Differences in Serum Cytokine Levels in Acute and Chronic Autoimmune Thrombocytopenic Purpura: Relationship to Platelet Phenotype and Antiplatelet T-Cell Reactivity

By John W. Semple, Youli Milev, Donna Cosgrave, Meera Mody, Adriana Hornstein, Victor Blanchette, and John Freedman

Patients with both acute and chronic autoimmune thrombocytopenic purpura (AITP) have in vitro lymphocyte defects in the form of platelet-stimulated proliferation and cytokine secretion. A blinded study was performed to determine if these defects are related to serum cytokine levels and/or platelet antigen expression. Compared with controls, 53% of children with chronic AITP, but only 9% of those with acute AITP, had increased serum interleukin-2 (IL-2), interferon-γ, and/or IL-10; however, none of the patients had detectable serum levels of IL-4 or IL-6, cytokine patterns suggesting an early CD4+ Th0 and Th1 cell activation. In children with chronic AITP, the levels of serum IL-2 correlated with in vitro platelet-stimulated IL-2 production. Few (17%) patients with AITP showed platelet activation, as measured by CD62 expression, or abnormal expression levels of platelet membrane glycoprotein (GP) IIb/IIIa, but abnormal GPIIb levels were observed in one-third of children with AITP. In contrast to normal controls and patients with nonimmune thrombocytopenia, a significant number of children with acute (80%), chronic (71%), or chronic-complex (55%) AITP had GPIIb- platelet counts expressing HLA-DR. HLA-DR was variably coexpressed on distinct smaller and larger-sized GPIIb- cell populations with CD41, CD45, CD14, CD80, and/or glycoporphin molecules. GPIIb- cells isolated from spleens of patients with chronic AITP had high expression (49% ± 30%) of HLA-DR and splenic T cells had a high level of in vitro platelet-stimulated IL-2 secretion compared with controls. Platelet HLA-DR expression correlated inversely with platelet count, but not with therapy, serum cytokines, or in vitro lymphocyte antiplatelet reactivity. The results indicate that platelet HLA-DR expression is a common occurrence in patients with immune thrombocytopenia, whereas a large subpopulation of children with chronic AITP can be identified by increased serum cytokine levels and in vitro platelet-stimulated IL-2 secretion by lymphocytes, suggesting that differences exist in the immune pathogenesis of acute and chronic AITP, particularly at the level of platelet reactive T cells. © 1996 by The American Society of Hematology.

A U T O I M M U N E thrombocytopenic purpura (AITP) is a common immune-mediated bleeding disorder in which platelets are opsonized by autoantibodies and prematurely destroyed by the reticuloendothelial system. In children, both acute and chronic forms of the disease can be distinguished. Acute AITP in children is often preceded by viral or bacterial infections and generally resolves spontaneously within 6 weeks. Approximately 20% of children with acute AITP progress to the chronic form of AITP, defined as persistence of thrombocytopenia (platelet counts <150 × 10^9/L) for greater than 6 months.1 In contrast, AITP in adults is generally chronic and often requires treatment with immunosuppressive therapy or splenectomy. Although both acute and chronic AITP are immune-mediated, different pathogenetic mechanisms may be responsible2; elucidation of such differences may permit identification of those children with acute AITP likely to develop the chronic form of the disorder.

Several studies have shown in vitro cellular immune defects in patients with both acute and chronic AITP,3,9 but little is known regarding serum cytokines in AITP and their relationship to the autoimmune pathogenesis. In addition, despite extensive study of platelet-associated immunoglobulins and complement, little is known of the platelet surface antigenic profile in AITP. Boshkov et al10 reported a patient with acute AITP who had 39% of his platelets expressing HLA-DR; this amount declined as platelet counts increased. We examined the hypothesis that platelet membrane abnormalities and abnormal serum cytokine levels may play a role in the immune pathogenesis in patients with AITP, particularly with respect to in vitro platelet-stimulated T-helper (Th) cell activation. It was found that enhanced platelet HLA-DR expression was common to all the forms of AITP, whereas increased levels of interleukin-2 (IL-2), interferon-γ (IFN-γ), and/or IL-10 reflecting Th0 and Th1 cell activation were found primarily in the sera of patients with chronic AITP. These results suggest that differences exist in the immune reactivity of Th cells in a significant number of children with chronic AITP compared with those with acute AITP and that such differences may be useful in distinguishing the two forms of the disorder.

