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Prospective Screening of 205 Patients With ITP, Including Diagnosis, Serological Markers, and the Relationship Between Platelet Counts, Endogenous Thrombopoietin, and Circulating Antithrombopoietin Antibodies

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Immune thrombocytopenia purpura (ITP) is characterized by destruction of circulating platelets and the presence of antiplatelet antibodies. Many of the current immunomodulatory therapies act by reducing platelet destruction and usually do not have a lasting effect. This prospective, exploratory study characterized patients with ITP by identifying their demographic and comorbid clinical factors, use of treatments, serologic markers of autoimmunity, and possible relationships between platelet counts, concentrations of endogenous thrombopoietin (eTPO), and the presence of circulating anti-TPO antibodies. Data including medical history and laboratory evaluations were collected at a single patient visit on 205 patients (19 children, 186 adults). Reported histories revealed a 5% rate of thrombotic/ischemic events. Autoimmune markers including direct antiglobulin test and antinuclear antibodies were found more frequently than in the normal population; antiplatelet antibody testing was not done. eTPO concentrations were comparable to concentrations found in healthy volunteers. Our study confirmed that no significant inverse correlation occurred between circulating concentrations of eTPO and platelet counts in patients with ITP (Spearman $r = -0.15$). Two of the 205 patients tested (1%) had neutralizing activity of recombinant human TPO in a biological assay; however, this activity was confirmed to be anti-TPO antibody in only 1 patient. Am. J. Hematol. 76:205–213, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Immune thrombocytopenia purpura (ITP) is a disease characterized by the presence of antiplatelet antibodies and the peripheral destruction of circulating platelets, although the ability to meaningfully measure these autoantibodies remains elusive. Patients with ITP may fail to achieve or maintain a durable remission when treated with currently available medical and surgical therapies, may experience significant side effects from treatment, or may need ongoing therapy to maintain an adequate platelet count [1]. A number of studies have suggested that patients affected by this disease

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may have inadequate platelet production as a contributing factor to their thrombocytopenia [2–4]. With the discovery of thrombopoietin (TPO), the major hormone involved in the stimulation of megakaryocyte and platelet formation, new tools have emerged to study thrombopoiesis and its alterations in thrombocytopenic disorders such as ITP [5–9]. Serum concentrations of endogenous TPO (eTPO) reflect its constitutive production by the liver and its clearance through binding and internalization by Mpl, the TPO receptor found on the surfaces of platelets and megakaryocytes [10,11]. Several studies have reported increased amounts of eTPO in patients with thrombocytopenia secondary to aplastic anemia, amegakaryocytic thrombocytopenia, and myeloablative chemotherapy [12–19]. In contrast, eTPO levels are not increased in patients with ITP, despite the markedly reduced number of circulating platelets [14–16,20]. In fact, the range of eTPO concentrations in patients with ITP overlaps with the range in healthy subjects [15–17,20,21]. A potential explanation is that patients with ITP have a rapid clearance of eTPO either by its binding to platelets which are being consumed and/or by binding to the increased number of bone marrow megakaryocytes present in this disease. These observations suggest that use of thrombopoietic factors could be effective as a treatment for patients with ITP.

We initiated a prospective, exploratory study of 205 patients with ITP who were of age >5 years and for whom other causes of thrombocytopenia had been excluded. The first goal was to identify demographic and comorbid clinical factors that might define clinically meaningful subsets of patients, including general information on autoimmunity, thrombosis, and pregnancy in patients with ITP. The second goal was to explore the relationships between platelet numbers, concentrations of eTPO, and the presence (if identified) of circulating autoantibodies that bind and/or neutralize eTPO in a population of ITP patients to gain further insights into the potential role for TPO-like agents in the treatment of ITP.

