Phenotypic and Clonal Analysis of T Lymphocytes in Childhood Immune Thrombocytopenic Purpura

By Russell E. Ware and Thad A. Howard

In an attempt to identify and characterize T-lymphocyte immunoregulatory abnormalities in immune thrombocytopenic purpura (ITP), we have performed phenotypic and clonal analysis on peripheral T lymphocytes from 23 children with ITP. Quantitation of lymphocyte subpopulations showed that children with acute ITP had higher numbers of CD45RA+ and lower numbers of CD45RO+ T cells than children with chronic ITP or controls, but these differences may be age related. Analysis of T-cell receptor variable gene usage identified 2 boys with chronic ITP and elevated numbers of Vα8+ T cells. Eight T-cell clones were established (6 CD4+, 4B4+ helper-inducer lines and 2 CD8+ lines) that showed in vitro proliferation against allogeneic platelets. The addition of autologous antigen-presenting cells enhanced the proliferation of six clones, but not for two clones that coexpressed natural killer (NK) markers. Four of seven positive clones also had measurable interleukin (IL)-2 secretion following platelet stimulation, providing further evidence for T-cell reactivity. Our results provide the first evidence that patients with ITP may have platelet-reactive T lymphocytes identifiable at the clonal level, supporting the hypothesis that autoreactive peripheral T lymphocytes may mediate or participate in the pathogenesis of this disorder.

MATERIALS AND METHODS

Patient population. Children with acute or chronic ITP routinely received medical care at Duke University Medical Center. A total of 9 children with acute ITP, 14 children with chronic ITP, and 9 controls were studied. Seven of the 9 children with acute ITP were studied at the time of initial diagnosis, whereas two were not studied until after resolution of thrombocytopenia. The mean initial platelet count of these 9 children was 20,000/μL (median 8,000/μL) and the mean age at the time of analysis was 6.4 years (median 5.3 years, range 2.1 to 13.3 years). None of the children with acute ITP was receiving therapy at the time of lymphocyte immunophenotyping or clonal analysis. Two of the 14 patients with chronic ITP had undergone prior splenectomy with resolution of thrombocytopenia. Of the remaining 12 with persistent thrombocytopenia, 3 received periodic treatments of anti-DP (WinRho SD; Rh Pharmaceuticals, Winnipeg, Manitoba, Canada), 2 received maintenance intravenous Ig (IVIG) therapy, and the other 7 did not require therapy. The mean platelet count of these 12 children was 50,000/μL (median 49,000/μL) and the mean age at analysis was 12.3 years (median 13.3 years, range 3.2 to 18.9 years).

Semplé and Freedman recently reported antplatelet reactivity mediated by CD4+ T-helper lymphocytes in adult patients with chronic ITP. Furthermore, the subpopulation of CD4+ lymphocytes known as the suppressor-inducer subset was found to be quantitatively reduced in those ITP patients who showed antplatelet T-cell proliferative responses. Reduced numbers of suppressor-inducer T lymphocytes have been previously reported in other autoimmune disorders including systemic lupus erythematosus, Sjogren’s Syndrome, rheumatoid arthritis, and progressive multiple sclerosis.

In an attempt to identify and characterize T-lymphocyte immunoregulatory abnormalities in ITP, and better understand the differences between acute and chronic ITP in children, we have performed phenotypic and clonal analysis on T lymphocytes from 23 children with ITP. We have quantitated T-lymphocyte subpopulations, including the 2H4 and 4B4 subsets, which identify the CD4+ suppressor-inducer and helper-inducer lymphocytes, respectively. We have also measured T-cell receptor variable gene products in these patients to identify predominant usage within the T-cell receptor repertoire. Finally, we have established clones of T lymphocytes from children with acute or chronic ITP that demonstrate reactivity against platelets.
omy for idiopathic polycythemia. The mean age of controls was 25 years (range 1 to 33 years).