MATERIALS AND METHODS

Patients. Eleven children with acute idiopathic AITP, 23 children with chronic idiopathic AITP, and 11 children with chronic-complex AITP were tested. Twelve normal children and 13 normal adults were also tested. Eleven patients (2 with acute, 6 with chronic, and 3 with chronic-complex AITP) were examined on multiple occasions. The above samples were all tested blinded as to category/diagnosis. Table 1 summarizes the clinical data for the blinded study groups. Acute AITP was defined as thrombocytopenic purpura of abrupt onset, often within several weeks of a history of infection, in the absence of other identifiable causes of increased platelet destruction. Chronic AITP was defined as thrombocytopenia (platelet FROM THE DIVISION OF HEMATOLOGY, St Michael’s Hospital, and The Hospital for Sick Children, Departments of Pharmacology, Pediatrics, and Medicine, University of Toronto, Toronto, Ontario, Canada. Submitted June 5, 1995; accepted January 3, 1996. Supported by grants from the Medical Research Council of Canada (MT-11676) and The Hospital for Sick Children Foundation (XG 92-058). Address reprint requests to John W. Semple, PhD, Division of Hematology, St Michael’s Hospital, 30 Bond St, Toronto, Ontario, Canada, MSB 1W8.

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count <150 x 10^9/L) persisting greater than 6 months, normal or increased marrow megakaryocytes, and no secondary immune or nonimmune abnormality that could account for the thrombocytopenic state. Chronic-complex AITP was defined as chronic AITP associated with immune neutropenia and/or hemolytic anemia, often associated with other autoimmune disorders, eg, systemic lupus erythematosus, diabetes mellitus, dysgammaglobulinemia, or thyroid dysfunction. In addition, samples from 12 adult patients with nonimmune thrombocytopenia secondary to chemotherapy for acute leukemia were examined and samples from 16 normal healthy adult laboratory volunteers were tested as daily controls.

Preparation of peripheral blood mononuclear cells (PBMC), spleen cells, and platelets. For PBMC, heparinized blood was layered on a 1.077 g/mL Percoll cushion and centrifuged at 2,500g for 30 minutes and PBMC were aspirated from the top of the gradient and washed twice. Five children with chronic AITP underwent splenectomy during the study. Portions of the spleens were minced and crushed and fragments were allowed to settle in RPMI-1640. The spleen cell suspension in the supernatant was washed three times and platelets were analyzed directly from the suspension; splenic mononuclear cells were prepared by Percoll isolation as described above. For platelet antigen quantitation studies, peripheral blood was drawn into KEDTA and immediately transferred into tubes containing 0.5 mL of a 0.5% paraformaldehyde (PFA) solution in saline to inhibit in vitro platelet activation during test manipulations. PFA-fixed platelets were isolated from platelet-rich plasma (PRP) as previously described. For three-color analysis of platelet populations, platelets were analyzed in whole blood.

Table 1. Clinical Data of Study Subjects

<table>
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<tr>
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<td></td>
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<tr>
<td>11:2</td>
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<tr>
<td>Age*</td>
<td>29 ± 8 (24-50)</td>
<td>12 ± 4 (3-18)</td>
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<tr>
<td>Platelet count</td>
<td>244 ± 35 (187-303)</td>
<td>286 ± 71 (178-412)</td>
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</table>

* Age is in years (mean ± SD, with the range in parentheses).
† Platelets × 10^11/L (mean ± SD, with the range in parentheses), excluding patients in remission.

Flow cytometric analysis of platelets. For platelet antigen quantitation, PFA-fixed platelets isolated from PRP were incubated with the indicated FITC- or PE-labeled antibodies for 30 minutes in the dark at 22°C, washed, and analyzed using a FACScan flow cytometer (Becton Dickinson) equipped with an argon ion laser at 15 mW; 20,000 events were acquired. The number of antibody binding sites per cell was calculated with Simply Cellular beads (Flow Cytometry Standards Corp, San Juan, Puerto Rico) by translating the fluorescence intensity (MESF) of the labeled cells to the number of bound antibodies using the determined fluorescence/protein ratio. For three-color analysis, platelets from whole blood or the spleen cell suspensions were incubated with the indicated labeled antibodies and acquired using forward scatter (FSC) and a fluorescent (FL1) threshold set on the lowest fluorescent channel of positive reactivity with FITC-anti-GPIb (CD42b) or -GPIBIIIa (CD41a). Thirty thousand to 50,000 GPIb* or GPIBIIIa* events were acquired and analysis gates (R1 to R3) were set around the three distinct platelet populations. Analysis markers were set based on the appropriate isotype controls for all analyses.

