Monoclonal Antibody-Specific Immobilization of Platelet Antigens (MAIPA): A New Tool for the Identification of Platelet-Reactive Antibodies

By V. Kiefel, S. Santos, M. Weisheit, and C. Müeller-Eckhardt

The analysis of sera containing different platelet-reactive antibodies, e.g., autoantibodies, platelet-specific alloantibodies like anti-P²⁺, -P²⁺⁻, -Bak, and HLA antibodies, is still difficult. Recently, monoclonal antibodies against major platelet membrane constituents (glycoproteins IIb/IIIa and Ib and HLA class I molecule) have become available. In this report we describe a new assay that takes advantage of these highly specific reagents to investigate selectively platelet reactive antibodies against epitopes on different glycoproteins. The reliability and specificity of this assay is demonstrated with known platelet-reactive autoantibodies and alloantibodies (anti-P²⁺, -Bak, -Pen). The discovery of a P²⁺ antibody in a serum of a polytransfused patient underscores the efficiency of this technique. Possible applications of this assay are discussed in detail.

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MATERIALS AND METHODS

Human antibodies. Six human sera containing various platelet-reactive antibodies were selected for this study. Their origin and predetermined specificities are listed in Table 1. All sera had been prescreened against lymphocytes and platelets from a large panel of donors with known HLA, PL(1/2), and Bak antibodies by the lymphocyte-toxicity test (LCT), the platelet complement fixation test (PCFT), and the platelet adhesion immunofluorescence test (PAIFT).

Monoclonal antibodies. The following murine monoclonal antibodies (MoAb) were used: MoAb Gi5, raised and characterized in our laboratory, directed against the human platelet GP Ib/IIa complex; MoAb FMC25, a gift from Dr Zola, Adelaide, Australia, specific for GP IX; a glycoprotein tightly complexed to GP Ib; and MoAb w6.32 specific for a monomorphic epitope on the heavy chain of HLA class I molecules.

MoAb-specific immobilization of platelet antigens (MAIPA). Platelets were isolated by differential centrifugation from EDTA-anticoagulated blood and washed three times. A volume of washed platelets, stored at 4°C for at least 12 hours in isotonic saline containing 0.1% sodium azide, was pelleted to give a total of 1 x 10⁸ platelets. The pellet was resuspended in 50 μL phosphate-buffered saline (PBS), pH 7.2, supplemented with 2% bovine serum albumin (BSA). Ten to 40 μL of a platelet glycoprotein-specific MoAb diluted at a concentration of 0.02 mg/mL in PBS-BSA, and 5 to 200 μL of MoAb-specific immobilization of platelet antigens (MAIPA). Platelets were isolated by differential centrifugation from EDTA-anticoagulated blood and washed three times. A volume of washed platelets, stored at 4°C for at least 12 hours in isotonic saline containing 0.1% sodium azide, was pelleted to give a total of 1 x 10⁸ platelets. The pellet was resuspended in 50 μL phosphate-buffered saline (PBS), pH 7.2, supplemented with 2% bovine serum albumin (BSA). Ten to 40 μL of a platelet glycoprotein-specific MoAb diluted at a concentration of 0.02 mg/mL in PBS-BSA, and 5 to 200 μL of the serum to be investigated was added. The mixture was incubated at 37°C for 30 minutes. The platelets were then washed three times in isotonic saline and solubilized in 100 μL of 0.01 mol/L Tris-buffered saline (TBS) containing 0.5% Nonidet P40 for 30 minutes at 4°C. Then all samples were centrifuged at 15,000 g for 30 minutes at 4°C. Fifty microliters of the supernatants was diluted 1:5 in TBS wash buffer (TBS with 0.5% Nonidet P40, 0.05% Tween 20, and 0.5 mmol/L CaCl₂). One hundred microliters of the respective diluted supernatants was added to each well of a multwell plate (Greiner, Nürttigende, FRG) that had been coated with 100 μL goat antimouse IgG (Dianova, Hamburg, FRG; final antibody concentration, 3 μg/mL in 0.05 mol/L carbonate buffer) by overnight incubation at 4°C, washed, and blocked for 15 minutes at 4°C with 200 μL PBS wash buffer per well. The trays were incubated for 90 minutes at 4°C and washed four times, and 100 μL alkaline phosphatase-labeled goat antihuman IgG (Fc) (Dianova) diluted 1:5,000 in TBS wash buffer was added. After incubation for 120 minutes at 4°C the tray was washed six times, and 100 μL substrate solution (paranitrophenylphosphate in diethanolamine buffer, pH 9.8) was added. The color reaction was stopped after 30 minutes with 3 N NaOH and read at 405 nm in a Titertek photometer. All tests were run in duplicate. Results were expressed as ΔE values, i.e., the difference of extinction (optical density) values between the mean of test samples and blanks (wells devoid of platelet lysate). A positive color reaction indicates a reaction of a human antibody with an epitope on the same molecule recognized by the MoAb. Reactions with other membrane constituents give no visible signal.

