Ten patients with idiopathic thrombocytopenic purpura (ITP) were studied before and following a rise in circulating platelets subsequent to infusions of intravenous gammaglobulin (400 mg/kg/day × 5 days). We quantitated the amount of circulating IgG capable of binding to normal donor platelets in vitro using a 125I-monoclonal anti-human IgG assay, as well as the amount of IgG associated with the patients' platelets before and following therapy. We found no evidence for a decrease in platelet-specific IgG antibodies in these patients undergoing an acute response to therapy. These data suggest that the short-term efficacy of intravenous gammaglobulin is due to effects other than a substantive reduction in platelet reactive antibodies, such as the alteration of IgG-coated platelet destruction.

Since the initial report of Imbach et al in 1981, high-dose intravenous gammaglobulin has been increasingly used in the treatment of immune thrombocytopenic purpura. Childhood ITP, frequently a self-limited disease, generally responds more dramatically to IV gammaglobulin therapy than does the adult form of the disorder. However, the majority of all ITP patients respond with at least a transient increase in platelet count following a two-to-five-day course of 200-600 mg/kg/day IV gammaglobulin.1-4

The pathophysiology of thrombocytopenia in ITP is thought to involve the presence of increased amounts of IgG on platelet surfaces, leading to the premature removal of platelets from the circulation via IgG-Fc receptor binding by cells of the reticuloendothelial system (RES). There are at least two general mechanisms that have been proposed to explain the therapeutic effect of IV gammaglobulin: (a) inhibition of the RES binding and clearance of IgG-coated platelets2,3,6,7 and (b) suppression of platelet autoantibody production or binding.4,8,9

Using antibody-coated red cell targets as a model, both in vivo and in vitro studies have supported the hypothesis that high-dose gammaglobulin administration can interfere with the efficiency of RES IgG-Fc receptor function.2,3,4 We have recently shown that therapeutic gammaglobulin preparations can also impair human monocyte Fc receptor binding of IgG-coated platelets in vitro.7 An alternative mechanism of beneficial effect has been the hypothesis that gammaglobulin down modulates platelet antibody production.4,8,9 In this study, we sought evidence for suppression of platelet autoantibody production by estimating the amount of circulating plasma IgG antibodies, as well as platelet-bound antibody, before and following high-dose gammaglobulin therapeutic responses.

Materials and Methods

Human subjects. Patients were selected on the basis that they had ITP by previously described criteria,2-10 had received high-dose gammaglobulin with a dramatic increase in platelet count within one week of starting therapy, and had plasma samples stored before and following therapy.

Five subjects were childhood ITP patients entered into a randomized study of high-dose gammaglobulin therapy in Italy (group 1, Table 1). Following the diagnosis of active ITP, these subjects were observed for 10 days without treatment before initiating infusion of Endoblin (IMMUNO, Austria) at 400 mg/kg/day for five consecutive days.16

Five subjects were adult patients treated by the Hematology Service of the University of Alabama at Birmingham for ITP (group 2, Table 1). All had either relapsed or had no response following prior treatments with corticosteroids, vincristine, and/or splenectomy. These 5 patients received infusions of Sandoglobulin® (Sandoz, Basel), 400 mg/kg/day on five consecutive days, without concurrent additional treatment for ITP.

Platelet counts, direct platelet antibody assays, and plasma samples were obtained within 24 hours before the first gammaglobulin infusion ("pre" samples) and on the day following the fifth and last treatment ("post" samples). Plasma samples were stored frozen until further study.

Healthy adult volunteers (blood group O, RhD positive) employed at the University of Alabama at Birmingham were the sources of platelets used in the normal donor platelet antibody-binding assays.

Quantitation of platelet-associated antibody. Two different assays were used for measurement of platelet-associated IgG in platelet samples freshly obtained from patients. For group 1 patients (Italy), assay was by ELISA on platelet extracts, using the procedure described by Hedge et al.11 For group 2 patients (USA) the previously described 125I-monoclonal antibody assay was used with fresh, intact platelets.12,13 Normally, results from the latter assays are expressed as molecules IgG/platelet; however, for consistency in this report, data are presented as ng IgG/10⁶ platelets. With the 125I antibody assay, values of <0.10 ng/10⁶ (<400 molecules/platelet) are considered normal, between 0.10 and 0.20 ng/10⁶ (400 to 800 molecules/platelet) are slightly elevated, and >0.20 ng/10⁶ (>800 molecules/platelet) are significantly elevated, as previously documented.16 In contrast, assays of platelet extracts, which measure total (surface and cytoplasmic) platelet-associated
IgG, give values approximately 100-fold higher than those for surface IgG. All data are the means of triplicate measurements.

