Spleen Is a Primary Site for Activation of Platelet-Reactive T and B Cells in Patients with Immune Thrombocytopenic Purpura

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Spleen Is a Primary Site for Activation of Platelet-Reactive T and B Cells in Patients with Immune Thrombocytopenic Purpura

Masataka Kuwana, Yuka Okazaki, Junichi Kaburaki, Yutaka Kawakami, and Yasuo Ikeda

We have recently reported that in patients with chronic immune thrombocytopenic purpura (IMTP), circulating T and B cells that are responsive to gpIIb-IIIa can induce anti-platelet autoantibody production. In this study, the frequencies and activation status of gpIIb-IIIa-reactive T and B cells were evaluated in the peripheral blood and spleen obtained from nine IMTP patients undergoing splenectomy. There was no difference in gpIIb-IIIa-reactive T cell frequencies between peripheral blood and spleen (6.4 ± 2.6 vs 5.2 ± 2.4 per 10⁶ T cells), as determined by limiting dilution analysis, but activated T cells responsive to gpIIb-IIIa showing accelerated proliferation kinetics and those expressing CD154 were more frequent in spleen than in peripheral blood. The frequencies of anti-gpIIb-IIIa Ab-producing B cells, as determined by ELISPOT assay, were also similar in peripheral blood and spleen (61.2 ± 24.0 vs 77.7 ± 45.3 per 10⁶ B cells); however, an anti-gpIIb-IIIa Ab was spontaneously produced by splenocytes in vitro, but scarcely secreted by PBMCs. CD19+/CD38+/CD138+ plasma cells secreting anti-gpIIb-IIIa Ab were exclusively detected in the spleen. In serial analysis, the frequencies of circulating gpIIb-IIIa-reactive T and B cells were markedly decreased after splenectomy in patients with a complete response, but were unchanged in nonresponders. These findings indicate that an interaction between gpIIb-IIIa-reactive T and B cells inducing anti-platelet Ab production in IMTP patients occurs primarily in the spleen and that the significant number of gpIIb-IIIa-reactive T and B cells activated in the spleen are released into the circulation as memory cells. The Journal of Immunology, 2002, 168: 3675–3682.

Chronic (idiopathic) thrombocytopenic purpura (IMTP) is an autoimmune disease characterized by increased platelet clearance caused by anti-platelet autoantibodies (1). These autoantibodies bind to circulating platelets, leading to platelet destruction by the reticuloendothelial system. The major target of the anti-platelet Abs is platelet membrane glycoprotein IIb-IIIa (gpIIb-IIIa), a calcium-dependent heterodimeric membrane receptor for fibrinogen and other ligands, and anti-gpIIb-IIIa Ab is shown to be detected in >90% of IMTP patients when sensitive assays were used (2–4). We have recently found that CD4⁺ T cells responsive to gpIIb-IIIa in the peripheral blood from IMTP patients have a helper activity that promotes the production of IgG anti-gpIIb-IIIa Ab capable of binding to normal platelets in vitro (5, 6). However, peripheral blood B cells in IMTP patients did not secrete anti-gpIIb-IIIa Ab spontaneously in in vitro cultures, and the production of measurable anti-gpIIb-IIIa Ab required optimal stimulation in the presence of gpIIb-IIIa-reactive CD4⁺ T cells and Ag. These findings were obtained using an in vitro culture system, but strongly suggest that the circulating gpIIb-IIIa-reactive B cells are mostly memory B cells, which are not primarily involved in anti-gpIIb-IIIa Ab synthesis in vivo in IMTP patients.

The spleen is thought to play an important role in the pathogenesis of IMTP, because ~60% of IMTP patients achieve a stable increased platelet count after surgical splenectomy (7, 8). In chronic IMTP, the spleen is considered to be the primary site of both platelet destruction and anti-platelet Ab production (7). Destruction of the Ab-sensitized platelets by phagocytosis through FcR in the reticuloendothelial system, including the spleen, has been confirmed by the clinical benefit of anti-Fc blockade treatment using anti-Rho(D) Ig (9) or Abs to the FcR (10). In contrast, the evidence for anti-platelet Ab production by spleens from IMTP patients was previously shown by McMillan et al. (11) and Karpatkin et al. (12). These studies demonstrated that splenocytes from IMTP patients spontaneously produce IgG Abs capable of binding specifically to autologous and allogeneic platelets. The daily splenic production of anti-platelet IgG was shown to exceed the quantity required for maximum sensitization of platelets produced daily in the bone marrow (11). If this is the case, autoreactive T and B cells responsive to gpIIb-IIIa should be activated and expanded in the spleen rather than in the peripheral blood. To test this hypothesis, the frequencies and activation status of gpIIb-IIIa-reactive T and B cells were compared between PBMCs and splenocytes obtained from IMTP patients who received surgical splenectomy.

