HLA–depleted antiplatelet antibody (before adsorption, after adsorption, or with patient serum samples) or 1% BSA in PBS-azide as a conjugated control and incubated for another 30 minutes on ice.

The reaction mixtures were washed 3 times with 1% BSA in PBS-azide before the addition of FITC-conjugated goat antirabbit IgGs (dilution 1:50; DAKO) or FITC-conjugated rabbit antihuman IgGs (dilution 1:50; DAKO) in case of detection in patient serum samples. After incubation for 30 minutes on ice with light protection, the reaction mixtures were washed 4 times with the same buffer, before the addition of 500 µL of 0.5% paraformaldehyde, and analyzed by flow cytometry.

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Depletion of Anti-PAIG From Anti-HLA–Depleted Antiplatelet Antibody

Antibody specific to PAIG was adsorbed from anti-HLA–depleted antiplatelet antibody by using human IgG coupled to CNBr-activated Sepharose beads. Briefly, 1 g of CNBr-activated Sepharose 4B (Sigma, St Louis, MO) was washed and reswelled in 1 mmol/L of ice cold HCl. Before coupling, beads were primed with 5 mL of coupling buffer (0.1 mol/L NaHCO3 and 0.5 mol/L NaCl).

Human IgG were prepared from serum samples from 5 healthy volunteers by ammonium sulfate precipitation. The purity of the IgG was prepared demonstrated by SDS-PAGE.

Primed CNBr-activated Sepharose (5 mL) was added to 1.5 mL of purified human IgG (10 mg/mL) and gently mixed on an end-to-end rocking device for 2 hours at room temperature. The beads were recovered by centrifugation at 4°C, and the remaining active groups were blocked overnight at 4°C by using glycine (2 mL, 0.2 mol/L, pH 8.0). The mixture was centrifuged to discard excess protein and glycine before washing with 0.1 mol/L of sodium acetate buffer at pH 3.5 and coupling buffer.

Human IgG coupled to Sepharose beads (1.0 mL) were suspended in PBS, pH 7.2, and 1.5 mL of anti-HLA–depleted antiplatelet antibody (10 mg/mL) was added. The mixture was gently mixed on a rocking plate at room temperature for 1 hour, and the beads were removed by centrifugation. The supernatant was collected and affinity purified by using Melon Gel. The purified IgG was assayed to verify that the adsorbed antiplatelet antibodies (AAP) had no residual anti-PAIG activity. Human IgG were subjected to electrophoresis on 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane; the membrane was blocked with nonfat dried milk (5% in PBS, pH 7.2) for 1 hour at room temperature and washed 3 times with PBS, pH 7.2, containing 0.05% Tween-20; and AAP was added and incubated at room temperature for 1 hour and washed 3 times with the same buffer. Horseradish peroxidase–conjugated goat antirabbit IgG (5 mL, dilution 1:2,500; DAKO) was added and reacted for another hour at room temperature before washing. Finally, the reactions were washed twice and fixed with 0.5% paraformaldehyde in PBS, pH 7.2, before analysis by flow cytometry.

Modified SPRCA

Preparation of Platelet Lysate

PRP and PC were prepared from whole blood drawn from 5 healthy volunteers collected over EDTA. The PC was pooled and washed 3 times with tris(hydroxymethyl) aminomethane-buffered-saline (TBS), pH 7.4, and the platelet count was performed by using an automatic cell counter (Sysmex KX-21N). The PC pellet was then adjusted to 1 × 109 platelets/mL with 0.5% NP-40 (Sigma) in TBS, pH 7.4, and mixed thoroughly for about 30 minutes on ice. The mixture was then subjected to ultracentrifugation (40,000 rpm) at 4°C for 30 minutes. Supernatant was collected, protein concentration was determined by the Lowry method, and the supernatant was stored at −70°C for further assays.

