Development and Characterization of Monoclonal Antiplatelet Autoantibodies From Autoimmune Thrombocytopenic Purpura-Prone (NZW × BXSB)F1 Mice

By Hajime Mizutani, Robert W. Engelman, Yoshiyuki Kurata, Susumu Ikehara, and Robert A. Good

Male (NZW × BXSB)F1 (W/BF1) mice develop systemic autoimmunity involving autoantibodies, progressive thrombocytopenia, lupus nephritis, and degenerative coronary vascular disease with myocardial infarction. Platelet-associated IgG (PAIgG) on the platelet surface mediates platelet destruction by the reticuloendothelial system in the autoimmune thrombocytopenic purpura (ATP) of W/BF1 mice. Because the epitopes targeted in ATP by PAIgG have not been identifiable using serum from thrombocytopenic W/BF1 mice, we developed seven hybridomas secreting antiplatelet monoclonal antibodies (MoAbs) using splenocytes of thrombocytopenic W/BF1 mice. Epitopes recognized by three MoAbs were similar to those recognized by PAIgG, because eluted IgG from platelets of thrombocytopenic W/BF1 mice inhibited platelet binding by MoAbs in competitive micro–enzyme-linked immunosorbent assay. Hybridoma cells or purified Ig from the ascites of two clones (2A12 and 6A6), when injected into nude mice produced acute thrombocytopenia, elevated the levels of PAIgG, purpura, and megakaryocytosis. MoAbs of two clones also reacted with single-stranded DNA or double-stranded DNA, and one of these clones (4-13) bound to cardiolipin (CL) but was nonpathogenic in nude mice, suggesting that anti-CL and antiplatelet autoantibodies can be distinct. On immunoblotting analysis, antiplatelet MoAbs frequently bound a 100-Kd platelet protein. These MoAbs contribute to an understanding of the etiopathogenesis of ATP and the several antigens and autoantibodies involved.

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Materials and Methods

Animals. Male (NZW × BXSB)F1 mice and both female Balb/c and Balb/c nu/nu mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in AAALAC-accredited, specific pathogen-free conditions in accordance with Public Health Services/National Institutes of Health Publication No. 86-23.

Platelet counts. Blood (20 µL) from nude mice was diluted in buffer containing ammonium oxalate (Unopette kits; Becton Dickinson, Mountain View, CA). Platelets were counted using a hemacytometer under a phase-contrast microscope.

Platelet isolation. Whole blood from mice or humans was obtained in acid-citrate-dextrose (pH 4.5, 0.01 mol/L), as previously indicated this fact.

From the Department of Pediatrics, University of South Florida, All Children's Hospital, St Petersburg, FL; the Department of Blood Transfusion, Osaka University Hospital, Osaka, Japan; and the First Department of Pathology, Kansai Medical University, Osaka, Japan.

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Address reprint requests to Hajime Mizutani, MD, All Children's Hospital, 801 Sixth St S, St Petersburg, FL 33701.

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Platelets were isolated by differential centrifugation at 300g for 5 minutes to obtain platelet-rich plasma, and washed three times at 1,000g for 15 minutes. Contaminating red blood cells (RBCs) were removed by centrifugation at 100g for 5 minutes.

Preparation and screening of hybridomas. Spontaneous anti-platelet antibody-producing hybridomas were produced by fusing splenic mononuclear cells of 3-month-old, unimmunized, thrombocytopenic W/BF1 male mice with Sp2/0 murine myeloma cells (shown) did not.

Supernatants of hybridoma cultures were screened for PBIg by micro-ELISAs, whereas similar concentrations of platelet-unreactive MoAbs (⋯⋯) or polyclonal IgG or IgM (not shown) did not.

Table 1. Characteristics of Monoclonal Antiplatelet Autoantibodies

<table>
<thead>
<tr>
<th>Clone</th>
<th>RBC</th>
<th>MNC</th>
<th>PI</th>
<th>H PI</th>
<th>Inhibited† by Eluted PAlgG</th>
<th>Pathogenic in Nude Mice</th>
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<tbody>
<tr>
<td>2A12</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>4-13</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>6A6</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2B10</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>6C10</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>3H6</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* Binding to murine erythrocytes (RBC), splenic mononuclear cells (MNC), platelets (PI.), or human platelets (H PI.) was determined by competitive micro-ELISA.
† The addition of eluates of W/BF1 platelet-associated IgG (PAlgG) reduced platelet binding by MoAb in competitive micro-ELISA.

