Inhibition of Autoantibody Binding to Platelet Glycoprotein IIb/IIIa by Anti-Idiotypic Antibodies in Intravenous Gammaglobulin

By Peter Berchtold, George L. Dale, Patricia Tani, and Robert McMillan

Intravenous immunoglobulin (IVIgG) causes an acute rise in the platelet count in the majority of patients with chronic immune thrombocytopenic purpura (ITP), but the mechanism(s) of action is still unknown. We evaluated the ability of three different IVIgG preparations to inhibit the in vitro binding of autoantibody to platelet glycoprotein (GP) IIb/IIIa. ITP plasma, known to contain anti-GPIIb/IIIa antibodies, was incubated overnight with either IVIgG or bovine serum albumin (BSA) followed by measurement of the autoantibody titer. Binding of autoantibody from eight ITP patients was inhibited by IVIgG in proportion to the IVIgG concentration. Using 3.2% IVIgG, compatible with therapeutic concentrations expected in vivo, mean inhibition of autoantibody binding ranged from 20.2% to 41.3%. No inhibition by IVIgG of alloantibody binding to the same or different molecules was detected (five patients with anti-GPIIb/IIIa and two with anti-HLA alloantibodies). Fab fragments of IVIgG also inhibited the binding of both plasma autoantibodies and purified anti-GPIIb/IIIa autoantibodies prepared by elution from antigen affinity columns. A portion of the anti-idiotypic antibodies could be adsorbed from IVIgG using insolubilized, purified anti-GPIIb/IIIa autoantibody. These results show that IVIgG preparations from normal donors contain anti-idiotypic antibodies directed against idiotypes located on GPIIb/IIIa autoantibodies but do not have anti-idiotypes to platelet alloantibodies against the same or different molecules. The importance of these anti-idiotypic antibodies in the therapeutic response to IVIgG remains to be established.

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CHRONIC IMMUNE thrombocytopenic purpura (ITP) is characterized by increased platelet destruction due to antiplatelet antibodies. Platelet-associated autoantibodies against the platelet glycoprotein (GP) IIb/IIIa and GPIb/IX complexes have been demonstrated in 75% of patients with chronic ITP. Furthermore, in 58% of these patients, plasma autoantibodies of the same specificity can be detected.

The short-term beneficial effect of high dose intravenous immunoglobulin (IVIgG) in childhood and adult chronic ITP is widely accepted. In addition, a recent report showing remission or stabilization of the disease in some patients receiving long-term IVIgG therapy suggests that this agent may provide long-term benefits in some patients. Despite these clinical observations, the mechanism of action is still unknown. Fehr et al showed that administration of IVIgG was followed by Fc-receptor blockade (FcR), suggesting that decreased platelet clearance is one mode of action in chronic ITP. However, a response to IVIgG has also been reported in ITP patients without detectable FcR blockade. Others reported a decrease in platelet-associated IgG following IVIgG and suggested that antiplatelet antibody synthesis was reduced. As a third possibility, it was reported that IVIgG enhances suppressor T cell function in responding patients. Finally, the response to IVIgG may be due to the effect of anti-idiotypic antibody present in IVIgG. Anti-idiotypic antibody against anti-factor VIII autoantibodies has been demonstrated in IVIgG.

In the present study, we report that commercial IVIg solutions inhibit the in vitro binding of autoantibodies from patients with chronic ITP to platelet GPIIb/IIIa. This inhibition is most likely due to the presence of anti-idiotypic antibodies.