Preparation of Indicator Cells—Anti-D IgG Sensitized O, Rh+ Cells

Acid-citrate-dextrose whole blood samples from healthy donors with blood group O, Rh+ (R1r) were drawn. Red cells were washed 3 times with normal saline solution (NSS). Anti-D IgG (Thai National Red Cross, Bangkok) was serially diluted (1:32-1:256) with NSS and reacted with an equal volume of washed packed RBCs. The mixture was incubated at 37°C for 30 minutes and washed 3 times with NSS. Each sample containing sensitized RBCs was individually prepared to a 5% suspension in NSS, and a direct antiglobulin test was performed. The optimal indicator cell was the dilution giving 1+ positive with the direct antiglobulin test. Anti-D IgG-sensitized O, Rh+ cells (R1r) were prepared for further assay of SPRCA by sensitizing the O, Rh+ cells with optimized dilution. The working dilution of the indicator cells was 0.2% in Alsever solution.
Modifications to the SPRCA

SPRCA was modified to reduce its labor intensiveness and to increase assay cost-effectiveness to make these assays feasible for transfusion service laboratories. Modifications to this assay included the following: (1) using a different form of antiplatelet antibody (NAP or AAP) to immobilize both platelet antigens and HLA to the microtiter plate or only platelet antigens to the microtiter plate; and (2) replacing intact platelets with platelet lysate, which can be easily prepared in advance and stored for long periods for immobilization. By using these modifications, the screening and identification of antiplatelet antibodies in patient serum samples can be performed using a single assay in any transfusion service laboratory.

Various concentrations of NAP or AAP, diluted in 0.01 mol/L sodium carbonate/bicarbonate buffer, pH 9.6, were coated (100 µL/well) onto a microtiter plate and incubated at 4°C overnight to optimize the modified SPRCA. After washing 3 times with 0.5% BSA-PBS, pH 7.2, 5% BSA in PBS was added, 200 µL/well, and incubated for 1 hour at 37°C to block nonspecific binding sites. The microtiter plate was again washed 3 times with BSA-PBS. Next, 1% paraformaldehyde was mixed with BSA-PBS (1:1) and used to prepare various concentrations of platelet lysate before loading a final volume of 100 µL/well onto a microtiter plate and incubated for another hour at 37°C. The microtiter plate was washed again 3 times with BSA-PBS, and 1.9% glycine in BSA-PBS (100 µL/well) was added. Normal human serum (dilution 1:10; 50 µL/well) or anti-HLA positive control serum (dilution 1:10; 50 µL/well) was added; the normal and positive serum samples were used as negative and positive control samples, respectively. The reaction was allowed to take place for 1 hour at 37°C, and the plate was washed 3 times with the same buffer. Finally, 50 µL/well of various concentrations of Fc-specific rabbit antihuman IgG (DAKO) and 50 µL/well of 0.2% indicator cells were sequentially added. The microtiter plate was then centrifuged at 2,000 rpm for 2 minutes, and the agglutination of indicator cells was observed.

Screening of Antiplatelet Antibodies in Patient Serum Samples by M-SPRCA

Serum samples from 361 patients with various diagnoses and 50 samples from healthy volunteers were investigated for antiplatelet antibodies. All patients studied had a low platelet count with or without a bleeding disorder and platelets had been requested from the Blood Banking Service, Maharaj Hospital, Faculty of Medicine, Chiang Mai University. Serum samples were analyzed by M-SPRCA using optimized conditions and results compared with the PSIFT reference method.

