Detection of Platelet-Directed Immunoglobulin G in Sera Using the Peroxidase-Anti-Peroxidase (PAP) Slide Technique

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An immunohistochemical procedure for the detection of immunoglobulin G adherent to platelets is described. The peroxidase anti-peroxidase method is used to detect antibody activity directed against platelets from normal donors in the sera from 305 individuals. These subjects were divided into three groups: group 1, patients referred for tissue typing; group 2, healthy normal females; group 3, healthy normal males. In group 1, 28% of the sera were found to be positive; in most of these a history of prior transfusions was obtained. In group 2, 7.4% were found to be positive, most having previous pregnancies. Only 1% were found to be positive in group 3, and no reason for presensitization was found. Results from the indirect immunofluorescence technique served as a control and as a means to compare the sensitivity. Under the conditions chosen, the peroxidase anti-peroxidase test was two to eight times more sensitive than the immunofluorescence technique. Specificity of the peroxidase anti-peroxidase technique was demonstrated using a monospecific anti-PLA1 antiserum. It is concluded that the peroxidase anti-peroxidase slide technique may be a useful tool in the study of platelet-related immunophenomena.

Materials and Methods

Collection of Sera and Cells

Sera

Serum samples from 305 individuals were obtained and stored at −20°C for 1-36 mo, then separated retrospectively into 3 groups for evaluation. Sera from 61 patients referred for tissue typing and crossmatch procedures comprise group 1. Sera from 148 healthy female volunteers or relatives of the patients comprise group 2, and sera from 96 healthy male volunteers or male relatives of the patients are included in group 3. Sera from one patient with the clinical diagnosis of posttransfusion purpura were obtained at the time of her presentation and 9 mo later. This patient lacked platelet-specific antigen PLA1, and her serum from the time of presentation showed strong antiplatelet antibody activity as well as cytotoxic antibodies directed against HLA-A2.

Cell Preparation

Whole blood was collected in EDTA from three nontransfused male volunteers (blood group O) and from one patient with confirmed absence of PLA1 (blood group A) as described above. An initial centrifugation at 1000 g for 9 min was used to prepare platelet-rich plasma (PRP). Sodium azide (0.1%) was added to the PRP if it was necessary to delay the completion of the experiment. The PRP was then centrifuged in a Beckman microfuge-B for 2.5 min at approximately 10,000 g to form a pellet. The pellet was washed in EDTA-NKH buffer 5 times. EDTA-NKH buffer contained per 1000 ml 7.9 g NaCl, 0.4 g KCl, 3 g disodium EDTA, and 1 ml 1 M HEPES (Ultrol grade, Calbiochem-Behring Corp., La Jolla, Calif.) and was adjusted to pH 7.4 using 1 N NaOH. Finally, the platelets were resuspended in the same buffer and adjusted to a concentration of 30,000-100,000 cells/cu ml.

Immunocytochemical Staining

Slide Preparation

All further procedures were carried out in a wet chamber. Glass slides (size: 25 x 75 mm) were coated with a compound that repels aqueous solutions and protein-containing fluids (Rain-X, Unelco Corp., Chicago, Ill.). Twelve wells with a diameter of about 4 mm were prepared on each slide by application of a droplet of concentrated H2SO4 followed by 1 drop of 50% NaOH. The slides were washed after 5 min with distilled water and dried. A drop of poly-l-lysine (PLL) (Sigma Chemicals, St. Louis, Mo., MG 32,000, 100 μg/ml NKH buffer) was applied to each well. After 30 min at room temperature, the slides were washed with NKH buffer, and 10 μl of cell suspension was added to each well. The slides were incubated at room temperature for 1 hr to allow the cells to adhere to the glass. To reduce background staining, 10 μl of buffer containing 0.1% gelatin (J.T. Baker Chemical Co., Phillipsburg, N.J.) was then added to each well and incubated for 30 min. This 0.1% gelatin buffer was used for washing and dilutions of antisera as necessary.

PAP Assay

Wells were washed separately by dropwise addition of buffer followed by removal of buffer by suction. Thorough washing was
necessary after each of the following incubation steps. The optimal dilution and incubation time had to be determined for each antisera at each of the different steps. The best results were obtained by the following procedure:

Step 1: Incubation with human sera (serial dilution) for 45 min at room temperature.

Step 2: Incubation with rabbit anti-human IgG (dilution 1:50, Fc fragment, heavy chain specific, obtained from Cappel Laboratories, Cochranville, Pa.) for 30 min on ice. In the comparison study with the IF technique, fluorescein-conjugated goat anti-human IgG (Meloy Laboratories, Springfield, Va.) was used.