Results of platelet-unreactive MoAbs (⋯⋯) or polyclonal IgG or IgM (not shown) did not.

Fig 1. Platelet-reactive MoAbs (⋯⋯) bound to PFA-fixed platelet-coated wells in micro-ELISAs, whereas similar concentrations of platelet-unreactive MoAbs (⋯⋯) or polyclonal IgG or IgM (not shown) did not.

Fig 2. Binding of antiplatelet MoAbs to PFA-fixed murine platelet-coated wells is reduced in the presence of fresh murine platelets, as shown by competitive micro-ELISAs (symbols are the same as in Fig 1).

Purification of MoAbs. Ascites were developed by the intraperitoneal (IP) injection of hybridoma cells of PBIg-positive clones into male, 6- to 8-week-old Balb/c nu/nu mice. Antibodies of the IgG class were partially purified from ascites fluid by ammonium sulfate precipitation (50% saturation). The precipitate was dissolved in and dialyzed against 0.85% NaCl to remove residual ammonium sulfate. The IgM-containing euglobulin fraction was dialyzed against 2% borate buffer, dissolved in and dialyzed against saline, and filter sterilized. Antibodies of the IgG class were precipitated from ascites fluid with ammonium sulfate, adjusted to pH 8.0 using borate-buffered saline, and purified by affinity chromatography on a protein A-Sepharose column (Pierce, Rockford, IL). The optical absorbance was measured at 405 nm using a micro-ELISA auto-reader, MR 580 (Dynatech). Culture supernatant from nonreactive clones 3H6 (IgG isotype) or 2E10 (IgM isotype), which did not bind platelets, single-stranded (ss)-DNA, double-stranded (ds)-DNA, or cardiolipin (CL), were used as negative controls to detect nonspecific platelet binding. Polyclonal mouse IgG or IgM was also used in place of hybridoma culture supernatant as a negative control.

Hybridoma cells from cultures producing PBIg were cloned twice by limiting dilution. The isotype of the Ig secreted by PBIg-positive clones was determined using an isotyping kit (Amersham, Amersham, UK; RPN29).

Reactivity with fresh platelets, RBCs, and mononuclear cells (MNCs). Antibodies of PBIg-positive clones were assessed for reactivity with fresh human platelets or fresh murine platelets, RBCs, or splenic MNCs by PFA-fixed platelet micro-ELISA as described above, except that a 50 μL sample of either fresh platelets, RBCs, or
ANTIPLATELET MOABS FROM (NZW × BXSB) F, MICE

Fig 3. Additional reactivities of MoAbs with ss-DNA, ds-DNA, or CL and determination of Ig isotypes. The antiplatelet MoAb of clone 2A12 also bound ss-DNA and ds-DNA, and that of clone 4-13 bound ss-DNA, ds-DNA, and CL in EIA. Clones 3H6 and 2E10 secreted platelet-unreactive MoAbs.

<table>
<thead>
<tr>
<th>Mab</th>
<th>Isotype</th>
<th>ssDNA</th>
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<tr>
<td>2A12</td>
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<tr>
<td>2E10</td>
<td>IgM</td>
<td>K</td>
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</table>

Fig 4. The binding of antiplatelet IgG MoAb of clones 6A6, 4E7, and 2B10 to PFA-fixed murine platelet-coated wells is reduced in the presence of increasing concentrations of IgG eluted from W/BF1, platelets (---), but not in the presence of polyclonal IgG (----), as shown by competitive micro-ELISA. Data points represent the mean ± SD of three determinations. *P < .05.
Assessment of pathogenicity of MoAbs in nude mice. To test for pathogenicity, $2 \times 10^7$ hybridoma cells in 500 $\mu$L of physiologic saline were injected IP into three male, 6-week-old Balb/c nu/nu mice; nude mice were observed for the development of thrombocytopenia, PAIgG, purpura, and ascites. In addition, groups of 3 nude mice were injected intravenously (IV) with 200 $\mu$L of 50 $\mu$/mL purified ascites IgG developed from either platelet-reactive clones or platelet-unreactive clone 3H6 as a negative control; the mice were observed for the development of thrombocytopenia.