Cytokine analysis. Sera from the patients and controls were tested for the presence of IL-2, IL-4, IL-6, IL-10, and IFN-γ with commercial solid-phase enzyme-linked immunosorbent assays (ELISAs; Cedarlane Laboratories and Immunocorp, Montreal, Quebec, Canada). Briefly, the sera were diluted 1:2 in phosphate-buffered saline (PBS) and coated onto 96-well ELISA plates preadsorbed with an anticytokine antibody for 2 hours at 22°C. An enzyme-linked anticytokine antibody was then added for an additional 2 hours. Substrate conversion was measured at 450 nm. Standard curves were generated with titrations of recombinant cytokines and were used to quantitate the serum cytokines in picograms per milliliter ranges. In our hands, the sensitivity of the cytokine assays were as follows: IL-2, >62.5 pg/mL; IL-10, >31.25 pg/mL; IFN-γ, >50 pg/mL; IL-4, >12.5 pg/mL; and IL-6, >6.25 pg/mL.

In vitro antiplatelet reactivity. For determination of in vitro lymphocyte antiplatelet reactivity, 7-day antigen-presenting cell assays were performed. Normal platelets (or autologous platelets when available) were titrated into cultures containing 2 x 10^5 PBMC or splenic mononuclear cells in 96-well round-bottom plates and incubated at 37°C for 6 days. Human allogeneic platelets have no in vitro stimulatory activity for resting T cells derived from normal individuals (data not shown). Supernatants were removed for IL-2 determinations and the cells were pulsed with 1 µCi of [3H]thymidine for an additional 24 hours and incorporated radioactivity was measured. IL-2 production was measured by a bioassay using the IL-2-dependent cell line, CTLL, as previously described. The bioassay sensitivity was 0.1 U of IL-2 based on standard curves obtained with recombinant human IL-2 (GIBCO-BRL, Gaithersburg, MD).

Analysis of results. To correct for daily variations in the assays, samples from at least 3 normal healthy adults were processed identically to the blinded test samples and included with each day's testing. Results were analyzed as absolute values and are presented as either absolute values or corrected for test variation by subtracting the mean daily control value (the delta (Δ) value). Normal ranges were established from the mean ± 2 SD of results with normal children (N = 12). Nonparametric tests were used to evaluate differences in means between groups. Regression analysis was used to test the significance of correlations between parameters.

RESULTS

Serum cytokine levels. A solid-phase ELISA was used to quantitate the levels of various cytokines in the sera of
The serum cytokine levels in patients with AITP were determined by ELISA. Assay sensitivities were as follows: IL-2, >62.5 pg/mL; IL-10, >31.25 pg/mL; and IFN-γ, >50 pg/mL. Results are expressed as cytokine concentration (in picograms per milliliter) of the sera samples. IL-4 and IL-6 results were negative in all the patients and controls (ELISA sensitivities: IL-4, >12.5 pg/mL; IL-6, >6.25 pg/mL; data not shown).

Only 1 of 11 patients with acute AITP had significantly elevated levels of cytokines, i.e., IL-2 (1,750 pg/mL), IL-10 (320 pg/mL), and IFN-γ (500 pg/mL). However, in patients with chronic AITP, 9 of 17 patients had increased serum IL-2 (mean concentration, 360 pg/mL); 3 of the 9 also had elevated levels of IL-10 and 2 of these 3 patients had increased IFN-γ. Three other patients with chronic AITP had elevated serum levels of IL-10. Three of seven patients with chronic-complex AITP had increased serum IL-2; 2 of these 3 patients also had elevated levels of IFN-γ and 1 of the 3 had detectible serum IL-10. The differences in the number of patients positive for serum IL-2 levels between acute and chronic AITP were significant (P < .001). Two children with chronic AITP were examined for serum cytokine levels on two occasions and were found to have consistent levels at both testing dates (e.g., for IFN-γ, 300 and 340 pg/mL for one child and 110 and 130 pg/mL for the second child). IL-4 and IL-6 serum levels were undetectable in the serum of all patients and controls in this study.