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PLATELET ANTIBODY TESTING WITH MoAbs

Table 1. Clinical and Serological Data of Patients With Platelet-Reactive Antibodies

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Clinical Condition of Serum Donor</th>
<th>Antibody Specificity</th>
<th>Titers</th>
<th>LCT</th>
<th>PCFT</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Posttransfusion purpura</td>
<td>Anti-PIAl( Zw)</td>
<td>4/5*</td>
<td>1/29* neg</td>
<td>PIAl-negative</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Healthy mother of child with NIT</td>
<td>Anti-HLA (polyspecific)</td>
<td>5/7*</td>
<td>22/25* 7/19*</td>
<td>Mother, PIAl-positive</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Autoimmune thrombocytopenia</td>
<td>Autoantibodies</td>
<td>5/5*</td>
<td>neg</td>
<td>neg</td>
<td>Antigenic determinant on GP lb (immunoblotting)</td>
</tr>
<tr>
<td>4</td>
<td>Healthy mother of child with NIT</td>
<td>Anti-Pen</td>
<td>7/7*</td>
<td>neg</td>
<td>nt</td>
<td>Kindly provided by Dr R.H. Aster (Milwaukee)</td>
</tr>
<tr>
<td>5</td>
<td>Posttransfusion purpura</td>
<td>Anti-Bak + anti-HLA</td>
<td>1:32†</td>
<td>22/30* neg</td>
<td>Kindly provided by Dr A. Waters (London)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Malignant mastocytosis; multiple</td>
<td>Anti-HLA</td>
<td>5/5*</td>
<td>29/29*</td>
<td>Complement PIAl antigen-positive; RBC antibodies anti-inhibition Jk&lt;sup&gt;-&lt;/sup&gt;, C&lt;sup&gt;-&lt;/sup&gt;, S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>blood transfusions</td>
<td>(polyspecific)</td>
<td>1:128†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sera were characterized with PAIFT, LCT, and PCFT.
Abbreviations: nt, not tested; neg, negative; RBC, red blood cells.
* Number of positive cells per number of cells tested.
† Titer when tested with Bak<sup>-</sup>-negative cells.
§ Titer when tested with Bak<sup>+</sup>-positive cells.

Inhibition of calcium-dependent proteases in platelet lysates has no effect on antibody assessment. In experiments using leupeptin and sera no. 1 and 3, the results with platelets from four donors were identical whether platelets were lysed in the presence (1 mg/mL) or absence of leupeptin.

The intraassay and interassay reproducibility of results is very satisfactory. Representative figures (± 1 SD) for an antibody-containing serum compared with a negative control serum with platelets from the same donor were as follows: intraassay variability, 0.913 ± 0.041 v 0.03 ± 0.017 (n = 8); and interassay variability, 1.426 ± 0.240 v 0.06 ± 0.015 (n = 8).

RESULTS

Glycoprotein assignment of platelet antibodies. In a first series of investigations, the antigenic determinants on different platelet membrane molecules of operationally "monospecific" platelet antibodies were evaluated. The results of representative experiments are illustrated in Figs 1 and 2. When serum 1 containing pure PIAl antibodies was assessed against a panel of platelets with different PIAl/A2 types, it reacted only with determinants on GP IIb/IIIa complexes of PIAl-positive but not PIAl-negative platelets if MoAb Gi5 was used for immobilization. No reactivity was noted with molecules carrying HLA determinants immobilized with MoAb w6.32 (Fig 1). In contrast, serum 2, which solely held multispecific HLA antibodies, was strongly positive with HLA determinants immobilized by MoAb w6.32, irrespective of the PL<sup>A</sup> type of panel platelets used. There was no reactivity with GP IIb/IIIa determinants.

In Fig 2, the discriminatory capacity of the MAIPA with regard to epitopes on different platelet glycoproteins is demonstrated. Three platelet-specific antibodies (sera 1, 3, and 4) were assayed against antigens either on the GP IIb/IIIa complex or on the GP Ib complex, which were immobilized by MoAbs Gi5 or FMC25, respectively. Although serum 1 (known PIAl antibodies) reacted only with determinants of PIAl-positive donors on the GP IIb/IIIa complex but not on the GP Ib complex, the reverse pattern was seen with serum 3. Immunoblotting studies had shown
that this serum contained only platelet autoantibodies reacting with GP Ibα specificity.12 This was confirmed by MAIPA. Serum 4 (kindly provided by Dr R.H. Aster, Milwaukee) had Pen antibodies that reacted in PAIFT with a high-frequency platelet antigen. It detected antigenic determinants present on the GP Ibβ/IIa complex (immobilized by MoAb Gi5) but not on the GP Ib complex (immobilized by FMC29), which confirmed the results of Furuhata et al.3

Dissection of mixtures of platelet-reactive antibodies. Two typical examples are depicted in Fig 3. Serum 5, a gift from Professor A. Waters (London), was known to contain high-titered multispecific HLA antibodies (positive with 29/29 panel cells in LCT) and, in addition, Bak antibodies. In PAIFT, no definite distinction of these two types of antibodies was possible at lower dilutions. When using the MAIPA, both antibody specificities were clearly discernible: the Bak antibodies reacted in an antigen-specific fashion with their epitope on GP Ibβ/IIa immobilized by MoAb Gi5, whereas the HLA antibodies were positive with MoAb w6.32–immobilized HLA antigens.