**EIA of platelet-associated IgG.** PAIgG was quantified in nude mice after an IP injection of PBIg-positive clones using a solid-phase competitive EIA according to the methods of Tsukabio et al., with minor modifications. In this assay, a platelet sample from an MoAb-treated nude mouse in 50 $\mu$L and 50 $\mu$L of 1:1,000-diluted ALP-conjugated goat antimouse IgG were added simultaneously to individual mouse IgG-coated wells. The mouse IgG and the variable amount of PAIgG compete for reaction with goat antimouse IgG. With increasing amounts of PAIgG, and after washing and discarding platelets and platelet-bound antisera, less goat antimouse IgG is retained in the mouse IgG-coated wells. The ALP reaction is developed and the optical absorbance is determined. Amounts of PAIgG in platelet samples were determined in triplicate. A standard curve was developed with known nanogram amounts of mouse IgG and was used for calculating PAIgG levels.

**Histologic analysis.** Formalin-fixed tissues were sectioned at 2 to 4 $\mu$m and stained with either hematoxylin and eosin or periodic acid Schiff.

**Platelet protein lysates.** Platelet proteins were prepared for immunoblotting by solubilizing washed, packed Balb/c platelets or the platelets of healthy human volunteers (blood type 0) in 1% Triton X-100 in 0.01 mol/L Tris-EDTA for 30 minutes at 4°C. Insoluble material was removed by centrifugation at 100,000g for 30 minutes and the proteins were stored at $-80^\circ$C.

**Immunoblot procedure.** Immunoblotting was performed using a modification of described methods. Briefly, platelet proteins were electrophoresed in the presence or absence of reducing agents on 8% polyacrylamide mini-gels and transferred to nitrocellulose (NC) paper (0.45 $\mu$m; BioRad, Richmond, CA) in 25 mmol/L phosphate buffer (pH 6.5) using a 100 V current for 1.5 hours. To block nonspecific binding, NC papers were incubated with 3% BSA in PBS-T for 16 hours at 4°C. Supernatants were incubated with NC papers for 2 hours at room temperature. Papers were washed three times with PBS-T and antiplatelet antibodies were detected by the avidin-biotin-peroxidase complex method using biotinylated antihuman IgG or IgM.

**Statistical analysis.** Statistical analysis of data was performed using the Mann-Whitney U test, with $P < .05$ considered significant.

**RESULTS**

Of the hybridomas produced by fusion of W/BF$_1$ spleenocytes and Sp2/0 cells, seven secreted MoAbs that bound to PFA-fixed murine platelets on micro-ELISA (Fig 1). Of the hybridomas that secreted other irrelevant MoAbs unreactive with platelets, two, clones 2E10 and 3H6, served as negative controls in all analyses. Minimal background binding of second antisera to platelet-coated wells was detected. Competitive binding of MoAbs for PFA-fixed platelets coated on wells and fresh platelets added to the wells was
Fig 6. Megakaryocytosis was evident in both the bone marrow (upper left) and spleen (upper right) in nude mice injected with hybridoma cells secreting pathogenic antiplatelet MoAb of either clone 6A6 (shown here) or 2A12, but not in the bone marrow or spleen of nude mice injected with cells of platelet-unreactive clone 3H6 (lower left and right) (stained with hematoxylin and eosin, original magnification X 200).

evaluated (Table 1). The addition of fresh platelets to wells reduced MoAb binding to PFA-fixed platelet-coated wells with each of the seven clones (Fig 2). Binding to platelet-coated wells by antibody of clone 4E7 was reduced when fresh murine MNCs were added (Table 1). Binding to platelet-coated wells by MoAbs of clones 2A12, 4-13, 2B10, and 6C10 was reduced in the presence of human platelets (Table 1). RBCs as competitor in the micro-ELISA did not diminish the binding to platelet-coated wells of MoAbs secreted by any of the seven clones. Antibodies of two of the seven clones, 2A12 and 6A6, were shown to be pathogenic, producing thrombocytopenia in Balb/c nu/nu mice, as described in detail below.

The reactivity of MoAbs with ss-DNA, ds-DNA, or CL was assessed by EIA and considered positive if the optical density of the tested MoAb exceeded the optical density of the negative control MoAb (clones 3H6 or 2E10) by more than three standard deviations (Fig 3). MoAbs secreted by two clones (2A12 and 4-13) were of the IgM κ isotype and each bound ss-DNA and ds-DNA. Antibody of clone 4-13 also bound CL. MoAbs of three clones (4E7, 4F10, and 6C10) were of the IgG κ isotype. MoAb of clone 2B10 was of the IgG κ isotype and MoAb of clone 6A6 was of the IgG1 κ isotype. None of these platelet-reactive MoAbs of IgG isoforms reacted with ss-DNA, ds-DNA, or CL.