Materials and Methods

Patients and controls

Nine adult patients with chronic IMTP who received surgical splenectomy between July 1998 and December 1999 were enrolled in this study. All patients had thrombocytopenia (platelet count <50 × 10⁹/L) persisting...
longer than 6 mo, normal or increased bone marrow megakaryocytes without morphological evidence for dysplasia, and no secondary immune or nonimmune diseases that could account for the thrombocytopenic state (1). All patients received laparoscopic splenectomy because they were refractory to or could not tolerate corticosteroid therapy. The clinical response to splenectomy was assessed from the platelet count at 6 mo after splenectomy. Responders, including patients with a complete or partial response, were defined as having a platelet count ≥50 × 10^9/L, whereas nonresponders were defined as having a platelet count <50 × 10^9/L. Platelet samples were obtained on the day of splenectomy before the operation, and pieces of the spleen (total weight >10 g) were processed immediately after resection. In seven IMPT patients, peripheral blood samples were obtained serially at 3 days and 6 mo after splenectomy. Control samples were obtained from four patients with cancer (two with gastric cancer and two with esophageal cancer) who required splenectomy as a part of the dissection of tumor tissues. These spleens were confirmed to be free of tumor invasion and metastasis by histopathologic examination. All samples were obtained before the patients gave their written informed consent, as approved by the Keio University Institutional Review Board (Tokyo, Japan).

**Preparation of gpIIb-IIIa**

Human gpIIb-IIIa was purified from outdated platelet concentrates using affinity chromatography (5). Purified gpIIb-IIIa was dialyzed against PBS with 0.5 mM CaCl₂ (PBS-Ca) and stored in aliquots at −80°C until use. gpIIb-IIIa modified by treatment with trypsin was used and for T cell stimulation as described previously (5).

**Detection of anti-gpIIb-IIIa Abs**

IgG and IgM anti-gpIIb-IIIa Abs in plasma, platelet eluates (from 5 × 10⁹ platelets), and culture supernatants were measured by ELISA using affinity-purified gpIIb-IIIa as an Ag, as described elsewhere (4, 5). Ab units were calculated from the OD450, with the calculation results being based on the standard curve obtained from a serial concentration of mAb to gpIIb-IIIa (clone HPL1; Harlan Laboratories, Leicester, U.K.). All samples were tested in duplicate, and the results were calculated as the mean duplicate. Cutoff values for plasma and platelet-associated IgG anti-gpIIb-IIIa Abs were 5.1 and 3.3, respectively, on the basis of the mean plus 3 SD of 20 samples from healthy individuals.

**Flow cytometric analysis**

Cell staining was performed using anti-CD3, anti-CD4, anti-CD8, anti-CD27, anti-Ig κ, anti-Ig λ (BD Pharmingen, San Diego, CA), anti-CD19, anti-CD38 (Sigma-Aldrich, St. Louis, MO), anti-CD138 (Beckman Coulter, Fullerton, CA), and anti-CD154 (Ancell, Bayport, MN) mAbs. These mAbs were conjugated to FITC or PE, or were unconjugated. Cells were analyzed on a FACSCaliber (Becton Dickinson, San Jose, CA), and anti-CD154 (Ancell, Bayport, MN) mAbs. In some experiments, effects of CD4⁺ cell or CD154⁺ cell depletion on gpIIb-IIIa-induced T cell proliferation in cultures of PBMCs and splenocytes were examined (5). The results were expressed as the percentage of inhibition, which was calculated as the difference between the cpm incorporated in the cultures with and without treatment by the counts per minute incorporated in the culture without treatment.

**Detection and quantification of gpIIb-IIIa Ab-producing B cells**

PBMCs were isolated from heparinized venous blood using Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation, and resuspended in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Sterile spleen tissue in complete medium was washed twice to remove peripheral blood and crushed with a syringe plunger. All of the cells were dissociated, the cell suspension was filtered through a nylon mesh and subjected to Lymphoprep density gradient centrifugation. The recovered cells were suspended in complete medium and used as splenocytes. Freshly isolated PBMCs and splenocytes were used in the following experiments unless indicated otherwise.

In some experiments, T cells were isolated from PBMCs and splenocytes by passing them through a nylon wool column twice as described previously (4, 5). Flow cytometric analysis of the T cell fraction contained >95% CD3⁺ T cells. PBMCs and splenocytes were depleted of CD4⁺ cells, CD8⁺ cells, or CD19⁺ cells by mixing the cells with magnetic bead-conjugated mAbs (Dynal, Oslo, Norway), followed by magnetic removal of the bead-bound cells. CD27⁺, CD38⁺, surface IgG⁺, CD138⁺, and CD154⁺ cells were also removed by incubating the cells with these mAbs to these molecules, followed by incubation with goat anti-mouse IgG Ab conjugated to magnetic beads (Dynal). After the depletion treatment, <2% of the cells were positive for these markers, except for CD154⁺ cells, which made up <0.1% of the population.