PATIENTS AND METHODS

Patients

Eleven sites in North America participated in recruiting patients with ITP to the study. The institutional review boards of the participating medical centers approved the study protocol, and all patients gave written informed consent before entry. Patients were eligible if they had ever had a diagnosis of ITP, using criteria based on the American Society of Hematology practice guideline for ITP [22], and were >5 years of age. Children under 5 years of age were not considered appropriate due to the risk associated with phlebotomy. Patients with known underlying causes of thrombocytopenia, such as human immunodeficiency virus, systemic lupus erythematosus, drug-induced thrombocytopenia, bone-marrow disorders, and liver disease, were excluded from study enrollment. No exclusions were made for time since diagnosis, relapse/remission state, or for the use of current/past ITP treatments, including splenectomy.

Study Methods

At a single outpatient visit, patients with ITP enrolled in the study and completed a brief questionnaire to obtain standardized information on patient demographics, medical histories, and certain specific details of ITP history including ITP treatments and concurrent medications within 30 days of study. Questionnaires were administered by a trained study coordinator or the principal investigator. The study coordinator or investigator was available to answer questions from the patients regarding the questions included in the questionnaire. Source documentation was not required to be used to validate the information obtained. Dates for ITP treatments more than 30 days in the past were not collected. To further delineate the relationship between ITP, thrombosis, and miscarriage, a follow-up, IRB-approved questionnaire was sent to each center to obtain additional information from patients who recorded these events.

Blood samples were obtained once at the single visit and were evaluated by a central laboratory (Quest Diagnostics, Van Nuys, CA) to provide less variation across sites. The samples were processed within 24 hr of being drawn. Complete blood counts, including an automated leukocyte differential and platelet count; tests for immune markers, including rheumatoid factor, antinuclear antibodies, and direct antiglobulin test; and serum chemistries, including measurement of at least alanine transaminase, aspartate transaminase, and other renal and hepatic function tests, were measured. Additional samples were sent to Amgen Inc. (Thousand Oaks, CA) for assessment of serum eTPO and anti-TPO antibodies. Smaller blood volumes were obtained from children participating in the study.

Thrombopoietin Assay

Samples were assayed using a modification of a commercially available TPO ELISA Kit (R&D Systems, Minneapolis, MN). Briefly, microtiter plates and all other test reagents were purchased from R&D Systems with the exclusion of recombinant human TPO (Amgen Inc.) that was used as the standard. Amounts were determined using a colorimetric reaction (tetramethylbenzidine-HRP) (R&D Systems). Plates were read on a standard spectrophotometer at 450/650 nm wavelengths.

The lower limit of quantification was defined as the lowest quality control that met a ±20% coefficient of variation and a ±20% analytical recovery, and it was determined to be 0.02 ng/mL. The measurable analytical range for eTPO in normal human serum samples ranged from 0.02 to 0.64 ng/mL. The TPO assay has been validated for serum and plasma with matched samples from healthy subjects. No measurable differences were observed between sample types (data on file, Amgen Inc.). Samples for the assays were drawn under standard clinical conditions. The serum was separated, frozen, and shipped to the central laboratory for testing.

Cell-Based Assay for Detection of TPO-Neutralizing Antibodies/Activity

A bioassay to detect TPO neutralizing antibodies was used the 32D murine cell line (clone 23) [23] that had been transfected with the human gene encoding for Mpl, the receptor for TPO. Cells in this line are able to proliferate in response to recombinant human TPO and recombinant murine interleukin-3 (IL-3) [6]. Cells were incubated in 1% test or control normal human serum with recombinant human TPO (125 pg/mL) or recombinant murine IL-3 (20 pg/mL), a control for nonspecific inhibition of cell growth. All test samples were run in both culture systems (recombinant human TPO and recombinant murine IL-3), and cell proliferation was measured using tritiated thymidine (^{3}H) incorporation. The assay was read in counts per minute and expressed as the ratio of thymidine incorporation for the experimental sample compared with a normal pooled human serum control. A rabbit anti-human TPO polyclonal antibody (Amgen Inc.) was used as a positive control for neutralizing activity. The assay was sensitive to approximately 25 μg/mL of the control rabbit anti-human TPO antibody. Samples with a count-per-minute ratio of $\leq$0.49 (recombinant human TPO) and >0.53 (recombinant murine IL-3) were interpreted as positive for the presence of neutralizing activity with no nonspecific inhibition. To confirm that an antibody was responsible for the inhibition of cell growth, a new sample of patient serum was tested in the bioassay after immunodepletion to remove all classes of human IgG. The immunodepletion used batch absorption incubation of diluted serum samples at room temperature for 1 hr with protein-G Sepharose beads in excess (Pierce Biotechnology, Rockford, IL). Results were reported as antibody positive when no inhibitory activity was present after immunodepletion of the sample.