Quantitation of circulating platelet antibody. Stored plasma samples for each patient "pre" and "post" gammaglobulin infusion were subjected to ion-exchange chromatography for production of partially purified IgG fractions at the University of Alabama at Birmingham. Microgranular diethylaminoethyl cellulose (DE-52, Whatman Limited, United Kingdom) was equilibrated in 0.01-M sodium phosphate buffer, pH 8.0, according to the manufacturer's directions. Plasma samples were dialyzed overnight at 4°C against 

The final preparations were analyzed by Lowry protein assay and ranged from 14 to 45 mg/mL. Quantitative radioimmunoassays for human IgG (see below) indicated that on average 93% of protein in the DE-52 preparations was IgG.

As a control for these assays, we prepared a "mock" treated plasma sample spiked in vitro with Sandoglobulin. A 3-g vial of lyophilized Sandoglobulin was reconstituted with 30 mL of sterile distilled water and analyzed by Lowry protein assay. One hundred mg of Sandoglobulin protein was then added to one of two 10-mL aliquots of an adult ITP patient's plasma which was known to have IgG antibodies reactive with normal donor platelets. The treated and untreated plasma aliquots were then processed exactly as described above.

Normal donor platelets were obtained as previously described and used as a platelet concentrate (~10^9/mL) in autologous plasma. Aliquots of 5 x 10^7 platelets were incubated with the DE-52 plasma IgG fractions at doses of 0.5, 1.0, and 2.0 mg total protein for one hour at room temperature. Platelets were then washed three times in PBS, without Ca^2+ or Mg^2+, containing 1% (weight/volume) bovine serum albumin and 4 mM EDTA. Platelets were finally resuspended in the same buffer and 2 x 10^7 platelets were then assayed in triplicate by the 125I-monoclonal antibody assay for quantitation of IgG/platelet. We calculated the mean ng of IgG bound per 5 x 10^7 platelets at each concentration of IgG used. Results were analyzed in two ways. First, the amount of platelet antibody per mg of added plasma IgG was used to reflect the concentration of antibody "pre" and "post" therapy. Second, the amount of platelet antibody per mL of plasma was used to reflect the total circulating pool of platelet antibody at each sampling time.

Radioimmunoassay for total IgG. IgG concentrations in 0.2-μm-filtered plasma samples or in DE-52 fractions were determined by a competitive radioimmunoassay, measuring the inhibition of binding of 125I-monoclonal anti-human IgG to human IgG-coated beads in the presence of standard and unknown concentrations of soluble human IgG, as previously described. Assays were run in triplicate, using at least two dilutions of each unknown sample.

RESULTS

Table 1 presents clinical data for the 10 ITP patients treated with high-dose gammaglobulin. All responded to treatment, as demonstrated by an increased circulating platelet count on the day following completion of therapy. Seven of the 9 patients for whom we had complete data also demonstrated a reduction in the amount of platelet-associated IgG (ng/10^9 platelets), as measured by the assay performed on freshly obtained platelets. It should be pointed out that the direct assay for group 1 was performed by an ELISA assay on platelet extracts, which inherently gives higher base line (normal) levels of platelet IgG than the 125I-monoclonal antibody assay on intact platelets used for group 2.

The first two data columns of Table 2 demonstrate that all patients' plasmas had the expected increase in IgG concentration following IV gammaglobulin therapy, with a mean increase of 3.4-fold (from 6.8 to 23.3 mg/mL). The "mock"-treated ITP plasma had a similar increment, from 7.7 to 20 mg/mL IgG.