**Quantification of gpIIb-IIIa-reactive T cells**

The frequency of gpIIb-IIIa-reactive T cells was estimated using limiting dilution analysis as described previously (16, 17). Briefly, aliquots containing serial numbers of PBMC- or splenocyte-originated T cells (1, 2.5, 5, 10, 25, and 50 × 10⁵) were cultured with irradiated (40 Gy) autologous splenocytes (10⁵) in the presence of modified gpIIb-IIIa (5 µg/ml) for 5 or 7 days. Each aliquot of T cells was dispensed into 96 wells. Control cultures without Ag were also set up in 12 wells. After a final 16-h incubation with 0.5 µCi/well of [³H]thymidine, the cells were harvested and [³H]thymidine incorporation was determined in a TopCount microplate scintillation counter (Packard Instrument, Meriden, CT). A positive well was defined as having cpm greater than three times the mean counts per minute of the 12 control cultures. Based on the assumption that the responding cells were randomly distributed in the culture wells, the frequency of the responding T cells could be estimated according to a Poisson distribution formula. Only data with statistical significance in a single regression model (p < 0.05) were adopted. Because activated T cells showed accelerated proliferation kinetics upon antigenic stimulation (5), T cells proliferating at day 5 were regarded as activated T cells.

In some experiments, effects of CD4⁺ cell or CD154⁺ cell depletion on gpIIb-IIIa-induced T cell proliferation in cultures of PBMCs and splenocytes were examined (5). The results were expressed as the percentage of inhibition, which was calculated as the difference between the cpm incorporated in the cultures with and without treatment by the counts per minute incorporated in the culture without treatment.

**Detection and quantification of gpIIb-IIIa Ab-producing B cells**

B cells producing anti-gpIIb-IIIa Ab were detected and quantified using an ELISPOT assay, which was developed for the detection of autoantibody-producing B cells (18). Briefly, polyclonally induced diluted-bottomed 96-well multiliter plates (Millipore, Bedford, MA) were coated with 30 µg/ml of purified gpIIb-IIIa in PBS-Ca. After incubation at 4°C overnight, the plates were washed three times with PBS-Ca and blocked with 1% BSA in PBS-Ca for 1 h at room temperature. Plates coated with 1% BSA in the absence of gpIIb-IIIa were used as a control. The Ag-coated plates were prepared fresh each time. PBMCs or splenocytes (10⁵ cells/well) in complete medium were incubated in the Ag-coated plates at 37°C in a humidified atmosphere of 5% CO₂ for 4 h. The membranes were then washed three times with PBS-Ca containing 0.05% Tween 20 and subsequently incubated with alkaline phosphatase-conjugated goat-human IgG or IgM (ICN Pharmaceuticals, Aurora, OR) diluted 1/1000 in PBS-Ca at room temperature for 2 h. After the membranes were washed four times with PBS-Ca containing 0.05% Tween 20 and once with PBS-Ca, Ab bound to the membrane were visualized as spots by incubation with nitro blue tetrazolium/5-bromo-4-chloro-indolyl phosphate. The number of spots were counted under a dissecting microscope. Each experiment was conducted in 10 independent wells, and the results represent the mean of the 10 values. The frequency of anti-gpIIb-IIIa Ab-producing B cells was expressed as the number per 10⁶ mononuclear cells (MNCs), that was calculated by subtracting the number of spots on the control membrane coated with BSA alone from the number of spots on the gpIIb-IIIa-coated membrane. In some instances, the frequencies of anti-gpIIb-IIIa Ab-producing B cells were expressed as the number per 10⁶ B cells, based on the proportion of B cells, including immature/mature B cells and plasma cells, in the MNCs. To evaluate B cell subsets producing anti-gpIIb-IIIa Ab, PBMCs and splenocytes that were depleted of CD4⁺, CD8⁺, CD19⁺, CD27⁺, CD38⁺, and surface IgG⁺ were also used in the ELISPOT assay.

**In vitro anti-gpIIb-IIIa Ab production with or without Ag stimulation**

In vitro production of anti-gpIIb-IIIa Ab in cultures of PBMCs and splenocytes was evaluated as described (5, 6, 11) with some modifications. Briefly, PBMCs or splenocytes (5 × 10⁶ cells) were cultured in 500 µl of complete medium in 48-well culture plates with or without Ag stimulation (10 µg/ml gpIIb-IIIa Ab) in the presence of PWM (1 µg/ml). The Ag-induction of anti-gpIIb-IIIa Ab synthesis was examined in cultures with modified gpIIb-IIIa in the presence of PWM (1 µg/ml). PBMCs and splenocytes that were depleted of CD4⁺, CD8⁺, CD19⁺, CD27⁺, CD38⁺, and surface IgG⁺ cells, were also examined. The levels of IgG and IgM anti-gpIIb-IIIa Abs in undiluted culture supernatants were measured by ELISA. All cultures were prepared in duplicate, and the anti-gpIIb-IIIa Ab concentrations were normalized. In some experiments, anti-gpIIb-IIIa Ab produced in culture supernatants was absorbed by pre-incubation with platelets, erythrocytes, or PBMCs obtained from two healthy individuals (5).
### Statistical analysis

All comparisons between two groups were tested for statistical significance using Fisher’s two-tailed exact test or the Mann-Whitney test. The correlation coefficient (r) was determined using a single regression model. Significance of serial changes in T and B cell frequencies and Ab levels was assessed using one-way factorial ANOVA.