The specificity of alloantibodies was confirmed by indirect immunofluorescence using pooled normal leukocytes as sources of HLA antigens as mentioned previously. Briefly, rabbit antiplatelet polyclonal antibodies (NAP and AAP; 20 µg/mL, diluted in 0.01 mol/L of carbonate/bicarbonate buffer, pH 9.6) were coated onto microtiter plates in parallel (100 µL/well) overnight at 4°C. The plates were washed 3 times with BSA-PBS before blocking with 200 µL/well of 5% BSA-PBS for 1 hour at 37°C. The plates were washed 3 times with the same reagent and 2.5 µg/100 µL/well of platelet lysate diluted in the mixture of 1% paraformaldehyde, and BSA-PBS (1:1) was added. The reaction was incubated for 1 hour at 37°C before washing 3 times with BSA-PBS. After washing, 1.9% glycine (100 µL/well) was added, followed by the addition of the same volume of patient serum samples, BSA-PBS (negative control sample), or anti-HLA serum (positive control sample). The microtiter plates were incubated for another 1 hour at 37°C. Fc-specific rabbit antihuman IgG (50 µL/well), followed by 0.2% indicator cells (50 µL/well), was added to determine the antigen-antibody reaction. The plates were finally centrifuged at 2,000 rpm for 2 minutes, and the resulting red cell agglutination was compared with positive and negative control samples. M-SPRCA was further verified for the presence and absence of certain antibodies by indirect immunofluorescence using human leukocytes and platelets, respectively.

The criteria used to interpret the specificity of antibodies included the following: (1) positive in both wells (P-P pattern), indicating antiplatelet alone or mixed antiplatelet/anti-HLA; (2) positive only in the NAP well (P-N pattern: indicating anti-HLA alone); and (3) negative (N-N pattern), indicating no antibody specific to platelet or HLA antibodies.

Results

Preparation of Polyclonal Rabbit Antiplatelet Polyclonal Antibodies (NAP)

Pooled blood group O platelets from 5 healthy volunteers were prepared to produce antiplatelet polyclonal antibodies. Platelets were adjusted to 2 × 10^9/mL in sterile PBS, pH 7.2, and rabbits were immunized weekly using a 500-µL intramuscular dose. Before each immunization, blood was drawn and serum was collected to follow the rising titer of antiplatelet polyclonal antibodies using PSIFT. The results indicate that the rabbit produced polyclonal antiplatelet platelets 8 weeks after initial immunization. These antibodies are a mixture of a variety of specific antibodies including antiplatelet-specific antigens, anti-HLA, and antiplatelet surface Igs that may cause interference in the M-SPRCA. Thus, the interfering antibodies need to be removed through an adsorption step.
Preparation of Anti-PAIG/Anti-HLA Depleted Antiplatelet Antibody (AAP)

Platelet membranes consist of specific antigens including human platelet-specific antigen (HPA),\textsuperscript{29,30} as well as nonspecific antigens including HLA class I, PAIG, and Fc receptor.\textsuperscript{31,32} Therefore, antiplatelet polyclonal antibodies contain a variety of antibodies, many of which may result in misinterpretation of an SPRCA assay. Anti-HLA was removed by adsorption to pooled leukocytes derived from the owner of platelets used to immunize the rabbit from which anti-HLA was derived. Fc receptors on leukocytes were blocked by final 10% heated-inactivated AB before adsorption to prevent the loss of platelet-specific antibody. The adsorption was performed on ice 3 to 4 times to eliminate anti-HLA. Fluorescence intensity shifted to the left, compared with preadsorbed antiplatelet antibody, following multiple adsorptions. Anti-PAIG was further diminished by using Igs coupled to CNBr-Sepharose beads and was demonstrated to be free of anti-PAIG by Western blotting. AAP was confirmed to still be present after multiple adsorptions, as required for determining antiplatelet activity by PSIFT. Because AAP still provided antiplatelet activity, it could still be used as a tool to modify SPRCA.

Optimization of the M-SPRCA

Various concentrations of NAP and AAP used to immobilize the platelet lysate were 20 µg/mL (100 µL/well) and platelet lysate was 2.5 µg/mL/100 µL/well. The human anti-D IgG used to sensitize Rh+ (R1r) RBCs and the Fc-specific rabbit antihuman IgG were also optimized. The results showed that the optimal concentration for these 2 antibodies were 1:128 and 1:1,000, respectively. The positive M-SPRCA resulted in agglutination of indicator cells that spread throughout the microtiter plate as a result of the immobilized platelet antigens. The negative control showed a cell button in the middle of the well after centrifugation.

