Thrombocytopenia caused by the development of antibodies to thrombopoietin

Junzhi Li, Chun Yang, Yuping Xia, Amy Bertino, John Glaspy, Michael Roberts, and David J. Kuter

Thrombocytopenia developed in some individuals treated with a recombinant thrombopoietin (TPO), pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF). Three of the subjects who developed severe thrombocytopenia were analyzed in detail to determine the cause of their thrombocytopenia. Except for easy bruising and heavy menses, none of these subjects had major bleeding episodes; none responded to intravenous immunoglobulin or prednisone. Bone marrow examination revealed a marked reduction in megakaryocytes. All 3 thrombocytopenic subjects had antibody to PEG-rHuMGDF that cross-reacted with endogenous TPO and neutralized its biological activity. All anti-TPO antibodies were immunoglobulin G (IgG); no IgM antibodies to TPO were detected at any time. A qualitative assay for IgG antibody to TPO was developed and showed that the antibody concentration varied inversely with the platelet count. Anti-TPO antibody recognized epitopes located in the first 163 amino acids of TPO and prevented TPO from binding to its receptor. In 2 subjects, endogenous TPO levels were elevated, but the TPO circulated as a biologically inactive immune complex with anti-TPO IgG; the endogenous TPO in these complexes had an apparent molecular weight of 95 000, slightly larger than the full-length recombinant TPO. None of the subjects had atypical HLA or platelet antigens, and the TPO cDNA was normal in both that were sequenced. Treatment of one subject with cyclosporine eliminated the antibody and normalized the platelet count. These data demonstrate a new mechanism for thrombocytopenia in which antibody develops to TPO; because endogenous TPO is produced constitutively, thrombocytopenia ensues.

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subjects in accordance with the Helsinki protocol, and blood samples were collected under these guidelines. Large volumes of plasma from subjects no. 1 and 3 were obtained from therapeutic plasmapheresis treatments.

Four healthy subjects and one subject with severe aplastic anemia served as controls. Serum and platelet-rich plasma were prepared as previously described.14

Materials
PEG-HuMGDF and rHuTPO were generous gifts from Amgen (Thousand Oaks, CA). Agarose-conjugated proteins A, G, and L, insoluble protein A, immunoglobulin G (IgG) subtype-specific anti sera, and Western blotting reagents were obtained from Sigma (St Louis, MO) and Novex (San Diego, CA). Polyclonal, affinity-purified goat IgG versus human TPO was purchased from R & D Systems (Minneapolis, MN). Affinity-purified rabbit IgG versus human TPO was a generous gift from Kirin Pharmaceutical (Tokyo, Japan).

Clinical assays
Assays for antibody to platelet antigens were performed at the Blood Center for Southeastern Wisconsin (Milwaukee, WI) by indirect immunofluorescence by flow cytometry, antigen capture ELISA (ACE) using GPIb/IX, GPIV, and class I HLA, modified ACE using GPIIb/IIIa, and GPIa/IIa. HLA and platelet phenotype were determined using both serology and polymerase chain reaction methods. DNA was harvested from whole blood samples from subjects and the TPO open reading frame sequenced by standard polymerase chain reaction methods using overlapping primers.