In vitro antiplatelet T-cell reactivity. To assess whether PBMC from patients and controls produce IL-2 when stimulated with platelets in vitro, 7-day antigen-presenting cell (APC) assays were used. Figure 2 shows the levels of in vitro IL-2 secretion by platelet-stimulated PBMC from the patients. PBMC from 5 of 8 of children with acute AITP,
from 12 of 19 of those with chronic AITP, and from 5 of 9 of those with chronic-complex AITP proliferated and secreted measurable IL-2 upon platelet stimulation. Of the in vitro responding cultures, patients with chronic AITP consistently had higher levels of IL-2 (mean, 3.6 U) in the culture supernatants, whereas lower amounts were seen in the APC cultures from those patients with acute AITP (mean, 0.7 U) or chronic-complex (mean, 0.6 U). In 6 of 10 adult patients with chronic AITP (not otherwise included in this analysis), PBMC secreted IL-2 upon in vitro platelet stimulation (mean, 6 U). Splenic mononuclear cells from 4 of 5 patients with chronic AITP had the highest in vitro platelet-stimulated IL-2 production (mean, 12 U/mL). Figure 2B shows that in vitro antiplatelet T lymphocyte IL-2 secretion significantly correlated with the presence of increased serum IL-2 (r = .975, P < .001).

Platelet phenotype. A number of patients in each group had abnormal platelet phenotype results, compared with the number tested. Increased platelet-associated (PA)-IgG was the most common Ig detected, being present in 73% and 82% of those with chronic AITP and increased PA-IgM and PA-IgA were less frequent (40% and 5%, respectively). Increased platelet-associated (PA)-IgM and PA-IgA were less frequently observed (40% and 5%, respectively).

Figure 3A shows the individual Δ values by which the level varied from daily normal controls; the mean number of GPIbIIIa molecules per platelet for the daily normal controls was 47.1 ± 2.8 × 10^3 molecules/cell (N = 16). Few patients with AITP (6 of 40 children overall) showed abnormal levels of platelet surface GPIbIIIa (CD41a). The overall mean ± SD levels (×10^3 molecules/platelet) of GPIbIIIa were 47.1 ± 3.2 for acute AITP, 45.8 ± 6.9 for chronic AITP, and 49.4 ± 3.9 for chronic-complex AITP; these levels were not different from the levels observed in normal children (47.0 ± 3.2) or normal adults (48.4 ± 1.6). Figure 3B shows the individual Δ values; the mean number of GPIb molecules per platelet for the daily normal controls was 19.9 ± 1.4 × 10^3. Fifteen of 43 children with AITP overall had abnormal levels of GPIb (CD42b) and this was most common in those with acute AITP (5 of 11 children). Mean ± SD levels (×10^3) of GPIb were lower in normal children than in normal adults (18.2 ± 1.3 vs 20.2 ± 1.7, respectively; P < .005). The mean GPIb levels were 19.8 ± 4.9 for children with acute AITP, 20.9 ± 3.1 for those with chronic AITP, and 20.6 ± 2.7 for those with chronic-complex AITP; these were not different from normal adult values, but for chronic and chronic-complex AITP, the mean GPIb levels were increased from those of normal children (P < .02).

Platelet activation was assessed by CD62 (P-selectin; GMP140) expression. Immediate fixation in PFA was used to prevent in vitro platelet activation. Figure 3C shows the Δ values for platelets expressing CD62 for each individual; in normal children, the mean percentage of platelets expressing CD62 was 1.0% ± 1.5%. Only 17% of children with acute AITP and 13% of those with chronic AITP had an increase in platelets expressing CD62; an increase in platelets expressing CD62 was observed in 25% of children with chronic-complex AITP. Excluding 1 patient who had 35% of his platelets expressing CD62, the differences between patients with AITP and normal patients were not significant. When platelets were not fixed with PFA, 10% to 50% of the platelets from normal controls and from patients had increased expression of CD62.