MATERIALS AND METHODS

Patient samples. We studied plasma from eight patients with chronic ITP known to have autoantibodies against platelet GPIIb/IIIa and seven patients with antiplatelet alloantibodies (two with Glanzmann's thrombasthenia and anti-GPIIb/IIIa antibodies, three with anti-Pi antibodies, and two with anti-HLA antibodies). Study patients were chosen on the basis of having high levels of autoantibody or alloantibody, which permitted accurate interpretation of the inhibition studies. Seven of the eight chronic ITP patients (Table 1) had severe disease that was resistant to several forms of therapy and did not have anti-idiotypes to platelet alloantibodies against GPIIb/IIIa.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Response to Treatment</th>
<th>% Inhibition of Anti-GPIIb/IIIa Binding</th>
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<tr>
<td></td>
<td>CS SPLX VINC CTX IVIgG No. Assays* Mean SD SEM</td>
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</tr>
<tr>
<td>ITP-1</td>
<td>PR NR — — TCR 4 41.3 8.3 4.1</td>
<td></td>
</tr>
<tr>
<td>ITP-2</td>
<td>NR NR NR NR NR 17 37.3 8.7 2.1</td>
<td></td>
</tr>
<tr>
<td>ITP-3</td>
<td>PR NR PR NR TCR 4 36.0 4.2 2.1</td>
<td></td>
</tr>
<tr>
<td>ITP-4</td>
<td>PR TCR PR PR TCR 4 31.8 5.9 2.9</td>
<td></td>
</tr>
<tr>
<td>ITP-5</td>
<td>PR TCR — — NR 4 31.2 10.9 5.4</td>
<td></td>
</tr>
<tr>
<td>ITP-6</td>
<td>PR TCR — — 4 28.9 10.5 5.3</td>
<td></td>
</tr>
<tr>
<td>ITP-7†</td>
<td>— — — — 4 21.1 1.9 1.0</td>
<td></td>
</tr>
<tr>
<td>ITP-8†</td>
<td>PR TCR PR PR 4 2.02 3.5 1.7</td>
<td></td>
</tr>
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</table>

Abbreviations: ITP, chronic ITP patient; PR, partial response—platelet count temporarily increased to >50,000/µL; TCR, temporary complete response—platelet count >150,000/µL after therapy with eventual relapse; NR, no response to therapy or an increase in the platelet count to <50,000/µL; SD, standard deviation; SEM, standard error of the mean.

*No. assays—number of separate assays performed on different days. Duplicate incubations were performed for all experimental and control conditions.

†Patient ITP-7 has received only danazol and maintains normal platelet counts on this therapy.
one patient (ITP-7) has thus far obtained a complete response with continued therapy with danazol.

**Purified autoantibodies.** Plasma from one patient with high titer (1/500) anti-GPIIb/IIIa antibody was incubated overnight at 4°C with purified GPIIb/IIIa coupled to cyanogen bromide-activated Sepharose.\(^1\)

After extensive washing, bound autoantibodies were eluted with 0.1 mol/L glycine, pH 2.5. After dialysis against phosphate-buffered saline (PBS), pH 7.4, the purified antibody was concentrated by ultrafiltration to a titer similar to the original plasma.

**Immunoglobulin preparations.** Commercial immunoglobulin preparations were used. Sandoglobulin (Sandoz Inc, East Hanover, NJ), Gamimune N (Cutter, Berkeley, CA), and Gammagard (Hyland, Glendale, CA) are all produced by cold alcohol precipitation of pooled human plasma followed by mild pepsin digestion (Sandoglobulin), preparation at pH 4.25 (Gamimune N) or absorption with DEAE-sephadex.\(^2\)

**Antigen preparation.** Anti-platelet autoantibodies were bound to platelets in vitro. \(^1\) Purified autoantibodies (Gamimune N) were used at a titer of 1:10 in a modification of a previously reported immunobead assay. We used a modification of a previously reported immunobead assay for measuring antiplatelet autoantibodies.\(^3\) Briefly, washed platelet\(s\)\((10^8)\) were incubated for 2 hours in either plasma-IVlgG or plasma-BSA solution (control). After washing four times in isotonic 0.05 mol/L nitric acid buffer, pH 6.2, the sensitized platelets were solubilized in 1% Triton X-100. Each lysate was then incubated with a single 6.4 mm polystyrene bead coated with murine monoclonal anti-GPIIb/IIIa antibody (2A9, provided by Dr V.W. Woods, Jr, University of California, San Diego). After washing, bound antigen-antibody complexes were detected by incubating the beads with 400,000 cpm of \(^3\)H-labeled monoclonal anti-human IgG (HB43; American Type Culture Collection, Rockville, MD). The results are expressed as percent inhibition of the control autoantibody results obtained using BSA instead of immunoglobulin. The percent variation from the mean of replicate samples (51 paired observations) was 4.0% ± 3.4%.