**Antibodies.** Directly labeled monoclonal antibodies (MoAbs) conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used in this study for single and double-color analysis of lymphocyte subpopulations. MoAbs included CD3-FITC, CD3-PE, CD4-FITC, CD4-PE, CD5-FITC, CD10-FITC, CD14-FITC, CD20-FITC, CD45-FITC, CD45RO-FITC and Class II MHC-FITC (Dako Corporation, Carpinteria, CA), as well as CD57-FITC and glycoprotein A-FITC from AMAC (Westbrook, ME). MoAbs CD45RA (2H4), CD29 (4B4), and CD56 (NKH1) conjugated to phycoerythrin were the kind gifts of Meryl Forman (Coulter Corporation, Hialeah, FL). Antibodies against the variable regions of the T-cell receptor a or b chains were obtained from T Cell Sciences (Cambridge, MA) directly conjugated to FITC and included Pan-TCR& Vcy2, Vp5.1, Vp5.2 + 5.3, Vp5.3, Vp6.7, Vp8, and Vb12.1. Control MoAbs included IgG-FITC and IgG-PE from Dako. All antibodies were used according to manufacturers’ recommendations.

**Cell preparation.** Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood using Ficoll-Hypaque density centrifugation as previously described.10 Platelets were obtained from healthy donors and isolated as previously described.11

**Immunophenotyping.** Approximately 0.5 × 10^6 PBMC were incubated with saturating amounts of one or two MoAbs for 30 minutes at 4°C as previously described.12 After washing with PBS with 2% bovine serum albumin (Sigma, St Louis, MO), cells were fixed in paraformaldehyde and stored at 4°C in the dark until analysis. Samples were analyzed on a Profile II flow cytometer (Coulter) using forward and side scatter lymphocyte gating and color compensation. The use of glycoprotein A-FITC allowed direct quantitation of contaminating erythrocytes, typically 2% to 5% of gated cells. Isotype control values (0.1 to 0.5%) were subtracted from the reported results.

**T-lymphocyte cloning.** Fresh PBMC from patients or controls were cloned as previously described.13 Briefly, PBMC were plated at 2 to 3 cells per well in 96-well flat-bottom plates (Costar, Cambridge, MA) in 200 µL RPMI 1640 with L-glutamine (GIBCO/Life Technologies, Grand Island, NY) and 15% human A serum (HAS; Worldwide Biologicals, Cincinnati, OH) in the presence of 1.0 µg/mL phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC), 50 U/mL interleukin-2 (IL-2) (kind gift from Chiron Corporation, Emeryville, CA), and 10^5 irradiated (4,000 rads) Epstein-Barr virus (EBV)-transformed feeder cells (JY cell line). After 2 to 3 weeks, growing clones were moved to 48-well plates and fed with additional feeder cells and IL-2. Clones were progressively expanded to the 12-well stage, then were immunophenotyped with CD3, CD4, and CD8 MoAbs. Clones that grew well in T-25 flasks (Costar) without exogenous IL-2 for at least 1 week were tested for reactivity against platelets.

**Proliferative responses by T lymphocytes.** T-lymphocyte clones were separated over Ficoll-Hypaque and washed in RPMI to remove any dead cells, feeder cells, and exogenous IL-2. Purified clones were counted and resuspended in RPMI with 15% HAS at 2 × 10^6 cells/mL. Fresh autologous PBMC obtained from the patients were irradiated (4,000 rads) for use as antigen-presenting cells (APC), and also resuspended at 2 × 10^6 cells/mL. Stimuli for the clones included either PHA at a final concentration of 2.0 µg/mL or platelets at 100 × 10^3/mL in RPMI with 15% HAS. For the proliferative assay, 50 µL of cloned cells were placed in triplicate into 96-well U-bottom plates (Costar) along with either 50 µL of media or irradiated APC and 100 µL of stimuli (either PHA or platelets). Media alone was used as a control, and fresh PBMC were also tested for bulk lymphocyte reactivity. For PHA stimulation, cells were pulsed on day 3 with 0.4 µCi ³H-thymidine (New England Nuclear, Boston, MA) for 4 hours before harvesting on a cell harvester (Cambridge Technologies, Watertown, MA) and counting on a β scintillation counter. For platelet stimulation, 50 µL of supernatant was removed on day 6 for IL-2 assay, and cells were pulsed for 24 hours before harvesting.

**IL-2 assay.** Secre ted IL-2 from clones was assayed using the HT-2 mouse T-cell line as previously described.14 Briefly, HT-2 cells were plated in a flat-bottom 96-well plate (Costar), along with supernatants obtained from clones stimulated with platelets. After 20 hours incubation, the cells were pulsed with 0.4 µCi ³H-thymidine for 4 hours before harvesting.

**Statistics.** The two-sample Student’s t-test was used to analyze the results. All statistics were performed using the Primer of Biostatistics software program (McGraw-Hill, Inc, New York).