Figure 3D shows the Δ values for the platelets expressing HLA-DR in children with AITP; in the daily normal controls, the mean proportion of platelets expressing HLA-DR was 0.08% ± 0.05%. Although in individual patients only small proportions of platelets expressed increased HLA-DR, most children with acute (80%), chronic (76%), or chronic-complex (55%) AITP exhibited a small but distinct increase in platelets expressing increased HLA-DR (defined as the mean channel fluorescence > 2 SD above the mean for the daily normal controls). In normal children and in normal adults, 0.09% ± 0.06% and 0.13% ± 0.12%, respectively, of platelets expressed HLA-DR (P < .01). In contrast, there was a 10-fold increase (1.1% ± 0.9%) of platelets from patients with acute, chronic (0.9% ± 0.8%), and chronic-complex AITP (0.9% ± 1.1%) expressing increased HLA-DR. None-theless, the majority of platelets remained HLA-DR-. Compared with normal children, the increases observed in platelets expressing HLA-DR were significant (P < .002) v acute AITP, P < .01 v chronic AITP, and P < .02 v chronic-complex AITP. In 1 patient with acute AITP (not included in the calculations above), 24% of the platelets expressed HLA-DR. None of 12 patients with acute leukemia and non-immune thrombocytopenia showed an increased number of platelets expressing HLA-DR, compared with normal controls (data not shown).

Figure 4A through D shows a typical two-color flow cytometric analysis of PRP platelets derived from a healthy child (Fig 4A and C) and a child with chronic AITP (Fig 4B and D) with 0.07% and 5.0%, respectively, of GPIb^+ platelets expressing HLA-DR. Further characterization of HLA-DR^+ platelets was performed using three-color fluorescence. Figure 4E shows that GPIb^+ cells derived from the whole blood of a child with chronic AITP could be gated into three distinct populations based on size (forward light scatter; FSC) and a FITC-anti-GPIb threshold: GPIb^+ intact platelets (gate R1), GPIb^+ macroparticles (gate R2), and GPIb^+ microparticles (gate R3). Microscopic examination of sorted populations showed that the macroparticle gate R2 was composed of single, nonadherent cells that on Giemsa staining were erythrocytes. Figure 4F shows results with PE-labeled anti-glycophorin and PerCP-labeled HLA-DR antibodies of the ungated GPIb^+ cells; greater than 98% of the glycoporphin-positive events were contained in gate R2. HLA-DR expression was virtually absent on the whole blood GPIb^+ cells from a healthy child control (0.06%; data not shown), but was increased (2.0%, P < .001) on the GPIb^+ cells from the child with chronic AITP (Fig 4F). Splenic GPIb^+ cells from the same child with chronic AITP had higher expression of HLA-DR (21%) compared with the whole blood platelets (2%). Splenics from 4 other children with chronic AITP had 49% ± 30% of their GPIb^+ cells expressing HLA-DR; each spleen showed higher HLA-DR expression than the corresponding peripheral blood platelets. Table 2 summarizes the three-color flow cytometric results of the three
SERUM CYTOKINE LEVELS IN AITP

Fig 3. Scatter diagram showing the (A) GPⅡbⅠⅢa, (B) GPⅠb, (C) CD62, and (D) HLA-DR Δ values for the individuals in each category. Positive or negative values are the differences between the number of molecules per platelet (A and B) or the percentage of platelets (C and D) for the patients in relation to the daily controls. The boxed areas depict the normal Δ value ranges (mean ± 2 SD). The mean Δ values for normal children were -2.3 ± 2.8 × 10^4 and 0.18 ± 1.1 × 10^4 molecules/platelet for GPⅡbⅠⅢa and GPⅠb respectively. The mean percentage CD62 Δ value for normal children was 1.0% ± 1.3% and the mean percentage HLA-DR Δ value for normal children was 0.09% ± 0.06%.

gated populations of GPIb⁺ cells shown in Fig 4E. The increased peripheral blood GPIb⁺ cell-associated HLA-DR expression in patients with AITP was associated with the larger-sized GPIb⁺glycophorin⁺ cells (gate R2; 6%), the smaller-sized GPIb⁺ microparticles (gate R3; 4%), and the GPIb⁺ intact platelets (gate R1, 3%). The GPIb⁺ cells in gates 2 and 3 also variably coexpressed CD45 (4% and 2%, respectively), CD14 (5% and 33%, respectively), and CD80 (4% and 22%, respectively) molecules. The glycophorin-positive events in gate 2 represented approximately 2% of total red blood cells in the blood sample. The observations on the peripheral blood platelets from patients with AITP were also seen, in greater degree, in platelets derived from the spleens of patients with chronic AITP, eg, a mean of 50% of gate 1 intact platelets expressed HLA-DR. Table 2 indicates that, in the other gated populations within spleen-derived GPIb⁺ cells, there were, however, several significant differences in the coexpression of HLA-DR and CD45, CD14, CD80, and glycophorin compared with peripheral blood GPIb⁺ cells, eg, in splenic-derived cells in gate 2, 93% of the GPIb⁺/HLA-DR⁺ cells also expressed CD14, 80% coexpressed CD80, and 64% coexpressed glycophorin. Coexpression on single cells was confirmed by examining sorted populations using fluorescent microscopy.