Statistical Analysis

This study was conducted as a survey to identify the demographics of the ITP population and was not expected to show differences between any demographic grouping. Due to the limited number of patients available, the sample size was targeted at 200 patients, which was expected to be a sufficient number of samples to provide adequate testing for the presence of anti-TPO antibodies and the evaluation of eTPO concentrations.

Results were summarized using frequencies and percentages for categorical variables and means, standard deviations, medians, quartiles, and ranges for continuous variables. Correlation analysis was used to explore the relationship between continuous variables, such as platelet counts and eTPO concentrations. Wilcoxon's rank sum test was used for group comparisons.

RESULTS

Patient Demographics

Eleven centers enrolled 205 patients into the study. Of the 205 patients, 19 (9%) were children (defined as aged 5 to 17 years) and 186 (91%) were adults (aged $\geq$18 years). The median age was 42 years, with 37 (18%) patients known to be $\geq$65 years of age and 64% women/girls (Table I).

Study Findings

Diagnosis of ITP. Twenty-one percent of pediatric and 47% of adult patients were diagnosed with ITP during a routine medical examination. In the remaining cases, bleeding symptoms were present (in 79% of pediatric patients and 52% of adult patients; data not available for 1% of adult patients) at the time of diagnosis, and the platelet count was obtained for clinical reasons. The diagnosis of ITP was made by hematologists/oncologists in 58% of cases, general practitioners in 22%, and internists in 12%. In 8% of patients, other specialists, i.e., obstetrician/gynecologists and rheumatologists, made the diagnosis.

ITP Treatment. Forty-one percent of the patients were receiving treatment for ITP at the time of study entry (Table I) with the most frequent current treatments corticosteroids (predominantly prednisone) in 31%, followed by sex hormones (predominantly danazol) in 10%. Eighty-four percent had been treated with prednisone at some time, and only 12% of the patients had never received treatment for ITP. Forty-five percent of the adults reported having received 3 or more types of treatment. Twenty-one percent of the pediatric patients and 45% of the adult patients had had a splenectomy for the treatment of ITP.

Comorbid Conditions. Among the 186 adults studied, 8 (4%) received treatment for diabetes and 18 (10%) for thyroid deficiency within 30 days of the study visit. No apparent association was seen between

TABLE I. Baseline Demographics of Patients Evaluated for the Study*

	Pediatric ($n = 19$)	Adult ($n = 186$)	All patients ($n = 205$)
Sex (n/%)			
Women/girls	11 (58)	120 (65)	131 (64)
Men/boys	8 (42)	66 (35)	74 (36)
Race (n/%)			
White	10 (53)	153 (82)	163 (80)
Black	2 (11)	11 (6)	13 (6)
Hispanic	4 (21)	14 (8)	18 (9)
Other	3 (15)	8 (4)	11 (5)
Age (years)			
Median	14	45	42
Range	9–17	18–90	9–90
Splenectomy (n/%)			
No	15 (79)	102 (55)	117 (57)
Yes	4 (21)	84 (45)	88 (43)
Prior therapy (n/%)[a]			
Any	16 (84)	165 (89)	181 (88)
Prednisone[a]	8 (42)	156 (84)	164 (80)
Anti-D Ig	13 (68)	44 (24)	57 (28)
IV Ig	14 (74)	105 (56)	119 (58)
Cyclophosphamide	0	21 (11)	21 (10)
Azathioprine	4 (21)	24 (13)	28 (14)
Danazol	1 (5)	60 (32)	61 (30)
Vincristine/vinblastine	3 (16)	28 (15)	31 (15)
Other	5 (26)	55 (30)	60 (29)
Current ITP therapy (n/%)			
Any	4 (21)	81 (44)	85 (41)
Prednisone[b]	2 (11)	61 (33)	63 (31)
IV Ig	1 (5)	10 (5)	11 (5)
Cyclophosphamide	0	4 (2)	4 (2)
Azathioprine	1 (5)	9 (5)	10 (5)
Danazol	0	21 (11)	21 (10)

