A Human Monoclonal Autoantibody to Platelet Glycoprotein IIb Derived From Normal Human Lymphocytes

By Gregory A. Denomme, James W. Smith, John G. Kelton, and David A. Bell

Tonsillar lymphocytes from an otherwise healthy nonthrombocytopenic male child were fused with the lymphoblastoid cell line GM 4672. Twenty of 472 (4%) hybridomas had antiplatelet reactivity detected using intact platelets in an enzyme-linked immunosorbent assay. One hybridoma (STO 171) reacted to platelet glycoprotein IIb (integrin αIIb) as determined by radioligand binding and immunoblotting. Antibody specificity was confirmed using immunodepletion experiments with isotypic antibodies derived from a multitransfused Glanzmann's thrombasthenic patient. The antibody reactivity was restricted to platelets and did not react with other integrin α-chain proteins expressed on granulocytes or cultured human brain-derived microvascular endothelial cells. These studies indicate that lymphocytes of normal, nonthrombocytopenic individuals have the genetic potential to produce antiplatelet autoantibodies.

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DIOPATHIC thrombocytopenic purpura (ITP) is an autoimmune disease caused by autoantibodies against platelet-specific antigens leading to the immune destruction of sensitized platelets by the cells of the reticuloendothelial system. Since Harrington et al. showed that ITP was caused by a transferable plasma factor, considerable work has lead to a better understanding of the characteristics and the targets of the autoantibodies responsible for the disease. In most patients, it is an IgG autoantibody that reacts with platelet specific glycoproteins (GP). Recent studies have shown that the platelet target is often GP IIb/IIIa, the most abundant platelet proteins.

Although the antibody and antigenic characteristics have been well studied, the mechanism responsible for the autoimmune response to platelets is not known. Recent studies have shown that normal individuals have the genetic capacity to produce autoantibodies to a variety of antigens. These studies suggest that normal nonthrombocytopenic individuals might have, within their B-cell repertoire, the capacity to produce antiplatelet autoantibodies.

Therefore, we studied the capacity of normal tonsil lymphocytes to generate hybridoma antiplatelet antibodies when fused with the lymphoblastoid cell line GM 4672. The results of these experiments indicate that normal lymphocytes, like those of ITP, can produce antiplatelet antibodies against platelet GPIIb.

MATERIALS AND METHODS

Production of Human-Human Hybridomas

Tonsillar lymphocytes were obtained immediately after routine tonsillectomy from a nonthrombocytopenic 6-year-old male without a past history of ITP. Except for the tonsillitis, the child was well (complete blood cell count [CBC], normal; platelet count, 346 × 10^9/L) and did not have an autoimmune disorder. The postoperative convalescence was uneventful. Informed consent was obtained before the use of the tonsilar tissue. The human fusion partner was GM 4672 (Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ). Lymphocytes and the GM 4672 cell preparation, the fusion, and postfusion culture conditions were performed as described by Cairns et al. with the exceptions that a 1:1 cell fusion ratio was used with plating after fusion at 2 × 10^4 cells per well. Hypoxanthine-aminopterin-thymidine (HAT)-selected hybridomas were screened for antibody reactivity to human platelets by the enzyme-linked immunosorbent assay (ELISA) described subsequently. Cloning and subcloning was performed at one cell per well in hybridoma growth medium (HGM) containing 1% Nutridoma (Boehringer Mannheim Biochemicals, Montreal, Quebec, Canada).

IgM Quantitation

The IgM was quantitated by an ELISA. Polystyrene microtiter plates (Dynatech Laboratories Inc, Alexandria, VA), coated with 50 μL of goat antihuman IgM (10 μg/mL; Jackson ImmunoResearch Laboratories Inc, West Grove, PA) in carbonate buffer, were washed (0.1% bovine serum albumin [BSA], 100 mmol/L Tris-HCl, pH 7.4) and blocked with 2% BSA, 100 mmol/L Tris-HCl, pH 7.4. Diluted culture supernatant (50 μL) was added to antibody-coated and control wells (no-anti-IgM) in triplicate and incubated at 4°C overnight. A standard curve was made using pooled human IgM (Cooper Biomedical, West Chester, PA) with a final concentration ranging from 0.01 to 2.0 μg IgM/mL. The IgM was detected with alkaline phosphatase-conjugated goat antihuman IgM (Zymed Laboratories Inc, San Francisco, CA). The conjugated antibody was detected in the same manner as in the platelet ELISA. Supernatant IgM levels were determined by extrapolation from a semi-log dose-response curve of the IgM standards.