Correlations between the results. Overall, there was an inverse correlation of platelet count with proportion of platelets expressing HLA-DR, as shown in Fig 5A (correlation coefficient [r] = -.5837, P < .01). In individual patients studied over time, as the platelet counts improved (with or without therapy), the number of platelets expressing HLA-DR was reduced (r = -.5531, P < .01; data not shown). On 9 of 10 occasions in which the platelet count increased, there was a decrease in platelet HLA-DR. On occasions in which the platelet count remained unchanged on subsequent testing, HLA-DR remained unchanged in 2 and increased in 1. One patient with chronic AITP (data not shown) was examined on 7 separate occasions (before and after IVIgG therapy, over the course of 7 months); the r value for relationship between HLA-DR expression and platelet counts in this patient was -.5730 (P = .023). Platelet HLA-DR expression was not correlated to serum IL-2 levels (r = -.11, P = not significant [NS]; Fig 5B), and it did not correlate with the other serum cytokine levels (data not shown).

In a number of cases, patients had received therapy within the month before testing. Therapies included IVIg, anti-D, and steroids in patients with acute AITP (N = 6); steroids, IVIg, and splenectomy in children with chronic AITP (N
Fig 4. Flow cytometric analysis of HLA-DR expression on platelet populations prepared from washed PRP or whole blood. The upper panels show an FSC versus SSC dot plot of PRP platelets derived from (A) a healthy child and (B) a child with chronic AITP. The middle panels (C and D) represent the corresponding dot plots for HLA-DR/GP Ib fluorescence of the ungated PRP platelet populations. Twenty thousand events were acquired in (A) through (D) and fluorescent markers were set on FITC- and PE-isotypic control antibodies. The lower panels show a typical three-color analysis of GP Ib⁺ cells from the peripheral blood of a child with chronic AITP. (E) shows cells acquired through an FSC versus GP Ib FL1 live gate. Gates (R1 through R3) were drawn around the clustered populations of cells. (F) shows the second (FL2; PE-antiglycophorin) and third (FL3; PerCP-anti-HLA-DR) colors of the ungated GP Ib⁺ cells from (E). A summary of three-color fluorescent results for each gated population is shown in Table 2. The numbers indicate the percentage of cells in each quadrant. For clarity, only 2,000 events in each panel are shown.

Table 2. Three-Color Analysis of Gated Platelet Populations

<table>
<thead>
<tr>
<th>Gate R1: Intact Platelets</th>
<th>Gate R2: Macroparticles</th>
<th>Gate R3: Microparticles</th>
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<tbody>
<tr>
<td>GP Ib⁺ DR⁺</td>
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<td>&gt;99</td>
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Results are expressed as mean percentage of total cells within each gate (R1-R3) shown in Fig. 4E. Fifty thousand events were acquired for analysis. The relative size of the cells within each gate are shown in Fig 4E (there was no significant difference in GP Ib⁺ cell size in control or patient groups).

Abbreviations: Co, GP Ib⁺ cells from whole blood of healthy control children (N = 10); Pat, GP Ib⁺ cells from whole blood of children with chronic AITP (N = 6); Pat Spl, GP Ib⁺ splenic cells from children with chronic AITP (N = 4); NT, not tested.
responsible for increasing the intensity of an immune re-
response or directing a tissue-specific immune response.16 Be-
tween serum IL-2 levels and HLA-DR expression on GPlb+ platelet populations in PRP for all patients tested for both parameters. A

![Image of correlation between platelet count and HLA-DR expression](image)

**Fig 5.** (A) Correlation of platelet count with HLA-DR expression on GPlb⁺ platelet populations in PRP for all children tested for both parameters ([8] patients; [C] normal controls). (B) Relationship between serum IL-2 levels and HLA-DR expression on GPlb⁺ platelet populations in PRP for all patients tested for both parameters.

