The Effect of Therapy on Platelet-Associated Autoantibody in Chronic Immune Thrombocytopenic Purpura

By K. Fujisawa, P. Tani, L. Piro, and R. McMillan

Chronic immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by antibody-induced destruction of platelets. Several forms of therapy have been used, including corticosteroids, splenectomy, danazol, and a variety of immunosuppressants. We studied the mechanism of action of some of these treatments by evaluating patients’ platelet-associated autoantibodies (PAAb) and platelet count before and serially following therapy. Treatment with corticosteroids, splenectomy, cyclophosphamide, and combination chemotherapy resulted in a progressive decrease in PAAb associated with an improvement in the platelet count, and appeared to act by primarily affecting autoantibody production. Conversely, PAAb levels either remained stable or increased during vincristine or danazol therapy despite improvement in the platelet count, suggesting that the major effect of these agents was decreased platelet removal by the reticuloendothelial (RE) system.

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MATERIALS AND METHODS

Patients

Characteristics of the patients are listed in Table 1. We studied 14 patients with chronic ITP and one patient with immune thrombocytopenia associated with chronic lymphocytic leukemia (CLL). Patients with chronic ITP satisfied the usual clinical criteria. One patient (ITP-1) also had autoimmune hemolytic anemia. The patient with CLL had CD5+ lymphocytosis and thrombocytopenia out of proportion to the severity of the CLL. All patients had high levels of PAAb against either GPIIb/IIIa or GPIb/IX as measured by the immunobead assay described below. Plasma autoantibodies were demonstrable in 13 of the 15 patients (Table 1). Approval was obtained from the Institutional Review Board for these studies. Patients were informed that samples were obtained for research purposes and that their privacy would be protected.

The effect of therapy was studied on the following numbers of patients: corticosteroids, five chronic ITP patients; cyclophosphamide, four on high-dose prednisone (40 to 80 mg/d) and one on high-dose solumedrol [one g/d for 3 days]; splenectomy, six chronic ITP patients (three attained a complete remission, three failed to respond to splenectomy); vincristine (2 mg/wk intravenously [IV]), one chronic ITP patient; danazol (600 to 800 mg/d), three chronic ITP patients; and combination chemotherapy with 4 monthly courses of CMOPP (cyclophosphamide 650 mg/m² on days 1 and 8, vincristine 2 mg IV on days 1 and 8, prednisone 40 mg/m² on days 1 to 14, and procarbazine 100 mg/m² on days 1 to 10) followed by 4-monthly courses of CEP (cyclophosphamide 650 mg/m² on days 1 and 8, prednisone 40 mg/m² on days 1 to 14, and etoposide 100 mg/m² on days 14, 15, 16), one chronic ITP patient, and with 4- and 5-monthly courses of CEP, one chronic ITP patient and one CLL patient, respectively. Two patients were studied during more than one type of treatment: ITP-5 during corticosteroids and splenectomy, and ITP-10 during splenectomy, danazol, cyclophosphamide, and combination chemotherapy.

In each patient, serial blood samples were obtained for PAAb and platelet counts before and at varying intervals following therapy. Plasma autoantibodies were measured in all patients when first seen (Table 1), but in most cases these values were either negative (two patients) or too low to allow accurate evaluation of changes with therapy. These serial plasma data will therefore not be included.

Autoantibody Assay

All incubations are at room temperature.

Immunobead preparation. Polystyrene immunobeads (Pierce Chemical, Rockford, IL, ¼ in) are coated with murine monoclonal antibody against either GPIIIa (2A9, provided by Dr Virgil Woods, University of California, San Diego) or GPIb (P3, provided by Dr Zaverio Ruggeri, Scripps Clinic) by incubating the beads to 60 minutes in 0.1 mol/L NaHCO₃ buffer, pH 8.2, containing 10 μg of monoclonal antibody per bead. After washing twice with 0.05% Tween 20 in phosphate-buffered saline (PBS-Tween), residual binding sites are blocked by incubation of the beads in 2% bovine serum albumin (BSA) in PBS-Tween for 60 minutes followed by four washes.