Ig eluates from platelets of thrombocytopenic W/BF1 mice were prepared and used in a competitive micro-ELISA with three biotinylated MoAbs of the IgG isotype. Polyclonal IgG substituted for eluted IgG and was added to some platelet-coated wells as a control, but did not diminish platelet binding by platelet-reactive MoAbs (Fig 4). The addition of W/BF1 platelet IgG eluates at concentrations 10 μg/mL and greater significantly reduced the binding of MoAbs of clones 6A6, 4E7, and 2B10 to platelet-coated wells (P < .05), showing that these MoAbs shared platelet-binding sites with autoreactive PAIgG.

The pathogenicity of MoAbs was assessed by both the IP injection of 2 x 10⁷ hybridoma cells and the IV injection of 10 μg of purified ascites IgG into Balb/c nu/nu mice. All six MoAb-injected mice (n = 3/clone) became thrombocytopenic and exhibited purpura within 3 days after the injection of hybridoma cells of either clone 6A6 or 2A12 (Fig 5A). The levels of PAIgG increased 10-fold within 5 days after the IP injection of these hybridoma cells. Mice developed ascites after 5 days and died between 14 and 21 days after the injection of hybridoma cells. Marked megakaryocytosis was evident postmortem in the bone marrow and spleen of nude mice injected IP with 6A6 or 2A12 hybridomas (Fig 6). IP injection of hybridoma cells of the platelet-unreactive clone 3H6 was not pathogenic in nude mice. A comparable sequence of pathology, including marked thrombocytopenia, followed the IV injection of 10 μg of purified ascites IgG from clones 6A6 or 2A12 (Fig 5B), indicating that the development of thrombocytopenic purpura in hybridoma-injected mice was attributable to MoAb-mediated platelet destruction.

The binding of platelet antigens by MoAbs that were platelet-reactive and pathogenic or nonpathogenic in nude mice or that were platelet-unreactive was examined using either Balb/c or human platelet lysates in immunoblotting analyses. Blots developed with either platelet-reactive (Fig 7, lanes 1 through 3) or platelet-unreactive (Fig 7, lane 4) MoAbs of IgG isotypes showed nonspecific binding to 200-, 120-, and 58-Kd platelet proteins (Fig 7, open arrowheads). One of these bands (200 Kd) may have been attributable to the nonspecific reactivities of the biotinylated second anti-
Fig 7. Immunoblots showing the interaction of MoAbs with murine or human platelet proteins. Platelet lysates were separated by electrophoresis on an 8% SDS gel under nonreducing conditions and transferred to nitrocellulose strips. Lanes 1 through 5 and 9 through 12, lysates of murine platelets (M.Plt.); lanes 6 through 8, lysates of human platelets (H.Plt.); lanes 1 through 4, IgG MoAbs added; lane 5, biotinylated antimouse IgG only added; lanes 6 through 11, IgM MoAbs added; lane 12, biotinylated antimouse IgM only added. Nonspecific reactivities of IgG MoAbs (lanes 1 through 4) identifying platelet proteins of 200, 120, and 58 Kd are indicated by open arrowheads. Unique reactivities of antiplatelet MoAbs are indicated by filled arrowheads. Lanes 1 and 2, MoAbs of clones 6A6 and 6C10 recognized mouse platelet proteins of 100 and 43 Kd; lane 3, MoAb of clone 2B10 showed no specific platelet protein binding; lane 4, platelet-unreactive MoAb of clone 3H6; lane 6, MoAb of clone 2A12 bound a 100-Kd human platelet protein; lane 7, MoAb of clone 4-13 bound 100- and 60-Kd human platelet proteins; lane 8, platelet-unreactive MoAb of clone 2E10; lane 9, MoAb of clone 2A12 bound 100- and 43-Kd mouse platelet proteins; lane 10, MoAb of clone 4-13 bound 200-, 100-, and 60-Kd mouse platelet proteins; lane 11, MoAb of platelet-unreactive clone 2E10.