Step 3: Incubation with the PAP immune complex (Cappel Labs) for 30 min at 4°C. Rabbit anti-IgG (Cappel Labs) was substituted in the comparison study.

Step 4: Incubation with the PAP immune complex (Cappel Labs) produced in rabbit (standard) or produced in goat (comparison study), for 30 min at 4°C.

Step 5: Staining with diaminobenzidine (DAB) (Fluka, Buchs, Switzerland); staining (0.05% DAB, 0.001% H2O2 in 0.05 M Tris-HCl-0.13 M NaCl pH 7.5) for 10 min under constant shaking at room temperature.

Step 6: Fixation with 2% OsO4 (Sigma Chemical Co.) (in distilled water) for 20 min in the dark.

Step 7: The slides were covered with 75% glycerol 0.1% glutaraldehyde and evaluated under a light microscope at 400- and 1000-fold magnification.

Indirect Immunofluorescence Assay

Slide preparation, attachment of the cells, and incubation with human sera was performed as described above. This was followed by incubation with fluorescein conjugated anti-human IgG (Meloy Laboratories) for 60 min at 4°C, followed by washing with buffer. Finally, the slides were covered with 50% glycerol and a coverglass and were examined with a Leitz Ortholux II microscope (reflected light illumination system, Mercury lamp HP 200, blue light excitation filter KP 490 and barrier filter K510 or K530). The results obtained from the immunofluorescence (IF) procedure were used as a control and to compare the sensitivity of the two techniques.

Characterization of Antibody

A complement-dependent lymphocytotoxicity (CDL) assay was performed as previously described21 to determine cytotoxic antibodies in sera with antiplatelet antibody activity using the lymphocytes from the platelet donors. In the sera from the patient with PTP (Table 1, 517) absorptions were made similarly to previously described methods21 to determine platelet specificity of this serum. One-hundred microliters of serum were incubated with 70 x 10⁶ lymphocytes (platelet depleted by thrombin treatment) for 90 min at 37°C. Eutions were performed by adding 0.1 M glycine buffer pH 3.0 to the cell buffer and readjusting the pH with 0.1 M phosphate buffer to pH 7.4.

Criteria of Specificity

Antisera

The specificity of the sera used in the PAP technique has been determined by the commercial supplier by immune electrophoresis. The specificity of the fluorescein-conjugated antisera has been determined by radial immunodiffusion by the supplier.

Staining Sequence

Controls were carried out using buffer or autologous serum as in step 1 and the sequential omission of the sera used in the different steps. A positive reaction was observed only when all steps were included and homologous sera was used.

Technical Observations

Longer incubation times or incubation at 37°C did not influence the reactivity in the IF and PAP procedure. We noticed a correlation between cell concentration and serum dilution, and a lower cell concentration can be used if weak or equivocal results are obtained. Storage of platelet-rich plasma results in an increasing number of dark cells that cannot be evaluated. No significant increase in sensitivity was noticed by pretreatment with proteolytic enzymes (pronase, bromelain, 100 µg/ml for 30 min at room temperature).23 Gel-filtered platelets24 and centrifuged platelets were not different in their reactivity in the IF and PAP. We felt that washing the platelets by centrifugation is a faster and more convenient way.

RESULTS

An example of a positive reaction using the PAP slide technique is demonstrated in Fig. 1A. A dark ring around the cell membrane is evidence of IgG attachment. A frequently observed phenomenon is the swelling of platelets under the influence of antibody-containing sera. Figure 1B represents surface IgG that cannot be removed by washing procedures described above. Figure 1C shows a negative reaction as a result of incubation with PAP only.

Antibody activity is defined as a positive reaction against blood group O platelets in the IF and/or PAP technique. In 17 (28%) of 61 patients in group 1, antibody activity against the platelets from our 3 panel members was found (Table 1). Prior transfusions, response to platelet transfusion therapy (determined by platelet counts 4 hr after transfusion and by the cessation of bleeding), presence of cytotoxic antibodies, and the serial dilution titers were also included in Table 1. Eleven (7.4%) of the 148 sera in group 2 showed platelet antibody activity. Of these 11 women with positive sera, 10 had previous pregnancies (Table 2) and no history was given for the other women. In the control group 3, 96 sera derived from healthy males were studied. Only one was found to be positive, and no cause for presensitization was found in this individual. Tables 1 and 2 compare the serial dilution titers found in the IF and PAP technique, suggesting that the PAP is at least as sensitive as the IF and at times is 2–8 titer steps more sensitive.