Patients with acute AITP differed from those with the chronic form of the disorder in that, in the former, few patients had increased cytokines, whereas many (53%) of the latter had increased levels of serum IL-2, IFN-γ, and/or IL-10. None of the patients tested had detectable serum lev-
els of IL-4 or IL-6. The increased serum IL-2 significantly corre-
related with in vitro platelet-stimulated IL-2 production by T helper cells. Thus, a population of children with chronic AITP may have abnormal in vivo T-cell activation causing accumulation of serum cytokines. Cytokine secretion patterns can distinguish CD4⁺ T-helper cells into Th1 and Th2 cells. Th1 cells primarily secrete IL-2 and IFN-γ and mediate DTH-like responses, whereas Th2 cells can secrete IL-4, IL-5, IL-6, and/or IL-10 and are superior in helping humoral responses, particularly IgE.21-23 A third group of Th cells is termed Th0 cells and is thought to be less differentiated than Th1 and Th2 cells, because they can secrete most or all of the cytokines made by either cell type, particularly IL-2 and IL-10.24 Our results suggest that the pattern of cytokine levels in chronic AITP may reflect an early Th1 cell activation, ie, primarily IL-2 secretion with some patients exhibiting Th0 or Th1 activation (IL2, IL-10, and/or IFN-γ). However, be-
cause IL-10 can be produced by other cell types25 and 3 of the patients with chronic AITP had elevated levels of only IL-10, it is unclear what the cellular source of this cytokine is in the patients with chronic AITP. Our in vivo results correlate with a recent report showing in vitro Th1 cytokine patterns in mitogen-stimulated CD2⁺ T cells from adult pa-
tients with chronic AITP.26 IL-2 plays a pivotal role in human immune responses and increased serum IL-2 is reported in various autoimmune diseases.25-26 Hypersecretion of endogenous IL-2 may lead to autoaggression by a number of mechanisms, eg, by by-passing the need for T cell co-stimulation,29 by upregulating costimulatory CD80 molecules on B cells,29 or by the induc-
tion of other cytokines such as IFN-γ and IL-10.30 Increased IL-10 was also seen in a number of patients with AITP. IL-10 has potent immunostimulatory effects on human B cells31 and suppressive effects on monocytes/macrophages that leads to downregulation of inflammatory cytokines such as IL-6.32 We found no detectable levels of IL-6 in these pa-
tients. The ability of IL-10 to suppress the production of inflammatory cytokines suggest that it may have a strong anti-inflammatory role in vivo33; although the role of IL-10 in chronic AITP is unclear, it may help in reducing antiplatelet reactivity and destruction. We are currently testing this hy-
pothesis, but, at the present time, it remains unclear whether the alterations in cytokines play a primary role in the etiology of AITP or are rather a reflection of an ongoing inflammatory and/or immune response.

We and others have previously reported that PBMC from patients with chronic AITP have enhanced platelet-stimulated IL-2 secretion in vitro. It was currently found that, in children with chronic AITP, serum cytokine levels correlated well with in vitro platelet-stimulated IL-2 production. In contrast, only 1 of 11 children with acute AITP had significant levels of serum IL-2, IL-10, and IFN-7, despite the fact that about one-third of these patients also have PBMC that mediated weak (0.7 U) in vitro platelet-stimulated IL-2 reactivity. These observations may relate to different immune pathogeneses in the various forms of AITP. Acute AITP may be due to a cross-reactive immune response directed against an infectious agent, e.g., a virus, whereas chronic AITP may be generated by a more platelet-specific autoimmune pathogenesis, possibly at the level of T cells. In acute AITP, the lymphocyte immune response, although slightly cross-reactive to normal platelets in vitro, may be due to a stimulus that does not generate the same serum cytokine profile as the autoimmune response directed at the platelets themselves in the chronic form of the disorder. The enhanced serum cytokine levels, the increase in activated T cells, and the strong in vitro antiplatelet T-cell response in patients with chronic AITP may be due to a continually platelet-stimulated autoimmune response.2 We are currently studying these platelet-reactive T-cell responses at the clonal level.