*Abbreviations: Ig, immunoglobulin; IV, intravenous.
[a]Because all therapies are included, the numbers do not add to number of patients.
[b]Predominant type of corticosteriod.

the presence of thyroid disease and red cell autoantibodies.

Thrombotic Events. Ten of 186 adult patients (5%) reported having 18 thrombotic or ischemic events (not miscarriages), 11 of which occurred after diagnosis of ITP. Five (50%) of these patients had had a splenectomy. No thrombotic or ischemic events were reported for pediatric patients. Five events, including 3 in one patient, were arterial, and 13 were venous. Three of the 10 patients were >65 years of age at the time of the events. Table II lists the patient-reported thrombotic events and the relationship of the events to the time of ITP diagnosis.

Pregnancies and Pregnancy Losses. Of the 120 women with ITP included in the study, 69% had had at least 1 pregnancy, with 29 women reporting having had elective terminations or miscarriages. Additional data were obtained about the lost pregnancies from 20 of the 29 patients. In 11 of these 20 women, 13 spontaneous miscarriages occurred and 9 women had an elective termination. Seven of the losses occurred after ITP was diagnosed, of which 3 were spontaneous miscarriages.

TABLE II. Relationship of Patient-Reported Thrombotic Events and ITP Diagnosis

Type of event reported (n)	Patients (n)	Patients with postdiagnosis events (n)
Any thrombotic event (18)	10	6
Deep vein thrombosis (9)	6	3[a]
Pulmonary embolism (2)	2[b]	1
Myocardial infarction (1)	1	0
Transient ischemic attack (3)	1	1
Stroke (1)	1	1
Phlebitis (2)	1	0

[a]One patient had deep vein thrombosis before and after diagnosis of ITP.
[b]One patient with pulmonary embolism also had deep vein thrombosis.

Laboratory Tests. Nine of the 205 patients (4%) were positive or weakly positive for rheumatoid factor, and 31 (15%) were at least weakly positive for antinuclear antibodies. Forty-five (22%) of the patients had a positive direct antiglobulin test. The four patients who took human Rho (D) immune globulin (anti-D) within 30 days of the study entry had positive direct antiglobulin test results (100%) compared with 21% in patients who did not take anti-D within 30 days ($P < 0.01$). Fifty-seven (28%) patients had previously been treated with anti-D, but the time of treatment compared with the day of study entry was undetermined except in 4 patients. Nineteen (10%) patients who had a positive direct antiglobulin test did not report having received anti-D treatment. Twelve patients (6%) tested positive for more than 1 of these 3 autoimmune markers (Table III).

TABLE III. Summary of Autoimmune Markers

	Pediatric ($n = 19$)	Adult ($n = 186$)	All patients ($n = 205$)
Antinuclear antibodies[a] (n/%)			
Negative	13 (68)	161 (87)	174 (85)
Weak positive	4 (21)	18 (10)	22 (11)
Positive	2 (11)	7 (4)	9 (4)
Rheumatoid factor[b] (n/%)			
Negative	18 (95)	176 (95)	194 (95)
Weakly reactive	1 (5)	1 (1)	2 (1)
Positive	0	7 (4)	7 (3)
Not available	0	2 (1)	2 (1)
Direct antiglobulin test (n/%)			
Negative	11 (58)	143 (77)	154 (75)
Positive	7 (37)	38 (20)	45 (22)
Not available	1 (5)	5 (3)	6 (3)

[a] <1:40, negative; 1:40 to 1:80, weak positive; >1:80, positive.
[b]0–39 IU/mL, negative; 40–79 IU/mL, weakly reactive; ≥80 IU/mL, positive.