We next analyzed the amount of IgG in each plasma sample which was capable of binding to normal donor platelets in vitro. Because of the wide range of plasma IgG concentrations, most notably between the "pre" and "post" treatment samples, we used IgG-enriched fractions of the individual plasma samples in order to assay comparable amounts of IgG in each platelet IgG binding determination. Table 2 (data columns 3 and 4) shows the amount of IgG bound per mg of total IgG incubated with a constant number of normal donor platelets. The majority of patient samples demonstrated a decrease in this value "post" treatment; the group as a whole had 32.9 ng of platelet IgG per mg of total IgG "pre" therapy and 15.1 ng of platelet IgG per mg of total IgG "post" therapy. This decrease probably reflects the passive infusion of gammaglobulin that does not contain platelet antibody (unpublished observation) and that dilutes out the specific activity of the patients' platelet antibodies. This is supported by results with the "mock"-treated ITP plasma, which underwent a similar reduction from 39 ng/mg of IgG to 22 ng/mg of IgG following in vitro addition of Sandoglobulin.
Since the total concentration of IgG per mL of plasma was quantitated "pre" and "post" therapy (columns 1 and 2, Table 2), the concentration of platelet antibody per mL of plasma could be calculated (data columns 5 and 6, Table 2). The patients' plasma samples as a group had a modest increase in circulating platelet antibody, from 254 ng/mL to 426 ng/mL of plasma. There was not a statistically significant difference between the values "pre" and "post" therapy (p = 0.068 by paired t-test analysis). The "mock"-treated ITP plasma sample yielded similar data, with a concentration of 300 ng/mL plasma "pre" therapy and 400 ng/mL "post" therapy (Table 2). These results do not support the hypothesis that therapeutic gammaglobulin produced a reduction in the circulating pool of platelet antibody.

Antibody bound to the platelet surface in vivo represents a second pool of platelet antibodies. As seen in Group 2 patients (Table 1), the amount of IgG antibody associated with the platelet surface was generally reduced following high-dose gammaglobulin therapy. The post-therapy values for platelet-surface IgG for patients BOK, SEH, and MKN were 0.16, 0.16, and 0.12 ng/10⁶ platelets, which reflect 640, 640, and 480 IgG molecules/platelet respectively. Patient CNI had a pretherapy value of 2.1 ng/10⁶ platelets (8,400 IgG/platelet) and six days later (post-therapy) 0.64 ng/10⁶ platelets (2,560 IgG/platelet). However, each of these patients had a dramatically increased number of circulating platelets post-therapy. If one multiplies the amount of platelet-associated antibody per 10⁶ platelets by the number of circulating platelets (Table 1), the total amount of platelet-bound antibodies has clearly not been reduced. This calculation is a rough estimate since it includes platelet-specific antibody as well as "nonspecific" IgG on the platelet surface, which amounts to 122 ± 5 molecules/platelet in our studies⁹ and to 79 ± 27 molecules/platelet as reported by George and Saucerman.¹⁴

### DISCUSSION

This study was performed in order to re-evaluate the possibility that modulation of platelet IgG autoantibody production is involved in the therapeutic efficacy of high-dose gammaglobulin therapy in ITP. Several authors have suggested that high-dose gammaglobulin is immunomodulatory in vivo, either by alteration of the anti-idiotype network or modulation of suppressor-T lymphocytes, thereby leading to depressed immunoglobulin production, including the production of any platelet-specific antibodies.⁴ Others have suggested that high-dose gammaglobulin may directly interfere with the binding of platelet-specific antibodies to the target antigens.⁶ In support of these hypotheses, many authors cite the frequently reported observation that as platelet counts increase during gammaglobulin therapy, measured amounts of platelet-associated IgG (eg, ng/10⁶ platelets) decrease. This inverse relationship between platelet count and platelet-associated IgG is not unique to gammaglobulin therapy. The same pattern is generally observed in ITP patients responding to splenectomy, corticosteroids, vincristine, or cyclophosphamide.⁵ If it is assumed that the rate of platelet autoantibody production remains constant during the response period of rapidly rising platelet counts, then a reduction in the average amount of IgG per platelet could be explained simply by the greater number of platelets available for antibody binding. Additionally, the total body load of platelet-specific antibody could increase (in the absence of an increased rate of antibody production) due to a decreased rate of removal of antibody-platelet complexes in the RES.

We undertook the present study to determine if we could find evidence for suppression of platelet antibody production during the acute response to high-dose gammaglobulin therapy by estimating the amount of platelet-binding IgG, both
in the plasma and associated with platelets, before and following a therapeutically effective treatment regimen with gammaglobulin. Our data do not suggest that the amount of platelet antibody was reduced by this therapy. We have previously published data to show that preparations of therapeutic gammaglobulin can down modulate human monocyte IgG-Fc receptor binding of IgG-coated platelets in vitro. Therefore, we conclude that in both pediatric and adult ITP patients, the short-term therapeutic effects of high-dose gammaglobulin are more likely to be mediated by inhibition of IgG-Fc receptor function in the RES or by additional factors other than reduction in the amount of whole-body platelet-reactive antibodies.

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