Immunochemical methods
TPO concentration was measured by using a sensitive enzyme-linked immunosorbent assay (ELISA) assay with a detection limit of 17 pg/mL. A total of 15 to 25 mCi/mg (555-925 mBq/mg) 125I-rHuTPO was prepared as previously described14 and retained its biological activity. Immunoprecipitation was performed by adding agarose beads conjugated with protein A, G, or L to 20 to 50 µL serum samples and incubating at room temperature for 30 to 60 minutes followed by centrifugation at 3500×g for 10 minutes. The isolated immunoprecipitate was washed once with phosphate-buffered saline (PBS) and the content of 125I-rHuTPO measured in a gamma counter. Anti-TPO IgG subtypes were measured by incubating 20 to 50 µL aliquots of subject sera with about 250 000 cpm of 125I-rHuTPO prepared as described previously and retained its biological activity. Immunoprecipitation was performed by adding agarose beads conjugated with protein A, G, or L to 20 to 50 µL serum samples and incubating at room temperature for 30 to 60 minutes followed by centrifugation at 3500×g for 10 minutes. The isolated immunoprecipitate was washed once with phosphate-buffered saline (PBS) and the content of 125I-rHuTPO measured in a gamma counter. Anti-TPO IgG subtypes were measured by incubating 20 to 50 µL aliquots of subject sera with about 250 000 cpm of 125I-rHuTPO prepared as described previously and retained its biological activity. Immunoprecipitation was performed by adding agarose beads conjugated with protein A, G, or L to 20 to 50 µL serum samples and incubating at room temperature for 30 to 60 minutes followed by centrifugation at 3500×g for 10 minutes. The isolated immunoprecipitate was washed once with phosphate-buffered saline (PBS) and the content of 125I-rHuTPO measured in a gamma counter.

TPO bioassay
A BaF3-mpl cell line was developed10 and 4 × 10^6 cells incubated for 48 hours in 100 µL culture medium containing various concentrations of interleukin-3 (IL-3), rHuTPO, or PEG-HuMGDF in the presence of or absence of IgG fractions from healthy or thrombocytopenic subjects. The extent of cell growth was measured by assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.15

Assays for antibody to TPO
All patients were subjected to frequent qualitative screening ELISA assays for detection of antibody to TPO16 during their participation in these clinical studies.

A more sensitive and quantitative assay for TPO antibody was developed to assess the thrombocytopenic subjects. Quantification of human anti-TPO antibody was based on the amount of 125I-rHuTPO that coprecipitated with subject IgG following treatment with protein A or G. In brief, subject samples or rabbit standard were incubated with about 250 000 cpm of 125I-rHuTPO and the total IgG content of the sample immunoprecipitated using insoluble protein A-agarose or protein G-agarose. After centrifugation, the pellet was washed with PBS, and its 125I-rHuTPO content was measured in a gamma counter. Test samples were compared with a standard curve generated with the rabbit anti-TPO antibody, and the amount of human anti-TPO antibody was expressed in micrograms per milliliter of the standard. This assay is reproducibly sensitive to 0.075 µg/mL rabbit anti-TPO IgG. Using 60 normal human serum samples, a normal cutoff (average + 3 SD for normal samples) of about 0.15 µg/mL has been established for this assay. This assay has a sensitivity of 100% and specificity of 96% for the clinical presence of antibody to TPO in humans.17

125I-rHuTPO competition binding assays
To measure the specificity of binding of IgG to TPO, 125I-rHuTPO was incubated with subject serum in the presence of various concentrations of PEG-HuMGDF, rHuTPO, or TPO peptides for 1 hour at room temperature. The TPO peptides tested contained the following TPO amino acid residues: 1-17, 12-28, 23-40, 24-32, 28-36, 32-40, 34-50, 45-60, 55-70, 65-81, 75-92, 87-104, 99-116, 111-127, 112-138, 133-149, 144-159, 154-163, 20-28, 24-32, 28-36, 32-40, 109-117, 114-122, 117-125, and 121-129. After addition of protein G-agarose beads, the reaction was incubated for another hour. The beads were washed twice, and their 125I-rHuTPO content was measured in a gamma counter.

To measure the effect of subject IgG on TPO binding to its platelet receptor, 125I-rHuTPO was incubated with 300 µL platelet-rich plasma in the presence of serial dilutions of subject serum for 1 hour at room temperature. The platelets were collected by centrifugation, washed twice with PBS, and their 125I-rHuTPO content measured. The supernatant was then collected, incubated with protein G-agarose beads for 1 hour at room temperature, the beads collected by centrifugation, washed, and their 125I-rHuTPO content measured.