Screening of Antiplatelet Antibodies in Patient Serum Samples

Serum samples (n = 361) from patients with various diagnoses (Table 1) and for whom platelet concentrate had been requested were collected and frozen at −20°C. Serum samples from 50 healthy volunteers were also included in this experiment. Screening for antiplatelet antibodies was performed by using M-SPRCA. The results for the samples from healthy volunteers showed no false-positives. Interpretation was based on 3 patterns of sensitized red cell agglutination including P-P, P-N, and N-N.
A problem associated with long-term platelet supportive care is the development of a state of refractoriness resulting in poor platelet transfusion increments. Nonimmunologic factors are frequently associated with reduced platelet survival after transfusion. Platelet survival may also be impaired by the presence of circulating antibodies directed against antigens expressed on the platelet membrane. HLA antibodies are the most common cause of immunologic platelet transfusion refractoriness. Thrombocytopenia and refractoriness, caused by the presence of antiplatelet antibodies, are known to be potential complications of transfusion therapy, but the presence of these antibodies is thought to be infrequent. Because thrombocytopenia and/or platelet refractoriness can result in severe adverse clinical outcomes for patients, regardless of the specificity of alloantibody, serologic evaluation of suspected...
Optimization of modified solid phase red cell adherence (SPRCA). Various concentrations of nonadsorbed antplatelet antibody (NAP) or adsorbed rabbit antihuman platelet antibody (AAP) were coated (100 µL/well) onto a microtiter plate and incubated at 4°C overnight to optimize the modified SPRCA. After washing with bovine serum albumin (BSA)–phosphate-buffered saline (PBS), 5% BSA in PBS was added, 200 µL/well, and incubated for 1 hour at 37°C to block nonspecific binding sites. The microtiter plate was washed again 3 times with BSA-PBS. Various concentrations of platelet lysate (100 µL/well) were added after washing and incubated for another hour at 37°C. Glycine (1.9% in BSA-PBS) was then added followed with 50 µL/well of normal human serum or anti-HLA+ serum as negative and positive control samples, respectively. The reaction was allowed for 1 hour at 37°C and washed with the same buffer. Finally, 50 µL/well of various concentrations of Fc-specific rabbit antihuman IgG and 50 µL/well of 0.2% indicator cells were added sequentially. The microtiter plate was then centrifuged at 2,000 rpm for 2 minutes, and the agglutination of indicator cells was observed. A, Agglutination observed in various concentrations of rabbit antihuman IgG used (1:500-1:2,500). B and C, Enlarged picture of agglutination when rabbit antihuman IgG 1:1,000 was used. a, Platelet lysate, 2.5 µg/100 µL/well, anti-HLA positive serum, 1:10; b, Platelet lysate, 5.0 µg/100 µL/well, anti-HLA positive serum, 1:10; c, Platelet lysate, 2.5 µg/100 µL/well, Normal serum control sample; d, Platelet lysate, 2.5 µg/100 µL/well, anti-HLA positive serum, 1:100; e, Platelet lysate, 5.0 µg/100 µL/well, anti-HLA positive serum, 1:100; f, Cell control.

Various patterns of agglutination for interpretation of modified solid phase red cell adherence. In every investigation, the cell control (the only sensitized red cell in the well), negative control (no serum), and positive control (anti-HLA positive serum, 1:10) were performed in parallel. The agglutination pattern in the test well was interpreted following the pattern of control. a, Cell control; b, Negative control; c, Positive control; d, e, and f, Positive in both wells; g, h, and i, Negative (no antibody specific to platelet or HLA).