## RESULTS

**Immunophenotype analysis.** Table 1 lists PBMC immunophenotype results for the children with acute ITP (n = 9), chronic ITP (n = 14), and normal controls (n = 9). PBMC from each child were analyzed on several occasions, with phenotype results remaining constant over time. Single color analysis showed no significant differences among the three groups in the percentage of PBMC that were T lymphocytes (CD3⁺), monocytes (CD14⁺), or B lymphocytes (CD20⁺). Similarly, there were no significant differences in the percentage of cells reactive with CD4, CD5, CD8, CD10, CD29 (4B4), CD56 (NKH1), CD57, or Class II MHC antibodies (Table 1).

All three groups had similar numbers of CD45⁺ cells (Table 1). However, the number of T lymphocytes in the CD45RO⁺ subset was lower in the children with acute ITP (12.1%) compared with chronic ITP patients (20.4%, P = .07) and normal controls (27.8%, P = .02). Conversely, the number of T lymphocytes in the CD45RA⁺ (2H4) subset was significantly higher in the children with acute ITP (61.8%) compared with chronic ITP (49.0%, P = .05) and normal controls (46.3%, P = .05).

**Double-color analysis of PBMC showed no increased**

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Acute ITP Mean ± SEM</th>
<th>Chronic ITP Mean ± SEM</th>
<th>Normal Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>69.6 ± 5.2</td>
<td>65.9 ± 3.4</td>
<td>70.3 ± 5.0</td>
</tr>
<tr>
<td>CD4</td>
<td>36.0 ± 6.6</td>
<td>31.8 ± 2.0</td>
<td>36.1 ± 1.9</td>
</tr>
<tr>
<td>CD5</td>
<td>71.9 ± 5.0</td>
<td>68.3 ± 3.9</td>
<td>78.5 ± 4.6</td>
</tr>
<tr>
<td>CD8</td>
<td>28.7 ± 3.0</td>
<td>28.6 ± 1.7</td>
<td>35.7 ± 3.5</td>
</tr>
<tr>
<td>CD10</td>
<td>0.9 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>CD14</td>
<td>10.3 ± 1.9</td>
<td>13.0 ± 1.6</td>
<td>8.9 ± 1.4</td>
</tr>
<tr>
<td>CD20</td>
<td>7.6 ± 3.0</td>
<td>3.8 ± 0.6</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>CD29 (4B4)</td>
<td>55.3 ± 7.1</td>
<td>66.4 ± 5.2</td>
<td>60.5 ± 3.5</td>
</tr>
<tr>
<td>CD45</td>
<td>95.4 ± 2.2</td>
<td>92.4 ± 1.7</td>
<td>92.5 ± 1.9</td>
</tr>
<tr>
<td>CD45RA (2H4)</td>
<td>61.8 ± 6.5</td>
<td>49.0 ± 2.6</td>
<td>46.3 ± 3.4</td>
</tr>
<tr>
<td>CD45RO</td>
<td>12.1 ± 2.8</td>
<td>20.4 ± 2.9</td>
<td>27.8 ± 5.5</td>
</tr>
<tr>
<td>CD56 (NKH1)</td>
<td>17.5 ± 2.8</td>
<td>25.0 ± 3.3</td>
<td>16.0 ± 2.8</td>
</tr>
<tr>
<td>CD57</td>
<td>15.0 ± 5.0</td>
<td>18.2 ± 2.0</td>
<td>19.8 ± 4.5</td>
</tr>
<tr>
<td>CD45</td>
<td>13.0 ± 4.6</td>
<td>6.2 ± 0.8</td>
<td>5.5 ± 0.9</td>
</tr>
</tbody>
</table>

Cells were stained with MoAbs as described in Materials and Methods. Results are the percentage of positive cells (mean ± SEM) for each group.

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T-lymphocyte (TCR) repertoire. To determine if children with acute or chronic ITP had abnormal TCRαβ variable gene region usage, PBMC were analyzed further using a panel of MoAbs directed against variable regions of the TCR. Results are listed in Table 2, and include the median and range of percent positive cells for each variable region. There were no significant differences among the three groups in the usage of TCR α gene Vα2 or TCR β genes Vβ5.1, Vβ5.2 + 5.3, Vβ6.7, or Vβ12.1. However, two teenage boys with chronic ITP had an elevated number of T lymphocytes expressing the TCR Vβ8 gene product (7.6% and 16.6% of CD3+ T cells, respectively). Lymphocytes from both patients were tested on at least three occasions over a 12-month period, and consistently showed this increase in Vβ8 usage. Double-color analysis showed that the Vβ8+ T lymphocytes from the first patient (SH) were predominantly CD4+ cells, whereas those from the second patient (CH) were CD8+ cells (data not shown).