Results
Clinical histories
Subject no. 1. A previously healthy 49-year-old female volunteer received 3 injections of PEG-HuMGDF at the times indicated in Figure 1A. Her platelet count rose to an equal extent after the first 2 injections but rose much less after the third. On study day 133 the platelet count fell below 180 × 10^9/L, and her platelet counts 1 and 12 hours after transfusion were 150 × 10^9/L and 54 × 10^9/L, respectively. The erythrocyte sedimentation rate was 39 mm/h, and the hematocrit and WBC count were normal. There was no splenomegaly. On study day 153, prednisone at 60 mg daily was initiated and continued for 25 days with no
she was well and received no platelet transfusions. The spleen was not palpable. On study days 749 to 751 she received daily infusions of 31 g IVIG, which was complicated by anaphylaxis, but she had no rise in platelet count. Over 772 study days, the hematocrit dropped from 40.2 to 25.9 and, because of excessive menstrual bleeding at a platelet count of $11 \times 10^9/L$, she underwent an uncomplicated dilation and curettage without platelet transfusion on study day 777. Postoperatively the hematocrit rose to 32.6 with iron supplementation. Despite normal vitamin $B_12$ and folic acid measurements on multiple determinations, the mean cell volume rose from normal (84.5-97.8 fL) to 115.9 fL with a mean cell hemoglobin of 40.7 pg/rbc (normal = 27.7-33.4 pg/rbc) by study day 832. Bone marrow chromosome analysis was normal. The prestudy WBC count of $5.7 \times 10^9/L$ to $7.3 \times 10^9/L$ also declined over this period to $3.3 \times 10^9/L$ to $4.4 \times 10^9/L$. Cyclosporine (125 mg twice daily) was begun on day 832; on day 869 the platelet count was $37 \times 10^9/L$, and by day 919 the platelet count was $175 \times 10^9/L$. Cyclosporine was discontinued on day 1066 at a WBC count of $7.7 \times 10^9/L$, hematocrit 41, mean cell volume 103 fL, and platelets $235 \times 10^9/L$.

**Subject no. 3.** A 61-year-old woman with stage IIIA non–small cell lung cancer and a history of scleroderma and Raynaud phenomenon was enrolled in a clinical trial in which she received 4 cycles of chemotherapy with paclitaxel (275 mg) and carboplatin (890 mg). Twenty-four hours after the administration of chemotherapy, she received PEG-rHuMGDF at a dose of 5 $\mu$g/kg subcutaneously daily for 7 days and filgrastim $5 \mu$g/kg subcutaneously daily for 14 days for all 4 cycles. Her platelet count fell after each administration of chemotherapy but rebounded less well after each cycle. After the fourth chemotherapy cycle, the platelet count fell below $20 \times 10^9/L$ on day 84. The hematocrit was 21 with an absolute neutrophil count of $0.6 \times 10^9/L$. With multiple RBC and platelet transfusions and filgrastim, her hematocrit and WBC count returned to a normal range, but the platelet count never rose above $20 \times 10^9/L$ without platelet transfusions. A screening test for antibody to PEG-rHuMGDF was positive on study day 92. On study day 152 the platelet count was $2 \times 10^9/L$ with an absolute neutrophil count of $6.4 \times 10^9/L$. Plasmapheresis was performed on day 148 with no improvement. Marked progression of cancer in the chest, abdomen, and pelvis was noted on study day 177. Brain metastases were subsequently irradiated, and the subject died on study day 226 due to disease progression.

**Clinical assessment of thrombocytopenic subjects**

All 3 thrombocytopenic subjects underwent bone marrow examination (on days 142, 777, and 146 for subjects no. 1, 2, and 3, respectively), and all had a marked decrease in bone marrow megakaryocytes. The 2 nonchemotherapy subjects, no. 1 and no. 2, had normal cellular marrows with no evidence of other hematologic disorders (and normal chromosome and flow cytometry analyses) but with megakaryocytes that were no more than 5% to 10% of that present in normal marrow (Figure 2A). This is markedly decreased from what would have been seen in the marrow of a subject with ITP and a similar extent of thrombocytopenia (Figure 2B). Moreover, the megakaryocytes that were present were small and hypolobulated. The marrow of subject no. 3 was also devoid of significant numbers of megakaryocytes and showed postchemotherapy changes of hypocellularity (5%-10%) but with normal maturation of myeloid and erythroid lineages.