Platelet preparation. Platelets from EDTA-anticoagulated blood were obtained from the patient (PAAb) or a normal donor (plasma antibody) and washed six times with 0.05 mol/L isotonic citrate buffer. Patient platelets are resuspended to a concentration of 10⁹/mL. To prepare antibody-sensitized platelets from patient plasma, 100 μL of washed normal platelets (10⁶) are incubated with 1 mL of patient or control plasma for 60 minutes, washed four times with 0.05 mol/L citrate buffer, and then resuspended in 1 mL of citrate buffer. Samples may be frozen at −20°C for later assay. Just before assay, the platelets are lysed by adding 111 μL of 10% Triton X-100 (1% final concentration) and then centrifuging for 5 minutes at 12,000g.

Assay. Solubilized platelets (900 μL) from each sample are then incubated for 60 minutes with a single immunobead coated with the appropriate monoclonal antibody to allow attachment of the glycoprotein and any bound autoantibody. After four washes with PBS-Tween, approximately 400,000 cpn of radioiodinated murine antihuman IgG (HB-43, American Type Culture Collection, Rockville, MD) in 1 mL of PBS-Tween is added and the mixture incubated for 60 minutes. After four washes, the bead-associated radioactivity is...
Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Antigen</th>
<th>Autoantibody*</th>
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<tbody>
<tr>
<td>ITP-1</td>
<td>20/F</td>
<td>GPllb/llla</td>
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<td>32/F</td>
<td>GPllb/llla</td>
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<tr>
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</tr>
<tr>
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<td>ITP-14</td>
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<tr>
<td>CLL-1</td>
<td>63/F</td>
<td>GPlb/lX</td>
<td>259.2 2.4</td>
</tr>
</tbody>
</table>

* Autoantibody data are expressed as a ratio of patient cpm to mean cpm of three normal controls. A ratio of greater than 2.0 is positive (>3 SD above the mean of normal control values).

determined. Assay of normal platelets results in bead-associated radioactivity ranging from 90 to 350 cpm depending on the date of labeling.

To evaluate the effect of therapy, serial samples of patient platelets were usually assayed on the same day. Net radioactivity was determined for each sample by subtracting the mean radioactivity of three control samples and the results were expressed as the percentage of the pretreatment value. In selected instances, where all samples could not be assayed on the same day, the results were corrected using a positive internal control sample, which was assayed with each day's samples. This positive sample consisted of an aliquot of $10^7$ platelets from the same ITP patient with high-level PAAb. Identical samples assayed on different days that were corrected using this method showed a mean variation of 10.8% ± 7.7% (19 observations).

Effect of In Vitro Incubation With Corticosteroids on PAAb

To evaluate the in vitro effect of corticosteroids on PAAb, washed platelets ($10^9$) in citrate buffer from a chronic ITP patient or a normal subject were incubated for 5 hours at room temperature or overnight at 4°C in varying concentrations of hydrocortisone sodium succinate (0.01 to 1.0 mg/mL) or in citrate buffer alone. After incubation, the platelets were solubilized in 1% Triton-X100 and assayed as described above.

RESULTS

Corticosteroids

Corticosteroid therapy, in each of the five patients tested (ITP-1–5), resulted in a decrease in PAAb levels and an increase in the platelet count (Fig 1). In the one patient tested (ITP-1), high-dose methylprednisolone (1 g/d for 3 days) resulted in a more rapid response when compared with the other patients treated with more conventional prednisone doses of 40 to 80 mg/d (Fig 2). Tapering of the corticosteroid dosages was, in each patient, associated with reappearance or worsening of the thrombocytopenia and an increase in PAAb levels (data not shown).

Because the decrease in PAAb levels after corticosteroids could be due either to decreased autoantibody production or to corticosteroid-induced interference of antibody binding to antigen, in vitro studies were performed in which washed patient platelets were incubated with varying concentrations of corticosteroids and then PAAb values before and after incubation were compared. PAAb results remained unchanged (Table 2).