### Results

#### Clinical characteristics of IMTP patients

The clinical findings and plasma and platelet-associated IgG anti-gpIIb-IIIa Ab levels in nine IMTP patients and four controls are summarized in Table I. The age at splenectomy tended to be younger for IMTP patients compared with control patients. The disease duration between diagnosis and splenectomy varied and ranged from 7 to 221 mo. All patients were on low-dose corticosteroids (<10 mg prednisolone/day) at the time of splenectomy. Intravenous Ig was administered as a pretreatment for splenectomy in all IMTP patients except patient P1, but none of the patients received a platelet transfusion at the splenectomy. The platelet count at 1 wk before splenectomy ranged from 7 to 42 × 10^9/L in the IMTP patients, whereas all of the control patients had normal platelet counts. Based on the platelet count at 6 mo after splenectomy, seven patients were responders while two were nonresponders. Plasma and platelet-associated IgG anti-gpIIb-IIIa Abs were detected in two and eight IMTP patients, respectively, but in none of the controls. IgM anti-gpIIb-IIIa Ab was not detected in any of the subjects.

#### Quantification of T cells responsive to gpIIb-IIIa

Limiting dilution analysis was performed to quantify T cells responsive to gpIIb-IIIa in PBMCs and splenocytes. As shown in Fig. 1A, the frequency of gpIIb-IIIa-reactive T cells that proliferated at day 7 was significantly greater in IMTP patients compared with controls in the peripheral blood (6.4 ± 2.6 vs 2.2 ± 0.8 per 10^5 T cells; p = 0.02) and in the spleen (5.2 ± 2.4 vs 1.5 ± 0.4 per 10^5 T cells; p = 0.007). Contrary to our initial expectation, there was no difference in the frequency of gpIIb-IIIa-reactive T cells between PBMCs and splenocytes in IMTP patients. However, when limiting dilution analysis was conducted in 5-day cultures, the frequency of gpIIb-IIIa-reactive activated T cells was significantly higher in splenocytes than in PBMCs (3.4 ± 2.0 vs 1.2 ± 0.4 per 10^5 T cells; p = 0.004; Fig. 1B).

To further confirm the increase in activated T cells responsive to gpIIb-IIIa in IMTP spleens, the effects of CD154− T cell depletion on gpIIb-IIIa-induced T cell proliferation was examined using the PBMCs and splenocytes of IMTP patient P5 (Fig. 2A). CD154, a ligand for CD40, is expressed transiently on T cells upon activation by Ag stimulation (19). CD4+ cell-depleted or mock-treated fractions were also analyzed as controls. gpIIb-IIIa-induced T cell proliferation was completely inhibited by depletion of CD4+ cells in both PBMCs and splenocyte cultures. In contrast, suppression of the T cell proliferation by CD154+ cell depletion was minimal in PBMCs, but prominent in splenocytes. As shown in Fig. 2B, the mean percentage of inhibition of the T cell proliferation by CD154+ cell depletion in splenocyte cultures was significantly greater than that in PBMC cultures (49.1 ± 12.0 vs 13.7 ± 7.1; p = 0.004). The inhibitory effect by CD154+ cell depletion in splenocyte cultures tended to be smaller in the two nonresponders than in the seven responders.

### Table I. Clinical findings and plasma and platelet-associated anti-gpIIb–IIIa Ab in IMTP patients and controls

<table>
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<th>No.</th>
<th>Age at Splenectomy (years)</th>
<th>Disease Duration at Splenectomy (months)</th>
<th>Current and Previous Treatment</th>
<th>Pretreatment for Splenectomy</th>
<th>Platelet Count (×10^9/L)</th>
<th>Response to Splenectomy</th>
<th>Anti-gpIIb–IIIa Ab (U)</th>
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<th>Platelet-associated (normal &lt; 3.3)</th>
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<tr>
<td>P1 F</td>
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<td>Responder</td>
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*PSL, prednisolone; dz, danazol; dap, dapsone; CPA, cyclophosphamide. Previous treatment is shown in parenthesis.

*NT, not tested.

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**Figure 1.** Frequency of gpIIb-IIIa-reactive T cells in PBMCs and splenocytes from IMTP patients and controls determined by limiting dilution analysis. Serial numbers of PBMC- or splenocyte-originated T cells were cultured with APCs in the presence of modified gpIIb-IIIa for 3 (A) or 5 days (B), and frequencies of gpIIb-IIIa-reactive T cells were estimated by a single regression model. Comparisons were made by the Mann-Whitney test. Closed and open circles in IMTP patients denote a responder and a nonresponder to splenectomy, respectively. NS, not significant.
Detection and quantification of anti-gpIIb-IIIa Ab-producing B cells