The immunologic status of these subjects was analyzed for host factors that might have contributed to the development of antibodies to TPO (Table 1). Only subject no. 3 had any history of

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**Figure 1.** Platelet counts and anti-TPO antibody concentration. The platelet count (closed circles) is inversely related to anti-TPO antibody concentration (open circles) in thrombocytopenic subjects no. 1 (A), 2 (B), and 3 (C). PEG-rHuMGDF treatment is indicated by vertical lines and chemotherapy by the arrows. Horizontal line denotes normal cutoff of assay for anti-TPO IgG. In panel B, the solid bar indicates cyclosporine administration and solid triangles denote negative anti-TPO antibody screening tests. The anti-TPO antibody titer on day 56 for subject no. 1 (A) is slightly above the cutoff value.
autoimmune disorder. HLA class I and class II antigens and platelet antigens showed no consistent pattern. Four different tests for antiplatelet antibodies were used, and only one was positive: Subject no. 1 had a modest elevation in platelet-associated antibody that was consistent with prior pregnancy or transfusion.

To determine whether these subjects had a polymorphism of their TPO coding sequence, complementary DNA (cDNA) was prepared from leukocytes from subjects no. 1 and 2 and the TPO cDNA sequenced. Both showed a wild-type TPO coding sequence identical to that found in PEG-rHuMGDF and rHuTPO.

Assessment of TPO levels

Median normal levels of endogenous TPO are approximately 179 pg/mL (range, 45-400 pg/mL). In patients with aplastic anemia and thrombocytopenia to the degree seen in the subjects studied, TPO levels range from 1500 to 2500 pg/mL. Thrombocytopenic subjects no. 2 and 3 had inappropriately normal or low levels of TPO in the circulation, but subject no. 1 had a marked elevation of TPO (Figure 3A).

To assess whether a factor neutralizing endogenous TPO might be present, a screening test was performed in which 1500 pg rHuTPO was added to 1 mL samples from each of the subjects and the total TPO concentration measured again. In 4 healthy individuals, the addition of 1500 pg rHuTPO increased the total TPO level from 57 ± 34 pg/mL to 1314 ± 67 pg/mL, as expected. Although the total TPO level in subject no. 2 rose by an extent similar to the healthy subjects, the total TPO concentration in subjects no. 1 and 3 rose much less or not at all, suggesting the presence of a neutralizing activity.

Presence of TPO-IgG immune complexes

To explore the etiology of the elevated endogenous TPO level in subject no. 1, a serum sample (day 469) was tested for its TPO content before and after treatment with beads conjugated with protein A. The protein A treatment specifically removed 90% to 93% of the IgG in the sample. As shown in Figure 3B, 87% of the endogenous TPO in subject no. 1 coprecipitated with the IgG.

**Table 1. Clinical characteristics of subjects**

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nt, indicates not tested.

*See "Materials and methods" for assay descriptions.
†Previously established normal levels.
(E1-E4) were analyzed. Lanes containing rHuTPO (T) and molecular weight markers for comparison. For each sample, 4 equivalent sequential column elution fractions from an aplastic anemia subject, and conditioned medium from HepG2 cells is shown methods.” Endogenous TPO isolated from plasma from a healthy subject, plasma content assessed by SDS-PAGE and Western blot as described in “Materials and full-length, endogenous TPO. Immune complexes were isolated and their TPO level and from a subject with a normal TPO level as well as serum of a subject with aplastic anemia who had a high endogenous line. Endogenous TPO is slightly larger than the rHuTPO that runs from the conditioned media of a TPO-producing hepatoma cell.

Upon addition of rHuTPO to a serum sample from subject no. 1, 81% of the total TPO coprecipitated with the IgG. Immunoprecipitation of IgG from normal control serum did not reduce the TPO content either before or after the addition of rHuTPO. In different serum samples from subject no. 3, the endogenous TPO content ranged from undetectable (Figure 3A) to modestly elevated (Figure 3B). When present in detectable amounts in subject no. 3, 85% of the endogenous TPO coprecipitated with IgG. These results suggest that endogenous TPO was circulating as a presumably inactive TPO-IgG complex in subject no. 1 and to a lesser extent in subject no. 3.