**Autoantibody absorption of IVlgG.** Affinity purified autoantibodies or BSA (control) (0.2 mg/mL) were biotinylated by adding NHS-biotin (50 \(\mu\)g NHS-biotin/mg protein). The mixture was incubated at room temperature for 2 hours and then dialyzed against PBS. The biotinylated protein was added to IVlgG at the same ratio used in the inhibition assay to allow binding of the autoantibody with anti-idiotypic antibodies if present. After incubation for 8 hours at 4°C, autoantibody anti-idiotypic complexes were removed by incubation of the mixture with Avidin coupled to polystyrene beads (1 mg Avidin/g beads) and the anti-idiotypic-depleted IVlgG was used for the inhibition assay.

**Statistical method.** Student's t test was used for all statistical comparisons between two groups of data.

**RESULTS**

Preincubation of plasma from eight chronic ITP patients with IVlgG before the immunobead assay reduced the binding of autoantibody to GPIIb/IIIa. Using IVlgG at a 3.2% (vol/vol) final concentration in the preincubation mixture (similar to in vivo concentrations noted during treatment with IVlgG), a mean decrease of autoantibody binding ranging from 20.2% to 41.3% was noted (Table 1, Fig 1). In contrast, no inhibition by IVlgG was seen when plasma from seven patients with platelet alloantibodies was tested (Fig 1). These alloantibodies, induced by transfusion of blood products, were directed in five patients against GPIIb/IIIa (two Glanzmann's thrombasthenia, three post-transfusion purpura) and in two patients against HLA antigens. Inhibition of autoantibody binding was proportional to the IVlgG concentration used and was similar using three different IVlgG preparations (Fig 2).

When the inhibition assay was performed with three times the usual number of platelets, the mean inhibition (±SD) by IVlgG decreased significantly from 35.7% ± 6.7% to 16.8% ± 7.8% (six studies, \(P < .01\)). In this experiment, the inhibitory activity in IVlgG (presumably anti-idiotypic anti-
body) is competing with the platelet antigen for autoantibody. These results show that the anti-idiotypic antibody is present in limited quantity and, in the presence of a greater amount of platelet antigen, the equilibrium is shifted from the anti-idotype (IVIgG) to the antigen (platelets) and the degree of inhibition measured in the assay is less.

F(ab')2 fragments of IVIgG were also examined for inhibitory activity. As shown in Table 2, inhibition of both plasma and purified anti-GPIIb/IIIa autoantibody binding to platelets was found when IVIgG or F(ab')2 fragments of IVIgG were used although the degree of inhibition by F(ab')2 fragments was significantly less than that seen with the intact IgG (P < .01).

IVIgG was adsorbed with biotinylated, purified autoantibody from one patient (ITP-2) with the aim of removing anti-idiotypic antibodies. As shown in Fig 3, this resulted in a significant decrease of the mean (±SD) inhibitory activity from 33.7% ± 3.8% to 24.8% ± 3.6% (five studies, P < .001) when compared with IVIgG incubated with biotinylated BSA as control.