B cells producing anti-gpIIb-IIIa Ab were detected and quantified using an ELISPOT assay. Fig. 3A illustrates a representative result. When PBMCs and splenocytes from IMTP patients were applied to the assay, clear spots were detected on the gpIIb-IIIa-coated membranes, but were rarely found on the control membranes coated with BSA alone. The sizes of individual spots were highly variable, indicating a difference in capacity of individual cells to secrete anti-gpIIb-IIIa Ab. Large spots were predominately found in IMTP splenocytes. Using this technique, the frequencies of anti-gpIIb-IIIa Ab-producing cells were quantified in PBMCs and splenocytes from nine IMTP patients and four controls (Fig. 3B). The number of IgG anti-gpIIb-IIIa Ab-producing cells per 10^5 B cells was greater in IMTP patients than in controls in the peripheral blood (61.2 ± 24.0 vs 1.3 ± 0.9; p = 0.007) and in the spleen (77.7 ± 45.3 vs 4.2 ± 2.5; p = 0.007), although there was no difference between PBMCs and splenocytes in IMTP patients. The frequencies of IgG anti-gpIIb-IIIa Ab-producing B cells in PBMCs and splenocytes were significantly correlated with the platelet-associated IgG anti-gpIIb-IIIa Ab levels in IMTP responders (r = 0.86, p = 0.002 and r = 0.83, p = 0.02, respectively). The frequency of IgM anti-gpIIb-IIIa Ab-producing B cells was <0.5 per 10^5 MNCs in all samples.

Correlations between the frequencies of gpIIb-IIIa-reactive T and B cells in the peripheral blood and spleen

As shown in Fig. 4A, the frequencies of gpIIb-IIIa-reactive T and B cells in individual IMTP patients were significantly correlated with each other within the peripheral blood and spleen. However, the gpIIb-IIIa-reactive T cell frequency was not correlated between the peripheral blood and spleen (p = 0.11), but was significantly correlated when the two nonresponders were excluded from the analysis (r = 0.72, p = 0.05; Fig. 4B, left). Similarly, the gpIIb-IIIa-reactive B cell frequency was not correlated between the peripheral blood and spleen (p = 0.14), but a significant correlation was obtained without the two nonresponders (r = 0.94, p = 0.004; Fig. 4B, right). These findings indicated that the frequencies of gpIIb-IIIa-reactive T and B cells in the spleen were relatively lower than in the peripheral blood in the two nonresponders.

In vitro anti-gpIIb-IIIa Ab production with or without Ag stimulation

PBMCs and splenocytes were cultured for 10 days, and the IgG and IgM anti-gpIIb-IIIa Abs secreted spontaneously into the culture supernatants were measured. As shown in Fig. 5A, PBMCs from IMTP patients scarcely produced IgG anti-gpIIb-IIIa Abs, but IMTP splenocytes spontaneously produced IgG anti-gpIIb-IIIa Abs in vitro. The amount of IgG anti-gpIIb-IIIa Ab produced in splenocyte cultures was significantly larger than that in PBMC cultures in IMTP patients (3.7 ± 2.3 vs 0.3 ± 0.1; p < 0.001). IgM
anti-gpIIb-IIIa Ab was not detected in any of the culture supernatants. IgG anti-gpIIb-IIIa Ab reactivity could be removed from the splenocyte culture supernatants by preincubation with normal platelets, but not by incubation with erythrocytes or PBMCs (data not shown), indicating that the IgG anti-gpIIb-IIIa Ab produced in vitro was capable of binding to normal platelets. The amounts of IgG anti-gpIIb-IIIa Ab produced in vitro in splenocyte cultures were significantly correlated with the frequency of IgG anti-gpIIb-IIIa Ab-producing B cells in splenocytes ($r = 0.91$, $p = 0.0007$). IgG anti-gpIIb-IIIa Ab levels produced in splenocyte cultures in the two nonresponders tended to be lower than those in the seven responders.

Ag stimulation of PBMCs with gpIIb-IIIa enhanced the in vitro anti-gpIIb-IIIa Ab production in all IMTP patients, whereas the enhancement of the Ab production was less prominent in splenocyte cultures (Fig. 5B). As a result, there was no difference in the amounts of anti-gpIIb-IIIa Ab produced upon Ag stimulation between PBMC and splenocyte cultures. The Ag-induced anti-gpIIb-IIIa Ab production in PBMC cultures was completely blocked by depletion of CD4$^+$ cells, indicating that enhancement of the Ab production was CD4$^+$ T cell-dependent (data not shown).