Table 3 illustrates the work-up of the antiplatelet antibody containing sera from a patient with post-transfusion purpura. It compares the results obtained from a platelet ⁵¹Cr release assay with the results from indirect IF and PAP technique performed in our laboratory. Positive reactions were found at dilutions of 1/60 in the ⁵¹Cr release assay using bromelain pretreated platelets, whereas positivity is found in the indirect IF at dilutions of 1/256 and in the PAP at a dilution of 1/2048. In a serum sample obtained 9 mo later, residual antibody activity was detected in the
PAP TECHNIQUE FOR PLATELET IgG DETECTION

31Cr release assay when tested undiluted, in the IF at a dilution of $1/4$, and in the PAP at a dilution of $1/4$. In the serum obtained at the time of this patient's clinical presentation, cytotoxic antibodies directed against HLA-A2 were found and absorbed. After absorption, there was remaining antiplatelet antibody activity in the PAP and IF. The eluate showed anti-HLA-A2 activity and weak antiplatelet antibody reaction presumably due to platelet contamination of the lymphocyte suspension used for absorption.

<table>
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<tr>
<th>Code No.</th>
<th>PAP</th>
<th>IF</th>
<th>Cytotoxic Antibodies</th>
<th>Diagnosis</th>
<th>Prior Transfusions</th>
<th>Platelet Transfusion Response</th>
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AL, acute leukemia; AA, aplastic anemia; CML, chronic myelogenous leukemia; SA, sideroblastic anemia; PTP, posttransfusion purpura; RBC, red blood cells; PLT, platelets; D.Pos, doubtful positive.

Seventeen of sixty-one patients were found to have platelet-directed IgG. The strength of PAP, IF, and CDL reactions are shown in columns 1-3. History of prior transfusions and platelet transfusion response are included.
The strength of the reactions with the various techniques is shown in the table for clinical application to the problem of platelet matching. J Clin G adherent to normal platelets that cannot be removed by aggregometry in selection of compatible platelet donors. N Engl J Med 290:989, 1974

Eleven of 96 healthy females were found to have platelet-directed IgG. The information on previous pregnancies is included. When normal platelets were incubated with different sera, a high incidence of PAP and IF positivity was found in groups 1 and 2, with possible presensitization, and a low incidence of positive reactions in group 3, where presensitization was very unlikely.

We noticed a strong correlation when comparing IF and PAP. Results suggest a similar sensitivity and in some sera, a 2–8 times higher sensitivity for the PAP technique. Specificity of this procedure was checked with an antiserum known to be specific for PL^A^ antigen. According to our preliminary data, some patients with elevated serum IgG adherent to normal platelets show poor response to platelet transfusion therapy (Table 1). Unfortunately, we found positivity with all platelets from our volunteer donor panel, suggesting multispecificity and/or very common antigens, which the presensitized patients were lacking. However, the clinical application in platelet transfusion therapy has yet to be determined. In patients with immune thrombocytopenia, the correlation between serum antiplatelet antibody activity and the clinical response to therapy has been shown to be poor in contrast to IgG levels on circulating platelets. Further investigation is therefore necessary to determine the value of the PAP technique in detecting platelet-associated IgG in various immune-related platelet disorders.

We conclude that the previously described unlabeled immunoenzyme method is applicable to human platelets. Advantages of this slide technique are that (1) small amounts of antisera are needed, (2) all reagents are commercially available, (3) use of light microscopy and, thus, simultaneous visualization of the platelet morphology, (4) higher sensitivity than IF and ^5^Cr release assay under certain conditions, and (5) stability of stain for later evaluation.

The quantitative photometric evaluation of peroxidase-labeled antibody has been reported recently.25-27 Our results with a preliminary test suggest that the PAP slide technique described here may also be suitable for spectrophotometric quantitation.

**DISCUSSION**

An assay is described that detects immunoglobulin G adherent to normal platelets that cannot be removed by the washing procedure outlined above (Fig. 1B).

**REFERENCES**


10. Steffen C: Results obtained with the antiglobulin consum-
tion test and investigations of autoantibody eluted in immunohematology. J Lab Clin Med 55:9, 1960
Detection of platelet-directed immunoglobulin G in sera using the peroxidase-anti-peroxidase (PAP) slide technique

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