To determine whether the endogenous TPO in the immune complex in subject no. 1 contained full-length TPO or TPO degradation products, the immune complexes were collected and the associated TPO was purified on a TPO affinity column, electrophoresed, and subjected to Western blotting. As shown in Figure 3C, the immune complexes from subject no. 1 contained a TPO band migrating at a molecular weight of about 95 kD. This band was identical in size to the endogenous TPO purified from the plasma as a complex with IgG. However, upon addition of increasing amounts of serum from subject no. 3, the extent of platelet binding was reduced to 5% and the extent of IgG-associated 125I-rHuTPO rose to 95%.

IgG was the only antibody type that bound to TPO. Serum samples from all 3 subjects were incubated with 125I-rHuTPO and

PEG-rHuMGDF and rHuTPO (Figure 4). IgG from subject no. 3 bound to native rHuTPO and PEG-rHuMGDF on Western blots (Figure 4A) but did not bind to denatured and reduced HupTPO or PEG-rHuMGDF (data not shown). Purified IgG from subject no. 1 as well as from healthy subjects repeatedly failed to bind to either native or denatured PEG-rHuMGDF or rHuTPO on Western blots.

The purified IgG fractions were tested for their ability to inhibit the biological activity of TPO. A BaF3-mpl cell line15 dependent upon either IL-3 or TPO grew maximally in the presence of IL-3, rHuTPO, or PEG-rHuMGDF. The IgG fractions from subjects no. 1 and 3 had no inhibitory effect on the growth of these cells when stimulated by IL-3 (Figure 4B). However, in the presence of rHuTPO, IgG from subject no. 1 reduced maximal cell growth by 90% and IgG from subject no. 3 reduced cell growth by approximately 48%. When the cells were grown in the presence of PEG-rHuMGDF, IgG samples from both subjects reduced cell growth by 86% to 89%. IgG from healthy subjects did not affect the growth of cells in the presence of any of these hematopoietic growth factors.

Antibody to TPO neutralized the biologic activity of rHuTPO by preventing the binding of rHuTPO to its receptor. 125I-rHuTPO was added to platelet-rich plasma from healthy individuals and its binding to the platelet TPO receptor measured. As shown in Figure 5A, in the absence of serum from subject no. 3, all the 125I-rHuTPO bound to platelets and none could be immunoprecipitated from the plasma as a complex with IgG. However, upon addition of increasing amounts of serum from subject no. 3, the extent of platelet binding was reduced to 5% and the extent of IgG-associated 125I-rHuTPO rose to 95%.

Figure 3. Plasma from thrombocytopenic subjects contained antibody to TPO and circulating TPO-IgG complexes. (A) TPO levels before (light bars) and after (dark bars) addition of HupTPO. The TPO concentration was determined before and after the addition of 1500 pg/mL rHuTPO to serum samples from healthy subjects (N1-N4) and from subjects no. 1, 2, and 3. (B) Endogenous TPO circulates as a TPO-IgG complex. The TPO concentration was determined before (–) and after (+) immunoprecipitation with protein A beads, both before (gray bars) and after (black bars) addition of 1500 pg/mL rHuTPO to serum samples from a healthy control subject and subjects no. 1 and 3. The horizontal line indicates the detection limit for TPO ELISA assay. (C) The immune complexes from subject no. 1 contain full-length, endogenous TPO. Immune complexes were isolated and their TPO content assessed by SDS-PAGE and Western blot as described in “Materials and methods.” Endogenous TPO isolated from plasma from a healthy subject, plasma from an aplastic anemia subject, and conditioned medium from HepG2 cells is shown for comparison. For each sample, 4 equivalent sequential column elution fractions (E1-E4) were analyzed. Lanes containing rHuTPO (T) and molecular weight markers (M) are indicated. The arrow denotes the position of endogenous TPO.