Splenectomy

Six chronic ITP patients were studied. A complete remission from splenectomy was associated with the disappearance of PAAb in two patients (ITP-6 and ITP-7) and a reduction to less than 21.8% of pretreatment values in another patient (ITP-5). Conversely, in three patients achieving a temporary response after splenectomy followed by relapse (ITP-8, ITP-9, and ITP-10), PAAb levels either decreased initially as the platelet count increased followed by an increase in PAAb levels with recurrent thrombocytopenia, or remained elevated. Examples of a responsive and a nonresponsive patient are shown in Fig 3.

![Fig 1. Effect of corticosteroids on PAAb levels and the platelet count of an ITP patient. Data are from patient ITP-4. Autoantibody values (○) are expressed as net cpm/$10^9$ platelets after subtraction of mean control values (<250 cpm) and platelet counts (○) X $10^9$/μL.](image-url)
Fig 2. Effect of corticosteroids on PAAb levels. Data are expressed as the percentage of the autoantibody level just before therapy. The pretherapy autoantibody values (net cpm/10⁶ platelets after subtraction of mean control values) were ITP-1 (●), 87,158; ITP-2 (○), 36,887; ITP-3 (△), 2,583; ITP-4 (▲), 69,337; ITP-5 (○), 18,040. Control values were less than 250 cpm.

Vincristine and Danazol

In one chronic ITP patient (ITP-11), 3-weekly vincristine injections resulted in normalization of the platelet count without affecting the elevated PAAb levels (Fig 4). Similar results were noted with danazol therapy, where platelet counts increased in two chronic ITP patients (ITP-10 and ITP-12) with either no significant change or an increase in PAAb levels (Fig 5). In a third ITP patient (ITP-13), in whom no pretreatment PAAb testing was possible, danazol therapy was associated with high levels of PAAb (net cpm, 8,616; ratio, 22.2) 1 year after continuously normal platelet counts on treatment.

Cyclophosphamide and Combination Chemotherapy

In the one ITP patient treated with three courses of IV cyclophosphamide as a single agent (ITP-10), a rapid decrease in PAAb levels and normalization of the platelet count occurred within 10 to 14 days of each injection. This was followed in each instance by reappearance of thrombocytopenia, accompanied by elevation of PAAb levels (Fig 6).

Table 2. In Vitro Effect of Corticosteroids on PAAb Levels

<table>
<thead>
<tr>
<th>Corticosteroid Concentration (mg)</th>
<th>37°C for 4 Hours</th>
<th>4°C Overnight</th>
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<tr>
<td>0.01</td>
<td>12.6</td>
<td>14.3</td>
</tr>
<tr>
<td>0.1</td>
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<td>14.9</td>
</tr>
<tr>
<td>10</td>
<td>12.6</td>
<td>14.0</td>
</tr>
</tbody>
</table>

Washed platelets from patient ITP-10 were incubated in varying concentrations of hydrocortisone succinate or buffer alone at either 37°C for 4 hours or 4°C overnight. After incubation, duplicate aliquots (10⁶) were assayed for platelet-associated autoantibody. Data are expressed as a ratio of cpm patient samples to mean cpm of similarly treated control platelets (control cpm ranged from 165 to 295 cpm).

Fig 3. Effect of splenectomy on PAAb levels and the platelet count. Autoantibody values (○) are expressed as net cpm/10⁶ platelets after subtraction of mean control values (< 250 cpm) and platelet counts (□) × 10⁴/µL. (A) A patient who attained a complete remission (ITP-6), and (B) a patient who failed to respond to splenectomy (ITP-10).

Three patients were evaluated during combination chemotherapy. In one ITP patient (ITP-10), who received combination chemotherapy with CMOPP (four courses) followed by CEP (four courses), a complete, unmaintained remission was attained, which has persisted for 36 months after stopping therapy (Fig 7). This remission was associated with a progressive decrease in PAAb levels to approximately 5% of the values determined at diagnosis. These low-level PAAb persist. In the other two patients (ITP-14 and CLL-1), a progressive decrease in PAAb has also occurred following each course of therapy, with a decrease to 5.4% (CLL-1) of the pretreatment value in one patient and to 19.3% (ITP-14) in the second patient after five and four courses of CEP, respectively. Platelet counts are normal.