**Differentiation stage of anti-gpIIb-IIIa Ab-producing B cells**

Because the anti-gpIIb-IIIa Ab produced by the PBMCs and splenocytes from IMTP patients was exclusively of the IgG isotype, it is likely that the Ab-producing cells were memory B cells and/or plasma cells, which had been already activated via a cognate interaction with activated CD4$^+$ T cells. To determine the differentiation stage of the anti-gpIIb-IIIa Ab-producing B cells, ELISPOT and in vitro assays for anti-gpIIb-IIIa Ab production were conducted using PBMCs and splenocytes that had been depleted of CD19$^+$ cells (Fig. 6). CD19 is expressed on the majority of memory B cells but its expression is largely diminished after differentiation to plasma cells. Depletion of CD19$^+$ cells from PBMCs resulted in the complete loss of anti-gpIIb-IIIa Ab-producing cells and in vitro anti-gpIIb-IIIa Ab production. In contrast, anti-gpIIb-IIIa Ab-producing B cells were detectable in splenocytes even after the CD19$^+$ cells were removed. Moreover, CD19$^+$ cell-depleted splenocytes from six patients secreted a detectable level of anti-gpIIb-IIIa Ab into the culture supernatants. The frequency of anti-gpIIb-IIIa Ab-producing cells and levels of anti-gpIIb-IIIa Ab produced in vitro in CD19$^+$ cell-depleted splenocytes were significantly greater than in CD19$^+$ cell-depleted PBMCs ($7.0 \pm 4.8$ vs $0.3 \pm 0.2$; $p < 0.001$ and $1.2 \pm 0.9$ vs $0.2 \pm 0.0$; $p = 0.04$, respectively). These findings suggest that the anti-
gpIIb-IIIa Ab-producing cells in the peripheral blood are exclusively CD19\(^+\) memory B cells, whereas those in spleen include CD19\(^+\) plasma cells. It was of note that the depletion of CD19\(^+\) cells from splenocytes resulted in the loss of anti-gpIIb-IIIa Ab-producing cells and in vitro anti-gpIIb-IIIa Ab production in IMTP patients. The PBMCs and splenocytes from IMTP patient P5 were depleted of cells expressing CD19, surface Ig, CD38, CD4, or CD8, and these fractions were applied to an ELISPOT assay for the detection of IgG anti-gpIIb-IIIa Ab-producing cells (A) and in an in vitro assay for IgG anti-gpIIb-IIIa Ab production without Ag stimulation (B). The SD of the number of spots on the gpIIb-IIIa-coated wells in the ELISPOT assay was <20% of the mean in all cases. Results shown are representative of two experiments.

To further confirm the presence of anti-gpIIb-IIIa Ab-secreting plasma cells in the spleen from these patients, the presence of anti-gpIIb-IIIa Ab-secreting plasma cells in IMTP spleens, the PBMCs and splenocytes of IMTP patients were depleted of cells expressing CD19, surface Ig, CD38, CD4, or CD8, and these fractions were applied to ELISPOT and in vitro assays for anti-gpIIb-IIIa Ab production. CD38 is expressed on both the early progenitors of B cells and plasma cells, but not on most memory B cells (20). A representative result of IMTP patient P5 is shown in Fig. 7. In peripheral blood, the anti-gpIIb-IIIa Ab-producing cells were completely lost in CD19\(^+\) cell-depleted and surface Ig\(^-\) cell-depleted fractions, whereas depletion of CD38\(^+\), CD4\(^+\), or CD8\(^+\) cells had no effect. In contrast, the decrease in the number of anti-gpIIb-IIIa Ab-producing cells after the depletion of CD19\(^+\) cells or surface Ig\(^-\) cells was partial in splenocytes. Depletion of CD38\(^+\) cells from splenocytes resulted in a slight decrease in number of the Ab-producing cells, and a complete loss could be achieved by the depletion of both CD19\(^+\) and CD38\(^+\) cells. Results obtained from in vitro splenocyte cultures were principally concordant with those from the ELISPOT assay, but partial inhibition of the anti-gpIIb-IIIa Ab production by CD4\(^+\) cell depletion was observed exclusively in splenocyte cultures. Because this phenomenon was not observed in the ELISPOT assay, it is likely that splenic CD4\(^+\) T cells are involved in the production of anti-gpIIb-IIIa Ab from B cells in vitro, even in the absence of exogenous Ag. Similar results were obtained from two additional IMTP patients, P4 and P8.

The presence of plasma cells secreting anti-gpIIb-IIIa Ab in spleen, but not in peripheral blood, was further assessed by the effects of depletion of cells expressing CD138, a specific marker for plasma cells (14), on the anti-gpIIb-IIIa-producing cell frequency by ELISPOT assay using frozen PBMC and splenocyte samples of two IMTP patients (P4 and P5). The percentage of inhibition of anti-gpIIb-IIIa Ab-producing cell spot formation by this treatment was <3% in both PBMC samples, but was 47 and 48% in splenocytes, respectively. In addition, similar experiments were performed using PBMCs and splenocytes depleted of cells expressing CD27, a specific marker for peripheral blood memory B cells (21). The spot formation was almost completely inhibited by CD27\(^+\) cell depletion in both PBMC samples, but the percentage of inhibition in splenocyte samples of patients P4 and P5 was 67 and 74\%, respectively, confirming that nearly all anti-gpIIb-IIIa Ab-producing cells in circulation were CD27\(^+\) memory B cells.