Positive in both wells (P-P pattern) of 2 samples with a difference in antibody combination. A, Sample 293. B, Sample 187. a, Cell control; b, Negative control; c, Positive control.
cases requires techniques to detect platelet-specific and common alloantibodies. Most reference laboratories now use a combination of a sensitive screening assay such as indirect immunofluorescence in flow cytometry assay along with a solid phase assay for antibody identification, usually either the monoclonal antibody captured ELISA or monoclonal antibody–specific immobilization of platelet antigens assay, as well as genotyping techniques. However, general blood banks and transfusion services can often not perform flow cytometry or other standard methods for detection of platelet antibodies, thus impeding the recognition of their occurrence. Simple, on-site detection methods are required to reduce this cause of thrombocytopenia.

SPRCA is a fast and simple method that many hospital laboratories use for platelet antibody screening and cross-matching; although one disadvantage of SPRCA is its insensitivity, the major limitation is a need for fresh intact platelets to coat microtiter plates. Moreover, the requirement that assays be repeated using chloroquine-treated platelets to rule out the presence of anti-HLA antibodies in case of a positive result puts an additional burden on the users of conventional SPRCA methods. Flow cytometry, while sensitive, lacks the ability to differentiate between pathologic and nonpathologic platelet-associated IgG (PAIG). Many patients with nonimmune thrombocytopenia also have high PAIG. In addition, thrombocytopenia in some patients with septicemia may be related to the binding of IgG to platelets.

The procedure we report bears some similarities to conventional SPRCA but adopts some specific methodological steps that make it more practical and more informative.

[Image 7] Positive in both wells (P-P pattern) of serum sample 293, which had only antiplatelet antibody. The serum sample was further investigated by the platelet suspension immunofluorescent technique (A) for antiplatelet antibodies and indirect immunofluorescence (B) using pooled normal human leukocytes for anti-HLA antibodies as mentioned in the “Materials and Methods” section.FITC, fluorescein isothiocyanate; Ig, immunoglobulin.

[Image 8] Positive in both wells (P-P pattern) of serum sample 187, which had antiplatelet and anti-HLA antibodies. The serum sample was further investigated by the platelet suspension immunofluorescent technique (A) for antiplatelet antibodies and indirect immunofluorescence (B) using pooled normal human leukocytes for anti-HLA antibodies as mentioned in the “Materials and Methods” section. FITC, fluorescein isothiocyanate; Ig, immunoglobulin.

[Image 9] Positive only in the nonadsorbed antiplatelet antibody well (P-N pattern) of 3 samples that had only anti-HLA antibody. a, Cell control. b, Negative control. c, Positive control.
Platelet lysate, prepared from pooled normal human platelets, is used in place of intact platelets. This diminishes the need for fresh platelets and also reduces the time and cost associated with repeated collection, preparation, and preservation of platelets. Moreover, the use of pooled platelet lysate also increases the number of platelet antigens in the reaction well. Rabbit antihuman platelet antibodies were prepared that were free of anti-HLA and other nonspecific antibodies that could interfere with the assay. Various platelet antigens were captured onto microtiter plates as a result of the multiple specificities of this antiplatelet antibody. The agglutination pattern of sensitized RBCs thus corresponds to the presence of different antibodies in the serum sample, especially antiplatelet, anti-HLA, and antiplatelet plus anti-HLA antibodies.

The pooled leukocytes were prepared from the same 5 donors whose platelets were used to immunize the rabbits. Leukocytes were prepared by Ficoll-Hypaque gradient centrifugation (PBMCs were obtained) following with 6% dextran sedimentation (granulocytes were obtained); therefore, the preparation included lymphocytes (T, B, NK cell, and monocytes) and all cell types of granulocytes (polymorphonuclear neutrophils, eosinophils, and basophils). Because leukocytes were from the same source as platelets, alloantibodies and autoantibodies could be distinguished.

