Inhibition of Anti-idiotypic Antibodies in Intravenous Gammaglobulin

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

Intravenous immunoglobulin (IVlgG) 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 IVlgG preparations to inhibit the in vitro binding of autoantibody to platelet glycoprotein (GP) Ibb/IIIa. ITP plasma, known to contain anti-GPllb/IIla antibodies, was incubated overnight with either IVlgG or bovine serum albumin (BSA) followed by measurement of the autoantibody titer. Binding of autoantibody from eight ITP patients was inhibited by IVlgG in proportion to the IVlgG concentration. Using 3.2% IVlgG, compatible with therapeutic concentrations expected in vivo, mean inhibition of autoantibody binding ranged from 20.2% to 41.3%. No inhibition by IVlgG of alloantibody binding to the same or different molecules was detected (five patients with anti-GPllb/IIla and two with anti-HLA alloantibodies). Fab2 fragments of IVlgG also inhibited the binding of both plasma autoantibodies and purified anti-GPllb/IIla autoantibodies prepared by elution from antigen affinity columns. A portion of the anti-idiotypic antibodies could be adsorbed from IVlgG using insolubilized, purified anti-GPllb/IIla autoantibody. These results show that IVlgG preparations from normal donors contain anti-idiotypic antibodies directed against idiotypes located on GPllb/IIla autoantibodies but do not have anti-idiotypic antibodies to platelet alloantibodies against the same or different molecules. The importance of these anti-idiotypic antibodies in the therapeutic response to IVlgG remains to be established.

© 1989 by Grune & Stratton, Inc.

In chronic immune thrombocytopenic purpura (ITP) is characterized by increased platelet destruction due to antiplatelet antibodies. Platelet-associated autoantibodies against the platelet glycoprotein (GP) Ibb/IIIa and GPIIb/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 (IVlgG) 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 IVlgG 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 IVlgG was followed by Fc-receptor blockade (Fcr), suggesting that decreased platelet clearance is one mode of action in chronic ITP. However, a response to IVlgG has also been reported in ITP patients without detectable Fcr blockade. Others reported a decrease in platelet-associated IgG following IVlgG and suggested that antiplatelet antibody synthesis was reduced. As a third possibility, it was reported that IVlgG enhances suppressor T cell function in responding patients. Finally, the response to IVlgG may be due to the effect of anti-idiotypic antibody present in IVlgG.

The short-term beneficial effect of high dose intravenous immunoglobulin (IVlgG) 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 IVlgG 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 IVlgG was followed by Fc-receptor blockade (Fcr), suggesting that decreased platelet clearance is one mode of action in chronic ITP. However, a response to IVlgG has also been reported in ITP patients without detectable Fcr blockade. Others reported a decrease in platelet-associated IgG following IVlgG and suggested that antiplatelet antibody synthesis was reduced. As a third possibility, it was reported that IVlgG enhances suppressor T cell function in responding patients. Finally, the response to IVlgG may be due to the effect of anti-idiotypic antibody present in IVlgG.

**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-GPllb/IIla antibodies, three with anti-Pt 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 idiotype antibody against anti-factor VIII autoantibodies has been demonstrated in IVlgG.

In the present study, we report that commercial IVlgG 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.

**Table 1. Inhibition of Anti-GPllb/IIla Autoantibody Binding by Intravenous Gammaglobulin**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Response to Treatment</th>
<th>% Inhibition of Anti-GPllb/IIla Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS SPLX VINC CTX IVlgG</td>
<td>No. Assays*</td>
</tr>
<tr>
<td>ITP-1</td>
<td>PR NR — — TCR</td>
<td>4</td>
</tr>
<tr>
<td>ITP-2</td>
<td>NR NR NR NR TCR</td>
<td>17</td>
</tr>
<tr>
<td>ITP-3</td>
<td>PR PR PR PR TCR</td>
<td>4</td>
</tr>
<tr>
<td>ITP-4</td>
<td>PR TCR PR PR TCR</td>
<td>4</td>
</tr>
<tr>
<td>ITP-5</td>
<td>PR TCR — — NR</td>
<td>4</td>
</tr>
<tr>
<td>ITP-6</td>
<td>PR TCR — — —</td>
<td>4</td>
</tr>
<tr>
<td>ITP-7†</td>
<td>PR TCR PR PR —</td>
<td>4</td>
</tr>
<tr>
<td>ITP-8</td>
<td>PR TCR PR PR —</td>
<td>4</td>
</tr>
</tbody>
</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 dazanol.

**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.12

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 (Cutler, 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 (Gammagard). Stock solutions containing 40 mg/mL IgG were prepared and dialyzed against PBS before use.

**F(ab')2 fragments.** IVlgG was further purified by separation on DEAE Sepharose. The IgG fraction was dialyzed against 0.1 mol/L acetate buffer, pH 4.0. Pepsin (1 mg/mL) was added and the mixture incubated at 37°C for 18 hours. The digested protein was size fractionated on a G-150 Sephadex-column equilibrated with PBS. F(ab')2 fractions were pooled and concentrated to 27 mg/mL for use in the inhibition assay. The F(ab')2 preparation contained no Fc fragments or intact IgG on electrophoretic testing.

**Inhibition assay.** Before measurement of antiplatelet antibodies, plasma samples (200 μL) or the purified autoantibody eluate were preincubated with different concentrations of IVlgG or bovine serum albumin (BSA control) overnight at 4°C. If present, anti-idiotypic antibodies in the immunoglobulin preparation would bind to the plasma autoantibody and reduce the amount detectable in the autoantibody assay (see below).

We used a modification of a previously reported immunobead assay for measuring antiplatelet autoantibodies.2 Briefly, washed platelets (10^6) were incubated for 2 hours in either plasma-IVlgG or plasma-BSA solution (control). After washing four times in isotonic 0.05 mol/L citrate 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 125I-labeled mononclonal 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 μg 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-
or dose-dependent inhibition of autoantibody binding to antigen. Antiplatelet autoantibodies are present in IVIgG. We noted 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.

**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 IVIg 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 (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</th>
<th>F(ab')2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. % Inhibition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma autoantibodies</td>
<td>17</td>
<td>37.3 ± 8.7</td>
</tr>
<tr>
<td>Purified autoantibodies</td>
<td>7</td>
<td>37.6 ± 8.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>IVIgG</th>
<th>F(ab')2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Inhibition</td>
<td>23.5 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>% Inhibition</td>
<td>18.7 ± 4.7</td>
<td></td>
</tr>
</tbody>
</table>

### Fig 3. Adsorption of IVIgG with purified biotinylated autoantibody. N = number of separate experiments. The plasma used in each case came from the same patient (ITP-2).
REFERENCES

Inhibition of autoantibody binding to platelet glycoprotein IIb/IIIa by anti-idiotypic antibodies in intravenous gammaglobulin

P Berchtold, GL Dale, P Tani and R McMillan