Figure 4. IgG from thrombocytopenic subjects binds to TPO and neutralizes its biological activity. (A) rHuTPO (lanes 2,4,6) and PEG-rHuMGDF (lanes 1,3,5) were subjected to native PAGE (10% Tris-glycine, pH 8.3, without SDS, heat denaturation, or reduction) and blotted with IgG from subject no. 3 (lanes 1,2). IgG from a control subject (lanes 3,4), or a polyclonal goat antibody to TPO (lanes 5,6). (B) BaF3-mpl cells were grown in the presence of varying concentrations of IL-3 (gray bars), rHuTPO (black bars), or PEG-rHuMGDF (white bars) in the absence (–) or presence (+) of IgG from subjects no. 1 or 3 and the effect on cell growth measured.
PEG-rHuMGDF and rHuTPO compete equally for binding to anti-TPO antibody. IgG subtype-specific sera was omitted from the control (C) lane. (C) 125I-rHuTPO along with various amounts of unlabeled rHuTPO or 125I-rHuTPO was immunoprecipitated by the protein G and none upon removal of the IgG–protein G complexes by centrifugation, immunoprecipitated with protein G, which removes only IgG. Upon removal of the IgG–protein G complexes by centrifugation, the supernatant was then immunoprecipitated with protein L, which binds not only IgG but also IgM, IgA, IgD, and IgE. All of the 125I-rHuTPO was immunoprecipitated by the protein G and none by the protein L at any time point measured (data not shown), indicating that all of the antibodies to TPO were IgG.

The IgG subtype of the antibody to TPO was analyzed using subtype-specific monoclonal antibodies. As shown for subject no. 3, most of the anti-TPO antibody in this subject was IgG4 (Figure 5B, Table 1). This is in marked contrast to the normal IgG distribution in humans (Table 1). For subject no. 1 there was an increased amount of IgG4 but less than that seen in subject no. 3 (Table 1).

Anti-TPO antibody bound to epitopes within the first 163 amino acids of TPO. Subject serum samples were incubated with 125I-rHuTPO along with various amounts of unlabeled rHuTPO or PEG-rHuMGDF and the IgG-TPO complexes immunoprecipitated with protein A. As shown in Figure 5C, both unlabeled rHuTPO and PEG-rHuMGDF competed equally for antibody binding, suggesting that only the first 163 amino acids of TPO contained the epitopes recognized. A series of shorter peptides within this region failed to compete for binding and suggested that more complex, nonlinear epitopes were involved. Identical studies were also performed with sera from subjects no. 1 and 2 with the same results.

**Time course of anti-TPO antibody development in subjects.** Using a sensitive and specific assay for IgG antibody to TPO, the time course of appearance of the antibodies was quantified in all 3 subjects (Figure 1). In none of the subjects was antibody detected prior to the injection of PEG-rHuMGDF, and none showed any IgM antibody response. In subject no. 1 (Figure 1A), trace amounts of antibody first appeared on day 56 when the platelet count had just started to fall below baseline, was markedly elevated by day 137 at a platelet count of $53 \times 10^9/L$, and was maximal at day 147 at a platelet count of $21 \times 10^9/L$. The antibody concentration decayed over the next 686 study days, and with resolution of the thrombocytopenia no more antibody to TPO was detectable in the serum of this subject. For subject no. 2 (Figure 1B), the elevated antibody level, first quantified on day 202, slowly declined and following treatment with cyclosporine rapidly disappeared. For subject no. 3 (Figure 1C), measurable amounts of antibody were first detected on day 71 when the platelet count was $152 \times 10^9/L$, became markedly elevated when the platelet count failed to rise after the third cycle of chemotherapy, and remained at a very high plateau thereafter during the entire course of her thrombocytopenia despite plasmapheresis on day 148. Notably, the concentration of antibody varied greatly among the subjects: For the same degree of thrombocytopenia, subject no. 2 had a maximal antibody titer of approximately 2.1 µg/mL versus 8 µg/mL for subject no. 1 and 13 µg/mL for subject no. 3.