The laboratory tests of liver function in the patients were within normal limits in all patients except one who had an ALT value of 51 U/L (normal range, 0–45 U/L). One hundred ninety-five (95%) of the 205 patients had blood urea nitrogen (BUN) values in the normal range with 3% ($n = 6$) above normal and 2% ($n = 4$) below normal. One hundred ninety-five (95%) of the 205 patients also had creatinine concentrations in the normal range with 1% ($n = 2$) above normal and 4% ($n = 8$) below normal.

Complete blood count data were available for 195 of the 205 patients (95%). Platelet counts were available for 203 patients (Table IV). Fifty-three (26%) patients had platelet counts $> 150 \times 10^9$/L, the lower end of the normal range.

For red blood cell parameters, 171 (88%) patients had normal red blood cell counts, 153 (78%) patients had normal hematocrits, and 149 (76%) patients had normal hemoglobin concentrations. Twenty-two (11%) of the patients had red blood cell counts below the normal range, and 2 (1%) had counts above the normal range. Four patients in this study reported receiving anti-D therapy within 30 days of study, and 3 of these patients were among the 22 patients with red blood cell counts and hematocrit values below the normal range. Information pertaining to the timing of any bleeding events or specific diet issues was not collected in this study, and therefore the relationship to these abnormal red blood cell counts could not be further assessed.

White blood cell counts were normal for 152 (78%) patients. The counts outside the normal range for white blood cells comprised 10 (5%) patients who were below the normal range and 33 (17%) who were above the normal range. Twenty-two (27%) of the 81 splenectomized patients had white blood cell counts above the normal range compared with 11 (10%) of the 114 nonsplenectomized patients ($P = 0.001$). The use of steroids within the past 30 days was examined as it relates to white blood cell counts. Twenty-two (35%) of the 63 patients who took steroids had white blood cell counts above the normal range compared with 11 (8%) of the 132 patients who did not take steroids ($P < 0.001$).

TABLE IV. Summary of Platelet Counts and Corresponding eTPO Levels in Adult Patients

Platelet groups ($\times 10^9$/L)	Adult patients n (%)	Median eTPO (ng/mL)[a]
<30	50 (27)	0.047
≥30–100	61 (33)	0.038
≥100–150	25 (13)	0.031
>150	48 (26)	0.039

[a]The median eTPO concentration for adult healthy subjects ($n = 62$) in this assay was 0.046 (range: 0.026–0.209) ng/mL in a separate study by Amgen Inc. (Amgen Inc., data on file).

Note: The median platelet count and eTPO level for the study adult ITP patients ($n = 184$) are 74×10^9/L and 0.040 ng/mL respectively.

The median eTPO concentration for the 19 pediatric patients was 0.037 (range, 0.022–0.355) ng/mL and for the 186 adult patients was 0.040 (range, 0.022–0.487) ng/mL. Since only limited published data were available on eTPO concentrations in pediatric patients and a relatively small number of pediatric patients were enrolled in our study, further analyses of the eTPO data were restricted to the adult patients (Table IV). The median eTPO concentrations for the adult patients were similar to values observed in healthy adult volunteers who had platelet counts within the normal range (150–450) $\times 10^9$/L using the same assay (median, 0.040; range, 0.022–0.487) versus 0.046 (range 0.026–0.209) ng/mL, respectively). Three patients had eTPO concentrations greater than the highest observed eTPO concentration in the healthy adult volunteers (i.e., 0.209 ng/mL), and these 3 patients all had platelet counts $<20 \times 10^9$/L (17×10^9/L with eTPO at 0.215 ng/mL, 8×10^9/L with eTPO at 0.487 ng/mL, and 6×10^9/L with eTPO at 0.287 ng/mL).