Serial measurement of the frequencies of gpIIb-IIIa-reactive T and B cells

The frequencies of gpIIb-IIIa-reactive T cells and anti-gpIIb-IIIa Ab-producing B cells in peripheral blood were serially examined 3 days and 6 mo after splenectomy in seven IMTP patients, including five responders and two nonresponders (Fig. 8). The frequencies of gpIIb-IIIa-reactive T and B cells were significantly decreased after splenectomy in responders (\(p < 0.001\) and \(p = 0.004\), respectively, by one-way factorial ANOVA), but unchanged or increased in nonresponders. A decrease in the frequency of anti-gpIIb-IIIa Ab-producing B cells was detected 3 days after splenectomy, whereas a decrease in gpIIb-IIIa-reactive T cells was not apparent at this timepoint. The levels of platelet-associated anti-gpIIb-IIIa Ab were also decreased after splenectomy in responders (\(p = 0.03\) by one-way factorial ANOVA), but not in nonresponders.
Discussion

The present study demonstrates that the activation of autoreactive T and B cells to gpIIb-IIIa induces pathogenic anti-gpIIb-IIIa Ab production occurs primarily in the spleen in the majority of IMTP patients. This finding supports previous studies showing evidence for the production of a large amount of anti-platelet Abs by the spleen from IMTP patients (11, 12). gpIIb-IIIa-reactive T and B cells in the spleen included cells with an activated phenotype, while those in the peripheral blood were mostly memory cells, based on the following findings: 1) activated T cells with accelerated proliferation kinetics were more frequent in splenocytes than in PBMCs; 2) gpIIb-IIIa-induced T cell proliferation in splenocyte cultures was inhibited by the depletion of recently activated CD154+ T cells, but the inhibition was minimal in PBMC cultures; 3) a larger amount of anti-gpIIb-IIIa Ab was secreted in vitro without Ag stimulation by splenocytes compared with PBMCs; and 4) plasma cells secreting anti-gpIIb-IIIa Ab were exclusively found in the spleen. Taken together with a significant correlation between gpIIb-IIIa-reactive T and B cell frequencies in IMTP spleens, these findings indicate that an interaction between gpIIb-IIIa-reactive T and B cells and the resultant anti-gpIIb-IIIa Ab production is ongoing in vivo in IMTP spleens. However, frequencies of gpIIb-IIIa-reactive T and B cells were not different between the peripheral blood and spleen in IMTP patients. This is probably because the majority of gpIIb-IIIa-reactive T and B cells are released into the circulation after they are activated in the spleen, given that circulating gpIIb-IIIa-reactive T and B cells were markedly decreased after splenectomy and gpIIb-IIIa-reactive T and B cell frequencies were significantly correlated between the peripheral blood and the spleen in responders. Because PBMCs were able to produce anti-gpIIb-IIIa Ab whose quantity was comparable with that produced by splenocytes when Ag stimulation was provided (see Fig. 5B), it is possible that the peripheral blood in IMTP patients serves as a pool of gpIIb-IIIa-reactive memory T and B cells with the potential for secreting anti-platelet Ab.

Based on the findings in this study, we propose the following process of continual anti-gpIIb-IIIa Ab production in vivo in IMTP patients. gpIIb-IIIa-reactive memory CD4+ T cells in the circulation arrive in the white pulp of the spleen, then migrate to the outer edge of the periarterial lymphatic sheath, where they interact with APCs, including macrophages and dendritic cells, in the adjacent red pulp. gpIIb-IIIa-reactive T cells are then activated upon recognition of Ag peptides, and interact with gpIIb-IIIa-reactive memory B cells that have also arrived from the circulation, resulting in the production of anti-gpIIb-IIIa Ab. T cell-derived IL-6 is expected to play an important role in this process, as shown in our previous study (6). After gpIIb-IIIa-reactive B cells are activated, some differentiate into plasma cells that constitutively secrete a large amount of anti-gpIIb-IIIa Ab, but have a limited lifespan and eventually undergo apoptosis. In contrast, the majority of gpIIb-IIIa-reactive T and B cells are released into the circulation as memory cells. This model is supported by our recent finding that antigenic gpIIb-IIIa fragments recognized by T cells were principally concordant between PBMCs and splenocytes in the majority of IMTP patients (our unpublished observation). The dynamics of the interaction between gpIIb-IIIa-reactive T and B cells controlling the anti-gpIIb-IIIa Ab synthesis account for the primary role of the spleen in the pathogenesis of IMTP.

Because all but one patient received i.v. Ig as a pretreatment for splenectomy, we have to consider the possible influence of i.v. Ig on the T and B cell responses to gpIIb-IIIa. The mechanisms for the in vivo activity of i.v. Ig in IMTP patients remains unclear, although a variety of explanations have been put forward to account for this activity, including FcR blockade, attenuation of complement-mediated platelet destruction, and neutralization of anti-platelet Abs by anti-idiotype Abs (22, 23). A recent study using a murine IMTP model has indicated that i.v. Ig mediates its protective effect by inducing the expression of the inhibitory FcγRIIb on macrophages, resulting in suppression of the clearance of the sensitized platelets (24). In addition, Levy et al. (25) reported that anti-gpIIb-IIIa Ab levels in IMTP patients were not decreased, but even increased after i.v. Ig treatment. These findings imply that i.v. Ig primarily affects platelet clearance by macrophages, rather than anti-platelet Ab production.