**Table 2. Inhibition of Plasma and Purified Autoantibody Binding by IVIgG and F(ab')2 Fragments of IVIgG**

<table>
<thead>
<tr>
<th></th>
<th>IVIgG No.</th>
<th>% Inhibition</th>
<th>F(ab')2 No.</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma autoantibodies</td>
<td>17</td>
<td>37.3 ± 8.7</td>
<td>5</td>
<td>23.5 ± 6.3</td>
</tr>
<tr>
<td>Purified autoantibodies</td>
<td>7</td>
<td>37.6 ± 8.0</td>
<td>3</td>
<td>18.7 ± 4.7</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Our data show that anti-idiotypic antibodies reactive with antiplatelet autoantibodies are present in IVIgG. We noted a dose-dependent inhibition of autoantibody binding to antigen by IVIgG at plasma concentrations that are compatible with those expected during IVIgG treatment. Both IVIgG and its F(ab')2 fragments blocked anti-glycoprotein antibody binding although the F(ab')2 fragments inhibited binding to a lesser degree than the undigested IgG. Whether this was due to damage during the course of the low pH pepsin digestion or to other mechanisms is not known. In contrast to the autoantibody results, IVIgG has no inhibitory effect on the binding of antiplatelet alloantibodies specific for either the same molecules or different molecules. Using biotinylated, purified autoantibody from one patient with chronic ITP, we were able to adsorb a significant portion of the inhibitory activity from IVIgG further underlining the specific nature of our findings. However, the degree of adsorption was less than expected from the quantity of biotinylated autoantibody used. Several reasons could account for this: biotinylation of autoantibody may disturb the binding site for anti-idiotypes or the affinity of the anti-idiotypic antibodies may be too low to allow complete adsorption with this system.

The results of these studies indicate that IVIgG contains anti-idiotypic antibodies that bind to idiotypes on antiplatelet autoantibodies but do not react with determinants on alloantibodies. The fact that IVIgG inhibits different autoantibodies to a varying degree suggests heterogeneity among the autoantibody idiotypes.

The therapeutic issue of whether these anti-idiotypic antibodies are important in the response to IVIgG remains to be demonstrated. Treatment with IVIgG is usually followed by a rapid but transient rise of the platelet count.4-6 This initial effect of IVIgG is most likely due to blockade of the reticuloendothelial system (RES) preventing the elimination of antibody-coated platelets.6,12 Anti-idiotypic antibodies could be involved to some degree in this early response by binding to the patient's autoantibodies followed by the removal of the complexed antibodies by the RES, perhaps contributing to the Fc blockade. This could in part explain the slowed destruction of sensitized red blood cells (RBCs) as well as the decrease in platelet-associated IgG19 as is noted after IVIgG treatment. However, anti-idiotypes are not required and may not be important in this early response phase since patients with posttransfusion purpura due to antiplatelet alloantibodies respond quite well to IVIgG and our studies demonstrate no anti-idiotypic antibodies against alloantibodies. Obviously, other important mechanisms are operative during this early response phase.

However, in selected chronic ITP patients receiving IVIgG maintenance therapy, disease stabilization or long-term remissions are reported.4,6 Since RES blockade is transient, downregulation of the immune response with resulting decreased antibody production has been suggested as an alternative mechanism.9,11,13 These long-term remissions or stabilization of the disease could be due to a beneficial effect of anti-idiotypes on immunoregulation. Anti-idiotypes have two potential effects on immunoregulation: inactivation of idiotype-bearing B lymphocytes and activation of suppressor T cells.16-18 This is consistent with the findings after IVIgG treatment where synthesis of antiplatelet antibodies may decrease,4,11,13,18 and impaired suppressor T cell function may be corrected.13,19

In summary, our observations show that IVIgG contains anti-idiotypic antibodies to anti-GPIIb/IIIa autoantibodies. Infusion of these anti-idiotypes into chronic ITP patients could inhibit the binding of autoantibodies to platelet antigens and in some cases downregulate the immune response. Alternatively, they may be simply positive laboratory observations that are unrelated to the therapeutic response to this agent. Further studies are required to determine the importance of anti-idiotypic antibodies in this clinical situation.
REFERENCES

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