Serum 6 was unusual in that it so far had been considered to contain only strong multispecific HLA antibodies. On MAIPA analysis, an additional PtA2 antibody was detected that reacted with MoAb Gi5–immobilized GP Ibβ/IIa determinants in a PtA2–specific pattern. The PtA2 specificity was further confirmed in dosage determinations by using platelets of PtA2 homozygous and PtA1A2 heterozygous donors (data not shown).

DISCUSSION

The identification of platelet-reactive antibodies is still a notoriously difficult task. This is particularly due to the fact that most sera contain mixtures of antibodies. Because the majority of patients with immune platelet disorders are women and have been pregnant and/or received blood or platelet transfusions, they are often immunized against HLA-A, -B antigens and possibly other as yet unidentified antigens are often destroyed by sodium dodecyl sulfate treatment of the platelet lysate. This is corroborated by the observation that some monoclonal platelet antibodies fail to bind to immunoblots. Moreover, the use of panels of several solubilized platelet suspensions in immunoblotting is laborious and time-consuming, and its results are difficult to interpret.

The microtiter assay described by Woods et al8,9 used MoAbs fixed to a solid phase. It allowed us to demonstrate platelet autoantigens on GP Ibβ/IIa or GP Ib, respectively. However, in our experience this assay is rather insensitive, most likely as a consequence of low binding ratios. Even strong PtA1 antibodies yielded rather low binding ratios that were apparently caused by high background activity due to unspecific adsorption to the solid phase of components to be investigated.

![Fig 3. Analysis of sera containing mixtures of platelet-specific alloantibodies and HLA alloantibodies with a panel of known donor platelets.](image-url)

When using MoAb Gi5 for immobilization, platelet alloantibodies anti-Bak (A) and anti-PtA2 (anti-Zw) (B) were detected through their typical pattern of reactivity, whereas the antigens immobilized by MoAb w6.32 carried HLA alloantibodies present in the same samples.
Antibody detection by MAIPA as described here circumvents most of these difficulties. It is based on the detection of trimolecular complexes formed by a MoAb, the human platelet-reactive antibody, and a platelet membrane molecule carrying the respective epitopes of either antibodies. Because antibody binding of both the monoclonal and the human antibody occurs on the surface of the intact platelet before solubilization, all antigens are preserved. Washing platelets after the sensitization phase removes excess MoAb as well as unbound serum. This ensures specific immobilization of the MoAb-platelet antigen complexes to the solid phase as well as specific binding of the labeled antibody to the human immunoglobulins associated with them. Hence, the background is usually low, and therefore, the specific binding values (difference between test and blank sample) are high. By using a set of MoAbs for different membrane constituents and a platelet panel with known alloantigens, platelet-reactive antibodies can be characterized in a single experiment with regard to both localization of the epitopes (eg, HLA class I molecule, GP IIb/IIIa complex, GP Ib complex, ie, GP Ib and GP IX) and the serological specificities. A possible false-negative result might be expected if monoclonal and human antibody have the same or a closely adjacent epitope for which they compete and therefore mutually hinder their binding. In these cases a MoAb directed against a different epitope on the same molecule is recommended. However, we have not observed such an interference between MoAb Gi5 and the platelet alloantigens tested thus far (Pl\textsuperscript{A1}, Pl\textsuperscript{A2}, Bak\textsuperscript{*} (Lek\textsuperscript{*}), Pen, Yuk\textsuperscript{*}, Yuk\textsuperscript{b}). Other advantages of this assay relate to its simplicity: unfixed platelets may be stored for weeks at 4\textdegree C without a loss of reactivity. Performance of the assay only requires six to seven hours. No sophisticated equipment is necessary.

The method we have described is a practical and sensitive tool for detailed analysis of sera with ambiguous serological findings. It allows reliable typing of donors for platelet alloantigens (eg, Pl\textsuperscript{A1} (Zw\textsuperscript{*}), Pl\textsuperscript{A2} (Zw\textsuperscript{b}), Bak\textsuperscript{*} (Lek\textsuperscript{*}), Pen) even with those sera that do not discriminate between negative and positive cells in the platelet immunofluorescence test due to HLA antibodies. Moreover, MAIPA will be a suitable method for platelet compatibility testing before platelet transfusion because it gives information about both HLA-specific (including noncytotoxic) and platelet-specific alloantibodies.

This assay takes advantage of the unique properties of MoAbs for diagnostic purposes. We are convinced that it will allow us to elucidate many of the unclear findings so often encountered in platelet serology.

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