Analysis of eTPO concentrations, by splenectomy status in adult patients, indicated no differences between adults who had had a splenectomy ($n = 84$; mean ± SD, 0.058 ± 0.049 ng/mL; median [range], 0.040 [0.022–0.287]) and adults who had not had a splenectomy ($n = 102$; mean ± SD, 0.055 ± 0.054 ng/mL; median [range], 0.039 [0.022–0.487]) ($P = 0.648$). The eTPO concentration in this patient population did not show a significant relationship to platelet counts or to the duration of ITP (data not shown).

The eTPO concentrations for the 50 adult patients with platelet counts $<30 \times 10^9$/L, however, tended to be higher than the concentrations observed in patients with platelet counts $\geq 30 \times 10^9$/L (median, 0.047 vs. 0.037 ng/mL, $P = 0.099$). The group of patients with more severe thrombocytopenia and ITP (platelet counts $<30 \times 10^9$/L) was analyzed to determine if an inverse relationship existed between eTPO concentrations and platelet counts in these patients (Fig. 1), but again, no significant correlation (Spearman correlation coefficient $= -0.15$; $P = 0.30$) existed. This group was selected for analysis because the patients with platelet counts in this range are the most likely to have an increased level of circulating eTPO. Evaluation of the full population could mask an increase in the patients in the group with more thrombocytopenia.

Further analyses were done to determine if a relationship existed between ITP therapy and eTPO concentration because some of the therapies could affect bone marrow megakaryocytes and precursor cells.

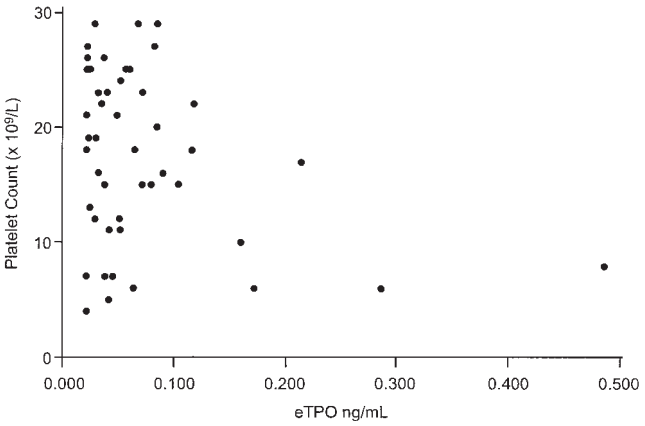

Fig. 1. Correlation between eTPO and platelet count for adult patient population ($n = 50$) with platelet counts < 30 × 10^9/L (Spearman $r = -0.15$; $P = 0.30$).

Sixteen of 23 patients (70%) receiving cytotoxic agents or immunosuppressive therapy (cyclophosphamide or azathioprine) within 30 days before phlebotomy had eTPO concentrations above the median for all patients (0.040 ng/mL), and 5 had eTPO concentrations in the upper 10% of values observed in the adult patients studied. The differences between the groups were not significant, however.

All 205 patients were tested for anti-TPO antibodies; 2 patients (1%) had neutralizing activity toward rHuTPO in the cell-based bioassay. One of these patients had confirmation of anti-TPO antibodies by the protein-G bead assay. The other patient was determined to have nonspecific inhibition because the serum also inhibited the recombinant murine IL-3, and this inhibition was not removed after incubation with protein G-coated beads. The patient whose sample had nonspecific inhibitory activity was a 40-year-old white man with a platelet count of 142 × 10^9/L and an eTPO concentration within the normal limits. His ITP was diagnosed in June 2000, and he had been treated with prednisone and IV Ig. He had no other significant medical history and was receiving prednisone within 30 days of study participation. The patient with the confirmed anti-TPO antibody was a 16-year-old Hispanic boy with a platelet count of 16 × 10^9/L and an eTPO concentration within the normal limits. His ITP was diagnosed in 1988 and subsequently treated with multiple cycles of prednisone and IV Ig in addition to vincristine, azathioprine, cyclosporin, anti-CD40, interleukin-11, and Cellcept® (mycophenolate). He had a splenectomy in 1992. He had no other significant medical history, but his mother as well as a female second degree relative had a history of autoimmune disease. Within 30 days of the study, he was receiving Cellcept® (mycophenolate) and Amicar® (aminocaproic acid).