Performance of the ELISA

Platelets from healthy nonthrombocytopenic volunteers were isolated from whole blood collected 6:1 (vol:vol) in acid citrate dextrose (73 mmol/L citric acid, 85 mmol/L trisodium citrate, 110 mmol/L dextrose [ACD], pH 4.5). The platelets were resuspended at 3 × 10^11/mL in phosphate-buffered saline (PBS) after four washes with 10 mmol/L ethylene-diamine-tetra-acetic acid (EDTA) PBS, pH 6.5.

To perform the assay, 50 μL of washed platelets was added to PVC microtiter plates (Dynatech) and allowed to sediment overnight. The wells were washed with 0.1% BSA, 10 mmol/L Tris-HCl saline, pH 7.4, and blocked with 2% BSA, 10 mmol/L Tris-HCl saline, pH 7.4. Culture supernatants (50 μL) were added to platelet

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Submitted May 6, 1991; accepted September 6, 1991.

Supported by a research grant (D.A.B.) from the Canadian Arthritis Society. G.A.D. is supported by a studentship award from the Canadian Arthritis Society.

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0006-4971/92/7902-0008$3.00/0

or control wells (no platelets) in duplicate and incubated at 4°C overnight. Antiplatelet antibody was detected using alkaline phosphatase-conjugated goat antihuman IgM, κ, or λ antibodies (Jackson ImmunoResearch Laboratories). The conjugated antibody was detected using p-nitrophenyl phosphate (1 mg/mL; Sigma Chemical Co, St Louis, MO) in diethanolamine buffer. As a positive control for the assay, rabbit antihuman platelet antiserum was used to monitor the assay (coefficient of variation [CV] = 9.5%).

Reactivity to endothelial cells was determined by ELISA using human brain-derived microvascular endothelial cell monolayers cultured in polylysine microwell plates and provided by Dr J. Rice (Department of Neurology, University Hospital, London, Canada). Endothelial cell cultures were verified by production of alkaline phosphatase and human von Willebrand factor (vWF). This assay was identical to the platelet ELISA except that initial washes before blocking contained 10 mmol/L EDTA-PBS.

Characterization of the Human Antiplatelet Antibody (STO 171)

Inhibition studies. The antiplatelet culture supernatants were preincubated at 4°C overnight with washed platelets (2.5 × 10^7/mL to 6.0 × 10^7/mL), red blood cells (RBCs; 1 × 10^7/mL to 4 × 10^7/mL), polymorphonuclear cells (PMNs; 1 × 10^7/mL to 4 × 10^7/mL), or peripheral blood mononuclear cells (PBLS; 1 × 10^7/mL to 4 × 10^7/mL). RBCs, PMNs, and PBLS were isolated as described. The samples were centrifuged at 12,000g for 5 minutes and the supernatants tested in triplicate for antibody reactivity using the platelet ELISA.

Radioimmunoprecipitation studies. Platelets from healthy nonthrombocytopenic volunteers were washed in 10 mmol/L EDTA-PBS, pH 6.5, and radioiodinated with ^125I using lactoperoxidase as described previously. For each test, 2 × 10^7 platelets were incubated at 4°C overnight with 3.5 mL of culture supernatant or a human serum containing allotypic antibodies anti-Bak'< and anti-Zav'. After centrifugation, the supernatant was removed and the platelets were solubilized in 1 mL of lysis buffer containing 1% Triton X-100. The lysates were added to 50 μL of protein A- sepharose beads (Pharmacia, Uppsala, Sweden) sensitized with affinity-purified rabbit antihuman IgM (2 mg/1.5 g beads; Jackson Immunoresearch). After 1 hour, the beads were washed in lysis buffer containing 0.5% Triton X-100 and the IgM-platelet protein complexes were eluted into 100 μL of sample buffer containing 5% β-mercaptoethanol. The supernatants and molecular weight markers (Bethesda Research Laboratories Inc, Gaithersburg, MD) were analyzed by 5% to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunoprecipitated radioiodinated proteins were detected by autoradiography using XRP-1 film (Eastman Kodak, Rochester, NY).