DISCUSSION

This report describes the development of the first monoclonal antiplatelet autoantibodies from ATP-prone W/BF1 mice. Epitopes targeted in ATP by PAIgG have not been identifiable using serum from thrombocytopenic W/BF1 mice in immunoblotting or immunoprecipitation analysis, hence necessitating the development of these MoAbs. Their development makes possible this initial description of the nature of the target antigens recognized by PAIgG in ATP-prone W/BF1 mice. Seven hybridomas that secrete antiplatelet MoAbs of either IgG or IgM isotypes were produced. Antibodies of each of these seven clones reacted with both PFA-fixed or fresh W/BF1 platelets, indicating that the epitopes recognized were not cryptic antigens that can be exposed or developed during platelet fixation.24

Competitive platelet binding between the IgG MoAbs and IgG eluted from platelets of thrombocytopenic W/BF1 mice shows that the platelet epitope(s) recognized by these MoAbs is similar to that recognized by pathogenic PAIgG. The development of acute thrombocytopenia and elevated levels of PAIgG, purpura, and megakaryocytosis in nude mice after the injection of either hybridoma cells or Ig purified from the ascites produced by each of two clones (2A12 and 6A6) shows that these MoAbs represent pathogenic PAIgG, and that PAIgG bound to the platelet surface is a critical mode of autoimmune platelet destruction in ATP. That some MoAbs competed with eluated IgG for binding of platelets, but were not pathogenic in nude mice (clones 2B10 and 4E7), may be attributed to reduced densities or diffuse as opposed to clustered distribution of the relevant antigenic determinants,25 or to differences in phagocytic efficiency of macrophages to engulf platelets coated with the MoAbs of different IgG isotypes.26 It is also possible that antiplatelet MoAbs of nonpathogenic clones can become pathogenic through synergism when combined in vivo, but this will require further investigation.
Antiphospholipid syndrome is characterized by the presence of anticardiolipin autoantibodies, thrombocytopenia, and thromboembolism.\textsuperscript{27} Monoclonal anticardiolipin autoantibodies, some of which also bind platelets, have been described in W/BF\textsubscript{1} mice and humans, but whether they induce thrombocytopenia has not been addressed.\textsuperscript{28,29} Only one of the seven MoAbs described here bound to CL (clone 4-13), and that MoAb did not induce thrombocytopenia in nude mice, suggesting that anti-CL and pathogenic anti-platelet autoantibodies can be distinct.

Autoantibodies can react with multiple antigens, as has been shown with human MoAbs established from patients with systemic lupus erythematosus (SLE), which react with both platelet surface antigens and DNA,\textsuperscript{30,31} and from patients with autoimmune thyroid disease, which cross-react with human thyroglobulin, microsomal antigen, and other nonthryroid antigens.\textsuperscript{32} MoAbs of our clone 2A12 reacted with antigen at multiple sites, perhaps due to shared antigenic structural motifs, because it not only bound platelet proteins of 100 and 43 Kd and induced thrombocytopenic purpura, but also bound ss-DNA and ds-DNA.

The nature of the target antigens recognized by W/BF\textsubscript{1} antplatelet MoAbs was further shown by immunoblotting analysis. IgM MoAbs commonly bound to a 100-Kd mouse platelet protein, MoAb of clone 2A12 also bound to a 43-Kd protein, and MoAb of clone 4-13 also bound 200- and 60-Kd platelet proteins, whereas IgG MoAbs bound to 100- and 43-Kd platelet proteins under both nonreducing and reducing conditions. Although similar in size to GP\textsubscript{II}a under nonreducing conditions, this protein's behavior under reducing conditions suggests that it is distinct from GP\textsubscript{II}a. The multiplicity of platelet antigens and antplatelet autoantibodies present in ATP is made evident by recent reports of autoantibodies in patients with ATP that bind platelet proteins of either 100 or 102 Kd molecular mass.\textsuperscript{23,32} The 100-Kd protein recognized by MoAbs in W/ BF\textsubscript{1} mice described here may be a platelet surface protein, the cytoplasmic domain of a platelet surface protein, or a cytoplasmic protein. Platelet cytoplasmic antigens are targeted by antplatelet antibodies in patients with either SLE or ATP\textsuperscript{33,34} and by human MoAbs developed from patients with ATP.\textsuperscript{35} The origin and function of the platelet proteins recognized by these MoAbs will be clarified in future investigations.

That MoAbs of other clones bound both PFA-fixed and fresh platelets, but did not bind any platelet protein on immunoblotting analysis, may be attributed to a conformational change in the targeted antigenic determinants during the preparation of platelet protein lysates with sodium dodecyl sulfate (SDS), or may suggest that these targeted antigens are present in platelet surface associated carbohydrate chains or are contained within three-dimensional structures.\textsuperscript{35}

The development and characterization of monoclonal antplatelet antibodies from W/BF\textsubscript{1} mice contributes to our understanding of the multiplicity of autoantibodies and targeted antigens involved in autoimmune disease, and ultimately contributes to development of a complete description of the etiopathogenesis of autoimmune thrombocytopenia.

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