DISCUSSION

This prospective, exploratory study characterized a large group of unselected patients with ITP seen at major referral centers. The goal was to delineate demographic, comorbid clinical factors, and laboratory parameters to better understand and explore patient characteristics that might be relevant when considering new therapies, especially those directed at increasing platelet production. This characterization revealed several novel observations. The diagnosis of ITP was established "incidentally" in approximately 50% of adults, and information was obtained on the therapies that many patients with ITP currently receive. A number of the patients with ITP who were in our study had comorbid diseases, including thyroid disease (10%), diabetes (4%), and thrombotic events (5%), the latter occurring both before and after their diagnosis of ITP. More than two-thirds of women with ITP had had pregnancies, with some experiencing pregnancy losses. The analysis of eTPO concentrations and the relationship to platelet counts provided the most detailed summary of this type to date and explores the relationship to such parameters as recent treatment and other demographic characteristics. The anti-TPO antibody observed in 1 of 205 patients with ITP in our study represents the first report of such an antibody in a patient with ITP.

Forty-seven percent of the adult patients studied reported that they had been diagnosed with ITP during a routine office visit. This finding suggests that more adult patients with ITP present with chronic, initially mild thrombocytopenia than with a precipitous "overnight" decline, as is typically seen in children. Not surprisingly, prednisone was by far the most common ITP treatment received by patients (84%). Most patients had received multiple treatments for ITP, with 45% of the adults having had a splenectomy. In addition, our data suggests that use of these treatments (specifically steroids and splenectomy) may result in white blood cell counts above the normal range. Substantial numbers of patients had persistent low counts and were receiving therapy, pointing to the lack of curative ITP treatments and a need for alternative therapies. Ten percent of patients were receiving treatment for thyroid disease (e.g., receiving thyroid replacement therapy), and 4% were receiving therapy for diabetes. This finding reflects a potential predisposition to autoimmune disorders and indicates the need to use ITP therapies, at least in a fraction of patients, that do not interfere with management of other diseases, i.e., glycemic control.

The observed prevalence of autoimmune markers in our patients was higher than the prevalence in the general population [24–26] but similar to data previously

reported in a series of patients with ITP [27,28]. In this study, the four ITP patients who received anti-D within 30 days of their study visit had positive direct antiglobulin test results. It is probable that additional patients may have positive direct antiglobulin test as a result of anti-D, as the dates of earlier treatments were not collected and the duration of the anti-D effect on the direct antiglobulin test is not precisely known and may be long-lasting. Although a positive direct antiglobulin test, not caused by previous anti-D, may identify patients with poor prognosis, i.e., Evans syndrome patients [29,30], the clinical significance of a positive direct antiglobulin test, without signs and symptoms of overt hemolysis, is largely unknown.

That only one patient had an increased ALT value (51 U/L with an upper limit of normal of 45 U/L) suggests that silent hepatitis, due to hepatitis C and other causes, is uncommon in patients with ITP. It is possible that physicians did not enroll patients with hepatitis (who may have been considered to have secondary ITP) in our study. The incidence of abnormal renal functions tests was relatively low.

Ten of the 186 adult patients (5%) had experienced 1 or more thrombotic/ischemic events, a rate, which is comparable to the 5% incidence of thrombotic events in patients with cancer, a group known to be at high risk for thrombosis [31,32]. The incidence of thrombotic events in the adult patients of age <65 years was as high as that in the overall group at 7/149 (5%). Etiologic factors, such as pregnancy, antiphospholipid antibodies, and sudden increases in the platelet count to high values, as specific predispositions to thrombosis were not addressed in this study. Furthermore, a number of thrombotic events occurred before the diagnosis of ITP. Potential pathogenic mechanisms resulting in thrombosis in patients with ITP include in vivo platelet activation, circulating platelet–leucocyte–monocyte aggregates, young aggressive platelets constituting a larger proportion of circulating platelets, and antiphospholipid antibodies. The mechanism responsible in patients remains unknown. Nonetheless, our data suggest ITP could be a risk factor for thromboembolic disease, if confirmed; this would have implications for the medical management of patients with ITP.