**Discussion**

Thrombocytopenia occurred unexpectedly in healthy volunteers and cancer chemotherapy patients treated with PEG-rHuMGDF. Thrombocytopenia developed after as little as 2 injections in the subjects studied here, and antibody to TPO was detected as early as 56 days after the initial injection. Except for easy bruising and increased menstrual bleeding, the subjects had no major bleeding episodes. Although there was a transient response to plasmapheresis in 1 subject, none of the subjects responded to corticosteroids or IVIG, and the 2 who received platelet transfusions had appropriate increases in the platelet count. Two subjects have recovered, and 1 died of her cancer. None of the 3 subjects had any unusual HLA or platelet phenotype, and all lacked evidence for antiplatelet antibody. Although polymorphism of the open reading sequence of TPO has so far not been described, the TPO cDNA from 2 subjects was sequenced and found to be normal.

All 3 subjects had a marked decrease in bone marrow megakaryocytes like that seen in homoygous knockout mice deficient in TPO or its receptor. As in those knockout animals, the megakaryocytes were small, hypolobulated, and had scant cytoplasm. Although formal platelet kinetic studies could not be performed in the subjects, their bone marrow appearance was consistent with decreased bone marrow production of platelets and differed distinctly from that in ITP. On average, the nadir platelet counts in all 3 subjects were 6% to 8% of baseline, somewhat lower than the 10% to 15% of normal found in the knockout animals. In one subject the WBC and RBC values also became modestly decreased. Assays could not be performed to see if these subjects had reduced levels of myeloid and erythroid progenitor cells, as has been reported for the knockout mice.
The thrombocytopenia was temporally related to the appearance of antibody to TPO, and the antibody disappeared in both subjects who recovered. Small amounts of antibody to TPO could be detected in 2 of the subjects when the platelet count fell below baseline and before it decreased below $10^9/L$. In general, the antibody level in any one subject was inversely proportional to the platelet count, but between subjects there was a variation in peak titer despite similarly low platelet counts.

Even using a very sensitive assay, no IgM response could be detected at early time points. All of the antibody to TPO was IgG despite similarly low platelet counts. All of the antibody to TPO was IgG titer despite similarly low platelet counts. Although naturally occurring antibody to GM-CSF is rare, 95% of the TPO biological activity using a TPO-dependent cell line. Finally, using an assay directly detecting the ability of IgG to bind to $^{125I}$-rHuTPO, all subjects had IgG that specifically bound $^{125I}$-rHuTPO.

The anti-TPO IgG from subject no. 3 bound both rHuTPO and PEG-rHuMGDF but only in their native forms. The lack of binding to denatured and reduced proteins suggested that simple, linear epitopes were not involved. IgG from subject no. 1 did not bind to TPO reproducibly on Western blots, probably due to the lesser titer of antibody present or the fact that much of the antibody existed as an IgG-TPO complex. Because PEG-rHuMGDF contains only the first 163 of the 332 amino acids in rHuTPO, the finding that rHuTPO and PEG-rHuMGDF both competed equally for antibody binding suggests that all antigenic determinants were located within the first 163 amino acids.

In 2 subjects the endogenous TPO level was inappropriately low for the degree of thrombocytopenia and reflected the binding of IgG to endogenous TPO and its subsequent removal by the reticuloendothelial system. However, endogenous TPO was persistently elevated in subject no. 1 but circulated as a presumably inactive IgG-TPO immune complex. The TPO component of the immune complex had a molecular weight identical to that found in endogenous TPO isolated from sera from healthy and aplastic anemia subjects. These data also serve to demonstrate for the first time that endogenous TPO has a molecular weight of about 95 kd, slightly higher than the approximate 90 kd rHuTPO made in Chinese hamster ovary cells and used in other clinical studies.