A number of surface platelet-specific and nonspecific antigenic markers were quantitated in children with AITP and controls. Although some of the children with AITP in each group did exhibit abnormalities, in general, children with either the acute or chronic forms of AITP had platelets with normal expression of GPIIbIIIa and the CD62 activation molecule. These results support those of Chong et al.,25 who reported that, in contrast to patients with consumptive thrombocytopenia, plasma P-selectin levels were not increased in patients with AITP. The low expression of surface GPIIbIIIa observed in some patients (Fig 3A) may have been due to blocking by high-affinity autoantibodies to GPIIbIIIa, which are frequently present in chronic AITP.25 An increase in GPIb expression, on the other hand, was present in one-third of the children with AITP and was more common in those with acute AITP; this may reflect a form of platelet activation, in which surface GPIb expression may be downregulated26 or upregulated.28 However, the observed increases, although statistically significant, were slight.

HLA-DR expression is normally primarily restricted to mature APC of the immune system, i.e., monocytes/macrophages, dendritic cells, B cells, and activated T cells. Although megakaryocytes have been shown to express class II molecules on their surface in vitro,29 platelets normally express only HLA class I and not class II molecules.30,31 However, the majority of the patients with AITP had a small but distinct increase in the proportion of platelets expressing HLA-DR. This was not seen in patients with nonimmune thrombocytopenia (data not shown) or in normal controls. Further three-color flow cytometric analysis indicated that the HLA-DR expression was coexpressed with GPIb and GPIIbIIIa on intact peripheral blood platelets and was also associated with CD14, CD45, and CD80 expression on platelet microparticles and macroparticles. These observations were particularly evident when splenic samples from patients with AITP were compared with the corresponding patients' peripheral blood platelets. Whereas the HLA-DR expression seen on GPIb-positive intact platelets may, as described above, have a role in the autoimmune pathogenesis of AITP, that seen on microparticles may reflect destructive mechanisms, i.e., platelet fragments may combine with leukocyte (monocyte) fragments, yielding microparticles expressing both platelet and leukocyte antigens. The amplified findings in the spleen samples may reflect the locus of destruction, with the peripheral blood samples reflecting only those cells not sequestered or destroyed in the spleen. However, the data presented on the splenic platelet HLA-DR expression need to be interpreted with caution because there were no control spleen samples available for analysis. Nonetheless, the observations in the spleen may have a potential relationship to platelet destruction in AITP and should be further studied. The inverse correlation of platelets expressing HLA-DR and the platelet count supports this hypothesis.

Although platelet HLA-DR expression was of low levels, 1 patient with acute AITP had a dramatic increase in platelet HLA-DR (24%) that was similar to that found by Boshkov et al.30 (39%) in acute AITP. However, because only 1 of 11 patients had a significant increase in platelet HLA-DR expression, it does not appear to be a common finding of acute AITP. The expression of HLA-DR on platelets was not correlated to serum cytokine levels. This may be due to either the number of patients being too low for statistical significance or it may indicate that other mechanisms are responsible for the expression. These mechanisms may include the passive adsorption of HLA-DR from membrane fragments of activated macrophages or possibly release of endogenous HLA-DR by the platelets themselves. However, we were consistently unable to show increased HLA-DR expression on normal platelets after thrombin or ADP stimulation and activation of the platelets (data not shown), suggesting that this pathway of endogenous release to the surface is not the likely mechanism. Furthermore, HLA-DR expression was not seen on young (as assessed by thiazole orange-detected RNA content) platelets from patients recovering from chemotherapy, suggesting that it is not the result of studying megakaryocytic platelets. On the other hand, the cells coexpressing GPIb and glycoporphin in patients with AITP may relate to the observations by Karpatic42 of increased red blood cell destruction in AITP. Approximately 20% of children with acute AITP will progress to the chronic form of the disease.1 It would be beneficial if markers of this transition were available. Although Ware and Howard found no major phenotypic differences in T cells between children with acute and chronic AITP, functional analyses showed that the majority of platelet-reactive T-cell clones were derived from the children with chronic AITP. None of the platelet phenotypic markers investigated in the current report clearly distinguished the different clinical forms of AITP; however, we are observing
these children to determine if these results may have a predictive value for determining progression to chronic AITP. Increased serum cytokines, together with in vitro platelet-stimulated IL-2 production, may be important tests in examining differences in the pathophysiology of the acute and chronic forms of AITP and may help predict which children with acute AITP will develop the chronic form of the disease.

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Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity

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