DISCUSSION

Chronic ITP is an autoimmune disorder in which thrombocytopenia results from autoantibody-induced platelet de-
EFFECT OF THERAPY ON ANTIPLATELET AUTOANTIBODY

Fig 4. Effect of vinristine on PAAb levels and the platelet count. Autoantibody values (△) are expressed as net cpm/10⁶ platelets after subtraction of mean control values (< 250 cpm) and platelet counts (Ο) × 10³/μL. Data are from patient ITP-11. Pretherapy autoantibody was 11,735 cpm; △, vinristine injection.

Fig 5. Effect of danazol on PAAb levels and the platelet count. Autoantibody values (△) are expressed as net cpm/10⁶ platelets after subtraction of mean control values (< 250 cpm) and platelet counts (Ο) × 10³/μL. Data are from patients ITP-12 (A) and ITP-10 (B). Pretherapy autoantibody values were ITP-12, 19,330 cpm, and ITP-10, 84,220 cpm.

assays as a measure of autoantibody. One group noted that the increase in platelet count in some ITP patients preceded normalization of PAIgG and postulated that this may reflect the corticosteroid-induced inhibition of phagocytosis occurring earlier than suppression of antibody production.

Additional support for a corticosteroid-induced suppression of antiplatelet antibody is provided by studies using a newly described mouse ITP model in which corticosteroid treatment resulted in normalization of the platelet count, a decrease in PAIgG, and an increase in the shortened platelet lifespan. Reduction of autoantibody levels in other autoimmune disorders has also been noted after corticosteroid therapy and this has also correlated with clinical improvement.

The reduction in autoantibody by corticosteroids may also influence platelet production by the bone marrow. Studies over the past 10 years have shown that a substantial number of chronic ITP patients have diminished platelet production in addition to shortened platelet survival. This is presumably due either to the effect of autoantibody on thrombopoiesis or to the intramedullary destruction of antibody-sensitized platelets before their release into the circulation.

Fig 4. Effect of vincristine on PAAb levels and the platelet count. Autoantibody values (△) are expressed as net cpm/10⁶ platelets after subtraction of mean control values (< 250 cpm) and platelet counts (Ο) × 10³/μL. Data are from patient ITP-11. Pretherapy autoantibody was 11,735 cpm; △, vinristine injection.

construction by the reticuloendothelial (RE) system. Successful therapy must affect one or more of the following: autoantibody production, binding of autoantibody to the platelet, or removal of antibody-sensitized platelets by the RE system.

The mechanism of action of corticosteroids is complex. In vivo studies in guinea pigs showed that corticosteroids decrease RE sequestration of red blood cells (RBCs) that had been sensitized with rabbit IgM or IgG antibody to guinea pig RBCs. This effect on phagocytosis was caused by a corticosteroid-induced decrease in Fc γ-receptor protein expression on the macrophage surface, rather than to an alteration of receptor mobility or clustering on the membrane.

In vitro and in vivo data on human subjects also show that corticosteroids suppress phagocytosis. The most compelling evidence in human subjects is the study by Shulman et al, who showed that the thrombocytopenia, induced by the infusion of ITP plasma into normal subjects, is partially inhibited by the prior administration of corticosteroids to the recipients. The effect could be overcome by increasing the dose of plasma. Therefore, there is no doubt that corticosteroids in high doses affect phagocytosis. However, if this were the only mechanism in chronic ITP, PAAb levels should either remain stable or increase during therapy, since the antibody-sensitized platelets would not be removed from the circulation.