The two IMTP patients who did not respond clinically to splenectomy had relatively low frequencies of gpIIb-IIIa-reactive T and B cells in the spleen compared with peripheral blood. In addition, gpIIb-IIIa-reactive activated T cells and anti-gpIIb-IIIa Ab-secreting plasma cells were barely detected in the splenocytes from the nonresponders. Although the number of patients examined was small, these findings strongly suggest that the spleen was not the primary site of gpIIb-IIIa-reactive T and B cell activation in these patients and alternate sites for activation are present outside of the spleen. In fact, in nonresponders, the frequencies of circulating gpIIb-IIIa-reactive T and B cells and the levels of platelet-associated anti-gpIIb-IIIa Ab were unchanged or even increased after splenectomy. Bone marrow is the most likely site for anti-gpIIb-IIIa Ab production in these patients, given that the bone marrow cells from IMTP patients have been shown to produce IgG Abs that bind to platelets in vitro (26). Therefore, the clinical response to splenectomy in IMTP patients appears to be dependent on the site of anti-platelet Ab production. In this regard, it has been reported that IMTP patients who have good response to i.v. Ig are likely to similarly respond to splenectomy (27). Because i.v. Ig exerts its therapeutic effect mainly through suppression of macrophage function, this clinical observation strongly suggests that two distinct pathogenic processes, anti-platelet Ab production induced by activated T and B cells and platelet clearance by macrophages, are closely related in IMTP patients.

Use of the ELISPOT assay enabled us to detect and quantify anti-gpIIb-IIIa Ab-producing B cells in PBMCs and splenocytes. The ELISA is a widely used assay for detecting the Ab, but the ELISPOT assay has several unique features. First, the ELISPOT assay is able to detect even a single cell of 10^5 cells, whose secretion level may be insufficient in detection by ELISA. In fact, in the majority of IMTP patients, anti-gpIIb-IIIa Ab-producing B cells were detected in PBMCs by the ELISPOT assay, but anti-gpIIb-IIIa Ab was not detectable in PBMC culture supernatants by ELISA. Second, de novo Ab production by B cells can be assessed by the ELISPOT assay, and spot formation may reflect ongoing Ab production in vivo. Regarding this point, the frequencies of anti-gpIIb-IIIa Ab-producing B cells in PBMCs and splenocytes detected by the ELISPOT assay were significantly correlated with the platelet-associated anti-gpIIb-IIIa Ab levels. Finally, the ELISPOT assay can detect the Ab produced by B cells without interference by binding to the Ag. In this regard, the majority of pathogenic anti-platelet Abs in IMTP patients are thought to be present as platelet-associated IgG (28), and it is necessary to use platelets as a source of Ab in the ELISA to detect the anti-gpIIb-IIIa Ab with high sensitivity and specificity (29). Therefore, the ELISA is not widely used for the detection of the anti-gpIIb-IIIa Ab in clinical settings. Because the frequency of circulating anti-gpIIb-IIIa Ab-producing cells was shown to be significantly higher in IMTP patients than in control cancer patients, the ELISPOT assay may be useful clinically for detecting platelet-specific Abs.

How are gpIIb-IIIa-reactive T and B cells activated in the spleen in IMTP patients? Because circulating memory T and B cells
readily produced anti-gpIIb-IIIa Ab when optimal Ag stimulation was given in vitro, we presume that the antigenic determinants of gpIIb-IIIa are efficiently presented by APCs in IMTP spleens. Our previous studies have shown that gpIIb-IIIa-reactive CD4+ T cells recognize antigenic peptides generated from chemically modified gpIIb-IIIa and recombinant fragments produced in bacteria, but not from gpIIb-IIIa in its native form, suggesting that the epitopes recognized by gpIIb-IIIa-reactive T cells are cryptic (5). We have recently found that gpIIb-IIIa-reactive T cells recognize immunodominant epitopes located within the amino-terminal regions of both gpIIb and gpIIIa (6), although the factors that induce the expression of these cryptic determinants in IMTP patients are unknown. The de novo presentation of a previously cryptic self-de- terminant has been proposed to be induced by up-regulated Ag presentation capacity and shifts in the peptide hierarchy (30, 31). Because of the association between the process of anti-platelet Ab production induced by activated T and B cells and the process of platelet destruction by macrophages in IMTP patients as mentioned above, it is likely that splenic macrophages that capture many sensitized platelets play a central role in this process in IMTP patients. Signaling through FcR would activate the Ag-processing pathway and up-regulate the expression of adhesion and costimulatory molecules (32). In addition, splenic macrophages would have the ability to concentrate and present to T cells a small quantity of cryptic determinants of gpIIb-IIIa, which would not be efficiently generated under normal circumstances without platelet sensitization. The efficient presentation of a small quantity of determinants to T cells was also reported in cross-reactive B cells that selectively capture the Ag via the B cell receptor (33). Further studies examining the mechanisms for activation of gpIIb-IIIa-reactive T and B cells in the spleen will be useful for clarifying the pathogenic process in patients with chronic IMTP and for developing a therapeutic approach that blocks pathogenic anti-platelet Ab production.

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References