Sixty-eight percent of the women studied with ITP reported at least 1 successful pregnancy, often before their diagnosis of ITP. The data did not allow an analysis of how often ITP first becomes apparent during pregnancy in women of childbearing age. Spontaneous miscarriage was identified in three women with active ITP; 10 other miscarriages had occurred before the diagnosis of ITP. Because data were not collected on age-matched controls, the exact implications for the reproductive health of women with ITP are unclear.

Generally, this study found that eTPO concentrations in the ITP population were within the range seen in healthy subjects, as previously reported [14–16,20]. No correlation was observed between eTPO concentration and platelet count, even in the subset of adult patients who had platelet counts $<30 \times 10^9$/L. These data are consistent with the removal of eTPO by Mpl on the increased numbers of megakaryocytes in the bone marrow and/or on platelets before their destruction by the mononuclear phagocyte system, preventing blood TPO concentrations from increasing even in severely thrombocytopenic ITP patients (Fig. I, Table IV). No relationship was seen between eTPO concentrations and platelet count in patients with ITP who were receiving therapies that affect bone marrow cellularity, such as cytotoxic drugs [14,15] but bone marrows were not examined. Interestingly, a similar dysregulation of endogenous cytokines has been observed in pediatric patients with autoimmune neutropenia, who have low concentrations of endogenous granulocyte colony-stimulating factor (G-CSF). Anecdotally, they may respond to treatment with exogenous G-CSF as might patients with ITP respond to exogenous TPO or a molecule with similar action [33].

Inadequate platelet production has been observed in as many as two-thirds of patients with ITP [2–4]. This finding led to the hypothesis that autoantibodies, such as anti-megakaryocyte or possibly anti-TPO antibodies, might contribute to their impaired platelet production. Thrombocytopenia has been linked to autoimmune anti-TPO antibodies in a patient with amegakaryocytic thrombocytopenic purpura [34]. Anti-TPO antibodies were observed in 23% of patients (23/100 studied) with systemic lupus erythematosus and thrombocytopenia, and they were generally observed in the patients with more severe thrombocytopenia [35]. In our study of unselected patients with ITP, however, we identified two patients (1%) with circulating inhibitors of TPO activity, using an assay that measures inhibition of a test cell line that is human TPO and/or murine IL-3 dependent. One patient (0.5%) had inhibitory activity attributable to anti-TPO antibodies. The ITP patient with the nonspecific inhibitory activity had recent therapy with several medications, and it is possible that one or more of these drugs was responsible for the patient's inhibitory activity. Taken together, our data suggest neutralizing anti-TPO antibodies rarely cause impaired platelet production in ITP. It is, however, possible that additional patients with ITP have non-neutralizing TPO antibodies not detectable in our assay that accelerate eTPO clearance in vivo. The observation that the patient with anti-TPO antibodies had eTPO concentrations within the normal range may seem inconsistent; however, increased eTPO values would have been expected to be associated with

thrombocytopenia, and therefore this normal value may indicate antibody-mediated clearance of TPO in this patient. The impact of these types of antibodies on patients' presentation, course, and response to ITP therapy is still unclear.

The medical histories of our unselected patients with ITP suggest that new and effective ITP therapies are needed, as the long-term response of many patients is not satisfactory with the currently available treatments. One patient with cyclic ITP has been treated successfully with a form of recombinant human TPO for more than 4 years [36], and 4 patients have responded to daily doses for up to 7 days of the same form of recombinant TPO [37]. These observations, and the low frequency of anti-TPO antibodies in patients with ITP, suggest that eTPO replacement therapy may be a potential therapeutic for both short- and long-term management of ITP.

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