Alternatively, corticosteroid therapy may also affect antibody production. Earlier studies show that patients who are receiving corticosteroids have low serum IgG and IgA levels, and, in chronic ITP patients, corticosteroid treatment is associated with the suppression of spontaneous IgG production by bone marrow cells. The present studies add direct support to the hypothesis that autoantibody suppression is an important result of corticosteroid therapy. Following corticosteroids, PAAb levels, in each of the five patients studied, decreased as the platelet count increased. Upon tapering the drug, the pattern reversed. Similar response patterns were noted in earlier studies using platelet-associated IgG (PAIgG)
Gernsheimer et al reported that increased platelet production was the major explanation for a corticosteroid response, rather than an increase in the shortened platelet lifespan, suggesting that this effect may be of particular benefit in some ITP patients. The effect of splenectomy in chronic ITP is also complex and is due to the combined effects of removing a site important to both antibody production and platelet destruction. In the present studies, complete remission after surgery resulted in either the complete disappearance of platelet-associated autoantibody or a marked but permanent reduction, whereas in splenectomy failure, autoantibody levels remained elevated. Similar results in PA IgG were observed after splenectomy in chronic ITP patients. These results suggest that, in chronic ITP patients responding to splenectomy, the spleen is the major, and in some cases the only, site of autoantibody synthesis, which confirms earlier studies showing that the spleen is an important autoantibody production site.

Splenectomy also removes an important site of platelet destruction. In the guinea pig model, referred to above, splenectomy resulted in reduced clearance of RBCs that were lightly sensitized with IgG antibody; this effect was lost as the level of antibody sensitization increased. A similar effect was noted in the in vivo human studies. Infusion of approximately fivefold more ITP plasma was required to cause the same degree of thrombocytopenia in splenectomized normal recipients as that needed in normal recipients with a spleen. The present results are compatible with these prior studies. Complete remission occurred in a setting where either no autoantibody was present or where light but stable autoantibody sensitization occurred. High levels of PAAb were associated with either no response to splenectomy or early relapse.

The third form of therapy that results in decreased PAAb levels is chemotherapy with either cyclophosphamide alone or combination chemotherapy. Treatment was associated with a rapid increase in the platelet count with a concomitant decrease in autoantibody levels, which reached a nadir in 7 to 10 days. In the patient treated with cyclophosphamide alone and in the early courses of combination chemotherapy, this pattern often reversed, with increasing antibody levels associated with the redevelopment of thrombocytopenia. In the patient treated with combination chemotherapy who was monitored serially through eight courses of therapy, the peak autoantibody values became progressively lower after each course and finally plateaued at levels less than 5% of those measured at the time of diagnosis. This has persisted for 3 years after stopping treatment. Similarly, two other patients treated with combination chemotherapy showed a progressive decrease of PAAb levels throughout their therapy. Long-term follow-up data are not yet available on these latter patients to evaluate the persistence of the response.

Conversely, patients treated with vincristine (one patient) and danazol (two patients) showed no significant decrease in autoantibody values despite normalization of the platelet levels. The effect of combination chemotherapy on PAAb levels and the platelet count. (A) Platelet counts $\times 10^9/\mu L$ and (B) autoantibody values are expressed as net cpm/10⁹ platelets after subtraction of mean control values (<250 cpm). Data are from patient ITP-10. Pretherapy autoantibody was 74,230 cpm.

\[\text{Platelet Count} \times 10^9/\mu L\]

\[\text{Autoantibody \ (cpm/10^9 platelets)}\]

\[\text{Days Post-therapy}\]
count, suggesting that the short-term mechanism of action of these agents is to affect platelet removal. These results are consistent with the findings of Schreiber et al, who noted a down regulation of the monocyte Fc receptor in ITP patients receiving these agents, but no effect on PAIgG.29 Since the follow-up period on our two patients treated with danazol was short, it is possible that these drugs may have other effects if administered over a long interval. Ahn et al noted normalization of elevated PAIgG levels in chronic ITP patients who achieved a complete unmaintained remission with danazol.30 We have studied one ITP patient after 1 year of normal platelet counts attributed to treatment with danazol. She continues to have high autoantibody values, although we did not have pretreatment samples to evaluate whether her autoantibody levels may have decreased during therapy. In summary, a therapeutic response to corticosteroids, splenectomy, cyclophosphamide, and combination chemotherapy is associated with decreased levels of PAAb, suggesting that a major mechanism of action of these therapeutic maneuvers involves suppression of antibody production. Conversely, vincristine and danazol result in little or no immediate decrease in PAAb levels and act by primarily suppressing platelet removal by the RE system.

REFERENCES

The effect of therapy on platelet-associated autoantibody in chronic immune thrombocytopenic purpura

K Fujisawa, P Tani, L Piro and R McMillan