We compared PSIFT and indirect immunofluorescence to be thorough because these 2 techniques are sensitive, and we attempted to show that our test was as sensitive as more expensive methods. Commonly found HLAs on platelet membranes include HLA-A, HLA-Bw4, and HLA-Bw6, and no HLA class II. While platelet-specific antigen is divided into 15 types, HPA-1a occurs in 99% of the population in Western countries (Europe and the United States) and Asia. From incidence reports, the antiplatelet antibodies in patients (if produced) are anti–HPA-1a and, in rare cases, anti–HPA-3 or anti–HPA-4 (often found in neonatal alloimmune thrombocytopenia). Furthermore, the antibodies in thrombocytopenic patients (including alloantibodies and autoantibodies) are anti-HLA. The 5 donors who donated serum samples had blood group O, Rh+, and, thus, there were no problems with blood group antigens. Standard SPRCA uses only 3 donors of blood group O to prepare intact platelets.

Agglutination in both wells indicates the presence of antiplatelet antibodies or a mixture of antiplatelet and anti-HLA antibodies in certain samples. We performed PSIFT as a reference method and investigated the presence of anti-HLA antibodies by indirect immunofluorescence testing using pooled human leukocytes. The results of our M-SPRCA correlated well with the reference method (κ = 0.90; 95% confidence interval, 0.89-0.94). No false-positives were observed in the normal samples that were tested. The pattern of 2-wells positive (P-P) could not be used to identify the specificity of antibody. However, it was a useful screening method for the antiplatelet antibody in patient serum samples. A 1-well positive pattern (P-N) indicates the presence of only anti-HLA in the serum sample. There were 3 cases from patients with idiopathic thrombocytopenia that showed the P-N pattern. These samples were confirmed by PSIFT and immunofluorescence with pooled normal leukocytes. With M-SPRCA, all samples showed the same pattern of weakly positive staining for platelets but strongly positive staining for leukocytes.

Of 361 clinical samples evaluated, 240 (66.5%) were positive. M-SPRCA indicated that patients with thrombocytopenia had antiplatelet antibodies (61.2%; range, 48%-74%; mean, 64.3%). The number of patients with both antiplatelet and anti-HLA antibodies was 37.5% (range, 16%-52%; mean, 34.1%), consistent with previous reports. More interesting, there were 3 cases of idiopathic thrombocytopenia (1.3%) that could be investigated and confirmed to have only anti-HLA by this modified technique.

By using M-SPRCA, we demonstrated the presence of antiplatelet antibodies in the serum samples of patients with various diagnoses and experiencing thrombocytopenia and for whom platelets had been requested. The advantages of M-SPRCA are the following: (1) Owing to its high sensitivity compared with conventional SPRCA, smaller blood samples are required from thrombocytopenic patients. The assay could be adapted easily to use as little as 50 µL of serum per assay. (2) Microtiter plates could be selectively coated with specific rabbit antiplatelet antibodies and stored at −70°C for as long as
as needed. (3) There was no need for freshly prepared intact platelets. (4) M-SPRCA could differentiate anti-HLA antibodies in a 1-step assay, overcoming the inconvenience of the conventional multistep SPRCA. The accuracy, feasibility, and simplicity of the M-SPRCA, if adopted, might provide a reduction in the unexpected high frequency of antiplatelet antibodies as a cause of thrombocytopenia and the poor response to platelet transfusions.

In the present study, we have tried to compare methods available in a general laboratory. There are many methods used to verify anti-HLA, such as the lymphocytotoxicity test. Although this test is better than ELISA, it requires many types of positive antibody control samples. ELISA is sensitive but requires every type of HLA to be coated onto microtiter plates, and there are always many molecules present that cross-react with the proteins in the serum samples and cause interference. Moreover, ELISA is very time-consuming and much more expensive because of HLA diversity. Currently, there are no products available that cover all this diversity and allow clinicians to screen and detect antibodies in serum samples so that patients can be treated correctly.

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