Antibody to PEG-rHuMGDF occurred much more frequently in the immunocompetent healthy volunteers (13 of 325) than it did in the immunocompromised chemotherapy patients (4 of 650). A similar finding has been observed following the administration of megakaryostim, a nonglycosylated granulocyte-macrophage colony-stimulating factor (GM-CSF) produced in Escherichia coli. Although naturally occurring antibody to GM-CSF is rare, 95% of immunocompetent patients who received megakaryostim developed antibody to GM-CSF whereas virtually no immunocompromised patients did.

In clinical studies with rHuTPO in more than 500 cancer chemotherapy patients, thrombocytopenia attributed to anti-TPO antibodies has not yet been reported despite the appearance of a partially neutralizing antibody in one subject. This may be due to the native structure of rHuTPO unlike the modified PEG-rHuMGDF. A second possible reason for the lack of thrombocytopenia with rHuTPO in these clinical studies was that rHuTPO was always given intravenously whereas PEG-rHuMGDF was always given subcutaneously. In experiments in rats, the subcutaneous but not intravenous injection of recombinant PEG-ratMGDF caused thrombocytopenia. Because the TPOs are potent stimulators of dendritic cells (J.L., unpublished observations, 1999), one possibility is that subcutaneous PEG-rHuMGDF has acted as an adjuvant for itself by stimulating antigen-presenting cells.

Platelet production may be especially sensitive to the appearance of antibody to TPO because TPO is constitutively synthesized in the liver and its rate of production does not rise during thrombocytopenia. Platelet production is regulated by a simple feedback loop whereby the TPO level is controlled directly by the circulating platelet count; the addition of an antibody-mediated clearance system would markedly alter this homeostatic mechanism. This is in contrast to RBC production where renal synthesis of erythropoietin (EPO) can be markedly increased and might be less sensitive to antibody to EPO. Only rare cases of pure red cell aplasia due to naturally occurring antibody to EPO have been described. Equally uncommon are antibodies formed against recombinant human EPO.

Low levels of antibody to G-CSF have been found in 11% of healthy adults and in 13% of cord blood samples but with no effect on the neutrophil count. These autoantibodies rose after administration of G-CSF but lacked clinical significance. This is in contrast to the aforementioned antibodies formed after the administration of megastim that reduced the half-life and biological effect of the recombinant drug but did not affect baseline WBC production.

The finding that antibody to TPO can cause significant thrombocytopenia has several major implications. First, it illustrates the wide variation in the rate of antibody formation to recombinant polypeptide therapeutics. Antibodies to recombinant EPO and G-CSF have been uncommon in patients treated with these hematopoietic growth factors and have rarely caused cytopenias. However, antibodies to recombinant α-interferon, factor VIII, and human insulin have been associated with clinical problems.

Second, these studies describe a new mechanism for thrombocytopenia in humans. Because TPO is constitutively produced, the appearance of a neutralizing antibody to TPO reduces platelet production by 95% and is similar to animals in which the TPO gene has been knocked out or in which autoantibodies to TPO have been generated. Although iatrogenic in the subjects described here, one spontaneous case of thrombocytopenia due to antibody to TPO has been described. Both this patient and our subject no. 2 responded to cyclosporine, which supports the use of this therapy. Whether other cases of acquired amegakaryocytic thrombocytopenia are due to antibody to TPO is unknown; loss of function mutations of the TPO receptor is a cause of congenital amegakaryocytic thrombocytopenia.

Finally, the long-term complications of persistent antibody to TPO on other lineages may be of concern. Subject no. 2 developed a reversible macrocytic anemia and a low WBC count as well as the marked thrombocytopenia. Because pluripotential stem cells and progenitors of all lineages depend on TPO to prevent apoptosis, depletion of early progenitor cells and concomitant pancytopenia might ensue, as has been seen in some children born with amegakaryocytic thrombocytopenia due to a defective TPO receptor. However, mice deficient in TPO or its receptor are thrombocytopenic but have normal levels of RBCs and WBCs despite reduced progenitor cells of all lineages.
References

Thrombocytopenia caused by the development of antibodies to thrombopoietin

Junzhi Li, Chun Yang, Yuping Xia, Amy Bertino, John Glaspy, Michael Roberts and David J. Kuter