Immunoblotting studies. Specific platelet protein binding was assessed using standard immunoblot technique. Washed test platelets from healthy donors at a concentration of 2 × 10^7/mL were mixed 1:1 with nonreducing 2x concentrated sample buffer (Bio-Rad Laboratories, Richmond, CA). After electrophoresis on a 7.5% SDS-polyacrylamide gel, the platelet proteins were transferred to nitrocellulose paper (Bio-Rad). Prestained molecular weight markers (Bio-Rad) were run in a 3 mm lane along with the platelet lysate. After blocking in 20 mmol/L Tris-HCl, 0.5 mol/L NaCl, 5% skim milk powder, pH 7.4, the nitrocellulose was cut into strips. The strips were incubated with culture supernatant for 3 hours at 22°C and the IgM bound detected using goat antihuman IgM followed by the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium chloride (BCIP/NBT; Bethesda Research Laboratories). An IgA murine monoclonal antibody (MoAb) (AP4) that binds to immunoblotted platelet GPIIb was provided by Dr T.J. Kunicki (The Blood Center of Southeastern Wisconsin, Milwaukee, WI).

RESULTS

Antiplatelet Antibody Screening and Cloning

Of 1,520 wells seeded from the fusion (termed STO) of tonsil lymphocytes with the lymphoblastoid cell line, 646 (43%) grew in the presence of HAT media. All hybridoma supernatants were screened for IgG and IgM antiplatelet antibodies by ELISA. Hybridomas with initial reactivity to platelets were selected for cloning. Twenty of 472 (4%) IgM hybridomas bound to human platelets. No IgG antiplatelet antibodies were detected and supernatant from the GM 4672 cell line did not show antiplatelet reactivity.

One of the cloned antiplatelet ELISA-positive hybridomas (STO 171) immunoprecipitated two platelet proteins with molecular weights (Mr) of 125 and 105 Kd (reduced) (Fig 1). A control antiplatelet ELISA-negative IgM hybridoma did not immunoprecipitate any specific platelet proteins nor did hybridoma growth medium or the fusion partner GM 4672 (Fig 1). The cell line showing this reactivity was subcloned and all tests were performed using supernatants from these subcloned cells. This antiplatelet antibody was an IgM κ and individual subcloned cell lines produced antibody that ranged in concentration from 0.9 to 6.2 μg IgM/mL. The cell culture growth was stable and antibody production has been maintained for more than 3 years. STO 171 was tested against platelets isolated from 17 different normal volunteers. The antibody did not show allotypic reactivity as identical results were obtained for all donors. To determine if the binding of STO 171 was dependent on EDTA, binding in the platelet ELISA was assessed with platelets washed in ACD/PBS or heparin/PBS. Identical binding occurred when either EDTA, citrate or heparin was used to isolate and wash the platelets (data not shown). This antibody bound well by ELISA at 4°C and room temperature, with minimal binding seen at 37°C (OD492nm < 0.15; data not shown).

The antiplatelet reactivity of STO 171 in the platelet ELISA could be inhibited in a dose-dependent manner with platelets. This inhibition was consistent using either the parent hybridoma or cloned and subcloned cell lines (Fig 2). Restricted reactivity to platelets was confirmed by showing a lack of inhibitory activity with RBCs, PMNs, or PBLS (data not shown). There was no detectable antibody reactivity when tested against endothelial cells (Table 1).
Table 1. Reactivity of STO 171 With Cultured Brain-Derived Microvascular Endothelial Cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>EC ELISA OD450nm*</th>
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<tbody>
<tr>
<td>STO 171</td>
<td>0.02</td>
</tr>
<tr>
<td>STO 6</td>
<td>0.01</td>
</tr>
<tr>
<td>R anti-H vWF (1:100)</td>
<td>0.94</td>
</tr>
<tr>
<td>NRS (1:100)</td>
<td>0.06</td>
</tr>
<tr>
<td>GM 4672</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: EC, cultured brain-derived microvascular endothelial cells; STO, the human hybridoma fusion described in this report; STO 171, antiplatelet GPIIb positive; STO 6, antiplatelet ELISA negative; R anti-H vWF, rabbit polyclonal antihuman vWF antibody; NRS, normal rabbit serum; GM 4672, the lymphoblastoid cell line partner used in the fusion process.

*ELISA optical density readings.

To confirm GPIIb reactivity, immunodepletion of GPIIb/IIIa from platelet lysate using isoantibodies against GPIIb/IIIa was performed. STO 171 was no longer reactive after immunodepletion (Fig 4).

DISCUSSION

The nature and the target antigen of the autoantibodies in ITP are reasonably understood. The majority of patients have increased platelet-associated IgG and at least some, but probably not all, of the autoantibody is bound to platelet-specific antigens. Studies have shown that platelet GPIIb/IIIa are the most frequent target antigens. In recent reports, autoantibodies to platelet GPIIb have been shown in the sera of patients with ITP.

In this study, lymphocytes from a child who did not have a history of autoimmune disorder were used to generate human hybridomas. Approximately 4% of the hybridomas had antiplatelet reactivity, as evaluated in an ELISA. It is possible that the high percentage of hybridomas reacting with platelets is due to the many potential antigens and epitopes presented in the ELISA. In addition, these cells were obtained from tonsil tissue that may provide an environment for the expansion of B cells, which react with bacterial antigens and also crossreact with platelet or other cellular constituents. Therefore, there may be a bias for the expression of antibodies that have crossreactivity with cell membranes.

One fusion product, designated STO 171, had reactivity against platelet GPIIb. The identity of the target antigen was shown by radioimmunoprecipitation using reduced conditions and then confirmed by immunoblotting using nonreduced conditions. As a final confirmation of the reactivity of STO 171 with GPIIb, we immunodepleted platelets using isoantibodies against GPIIb/IIIa. STO 171 was no longer reactive with immunodepleted platelets.

Most recently, Kunicki et al used Epstein-Barr virus-transformed lymphocytes obtained from a patient with chronic ITP to produce antiplatelet MoAbs. Although the patient had autoreactive anti-GPIIb/IX in the serum, the MoAb isolated reacted with platelet GPIIb. This observation could be interpreted as indicating that the patient produced antiplatelet autoantibodies with multiple different specificities. Alternatively, the patient had an inher-
ent genetic capacity to produce anti-GPIIb. The observations reported in this study suggest the latter possibility. The distinction is important as it relates to the issue of whether or not all individuals have the potential to generate autoantibodies. However, it does not exclude autoantibodies arising from the immune response to foreign antigens (common viral or bacterial antigens) that are crossreactive to self antigens. The thermal amplitude of this particular IgM suggests that the expression of it in the circulation would not be harmful, as virtually no binding was detectable at 37°C.

The MoAb STO 171 had a restricted reactivity to platelets and was nonreactive with human RBCs, neutrophils, and PBLs. The antibody bound equally well to EDTA, ACD, and heparin isolated platelets and therefore does not recognize cryptantigens exposed with the use of chelating agents. More importantly, STO 171 did not react with human endothelial cells. In addition, it is unlikely that our antibody binds to the α-chain of the platelet vitronectin receptor because individuals with Glanzmann’s thrombasthenia have normal amounts of this protein and serum from isoimmunized patients should not contain antibodies to it. Immunodepletion with an isotypic source of anti-GPIIb/IIIa was able to completely abrogate the reactivity of STO 171. These results suggest that, although GPIIb is a member of the integrin family of receptors, STO 171 does not recognize other GPIIb-like integrins. The MoAb against GPIII b that Kunicki et al isolated using lymphocytes from a patient with ITP had a similar restricted reactivity.

In summary, our studies show that normal lymphoid cells have the genetic potential to generate antiplatelet autoantibody specificities characteristic of chronic ITP. The disadvantage of this fusion procedure is that we were unable to

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**Fig 3.** Platelet immunoblotting studies (nonreduced). Immunoblotting was performed using STO 171 (lane A); antiplatelet-negative (ELISA) human IgM hybridomas from the same fusion (lanes B and C); antiplatelet-negative human IgM MoAb (lane D); the fusion partner GM 4672 (lane E); and AP4, a murine MoAb against GPIIb (lane F). Molecular weight markers are shown on the left in kilodaltons. These studies show that STO 171 binds to a protein with an Mr of 140 Kd, consistent with GPIIb (nonreduced).

**Fig 4.** Platelet immunoblotting studies after immunodepletion using isotypic antibodies to GPIIb/IIIa. Lanes A and C were sham immunodepleted (normal human serum IgG), and lanes B and D were immunodepleted using anti-GPIII b/IIIa serum. After immunodepletion, lanes A and B were reacted with STO 171 and lanes C and D were reacted with AP4, a murine MoAb against GPIIb. Molecular weight markers are shown on the left in kilodaltons. This experiment shows that STO 171 and AP4 react with a protein of the same molecular weight that is lost after the removal of GPIIb/IIIa.
obtain any IgG autoantibodies, whereas IgG autoantibodies predominate in ITP. Whether this finding reflects the fact that normal individuals regulate Igs of certain specificities and class remains unknown. However, an analysis of the variable region Ig gene sequence of the antiplatelet antibody described here is currently under study and should show whether the IgV gene used has retained a germline configuration. This would also provide the opportunity to compare the sequence with that of other antibodies both to self and foreign antigens. An analysis of the idotypic expression of our anti-GPIIb antibody, in comparison with the autoantibodies characterized by chronic ITP with autoanti-GPIIb may provide insight into the degree of similarity among these autoantibodies and also help assess the degree of restricted V gene usage.

REFERENCES

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