Bulk lymphocyte platelet reactivity. PBMC from patients with acute or chronic ITP, as well as from controls, were tested for proliferative responses to platelets (Fig 2). Four children with chronic ITP had substantial T-lymphocyte proliferation following exposure to platelets for 7 days. In contrast, the remaining children with chronic ITP, those with acute ITP, or normal controls had minimal proliferation.

Establishment and analysis of T-lymphocyte clones. Fresh PBMC from patients with ITP and normal controls were used to establish T-lymphocyte clones as described in Materials and Methods. The cloning efficiency, defined as the number of clones that were transferred from 96-well to 48-well plates, was approximately 10% for all three groups. One third of the T-cell clones initially transferred grew to the 12-well stage and were immunophenotyped; only strongly positive CD4+ or CD8+ clones were passed further. More CD4+ clones were established than CD8+ clones, although the CD4+/CD8+ ratio was similar for each group. Approximately one third of the phenotyped T-cell clones grew well enough to be analyzed for platelet reactivity.

A total of 168 T-cell clones were tested for reactivity against platelets in the 3H-thymidine incorporation assay. Eight clones were designated “positive”, defined as having reactivity at least threefold above background (Table 3). No positive clones were established from normal controls (n = 31), 2 positive clones (LD 2A3 and LD 1F11) were derived from two children with chronic ITP, and 6 positive clones (LD 2C1, LD 2C2, LD 2C3, LD 2C4, LD 2C5, and LD 2C6) were derived from 10 children with acute ITP.
**Table 3. Platelet-Reactive T-Lymphocyte Clones From Children With ITP**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Media</th>
<th>APC</th>
<th>Platelets</th>
<th>APC + Platelets</th>
<th>PHA</th>
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<tbody>
<tr>
<td>AS 4C6</td>
<td>964</td>
<td>448</td>
<td>25,126</td>
<td>37,641</td>
<td>61,508</td>
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<tr>
<td>LD 2A3</td>
<td>1,237</td>
<td>2,255</td>
<td>3,307</td>
<td>10,954</td>
<td>49,099</td>
</tr>
<tr>
<td>LD 1F11</td>
<td>1,820</td>
<td>2,594</td>
<td>9,372</td>
<td>12,107</td>
<td>27,747</td>
</tr>
<tr>
<td>TE 1404</td>
<td>1,027</td>
<td>1,071</td>
<td>937</td>
<td>9,055</td>
<td>33,777</td>
</tr>
<tr>
<td>SH 2D8</td>
<td>1,283</td>
<td>1,890</td>
<td>1,435</td>
<td>13,283</td>
<td>202,373</td>
</tr>
<tr>
<td>SH 11B7</td>
<td>3,834</td>
<td>4,430</td>
<td>72,575</td>
<td>66,004</td>
<td>26,308</td>
</tr>
<tr>
<td>CH 10B3</td>
<td>3,063</td>
<td>4,737</td>
<td>18,071</td>
<td>15,383</td>
<td>NT</td>
</tr>
<tr>
<td>CH 1588</td>
<td>1,138</td>
<td>2,937</td>
<td>11,740</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

Clones were stimulated with allogeneic platelets with and without APC (irradiated autologous PBMC) for 7 days. Clones were stimulated with PHA for 3 days as a control. NT, not tested.

**Table 4. Immunophenotype of Platelet-Reactive T-Lymphocyte Clones From Children With ITP**

<table>
<thead>
<tr>
<th>Clone</th>
<th>TCRαβ</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD29</th>
<th>CD45RA</th>
<th>CD56</th>
<th>CD57</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS 4C6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LD 2A3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LD 1F11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>SH 2D8</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>SH 11B7</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH 10B3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH 1588</td>
<td>+</td>
<td>+</td>
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</table>

Clones were stained with MoAbs as described in Materials and Methods.

**DISCUSSION**

Most children with ITP will have an acute clinical course defined as thrombocytopenia resolving within 6 months, whereas 10% to 20% of affected children will develop a clinical course similar to adults with ITP, with chronic thrombocytopenia often requiring long-term therapy. As a viral-like prodrome often precedes acute ITP, this disorder may involve "innocent bystander" destruction of platelets from circulating immune complexes. As with other viral-like diseases, the long-term sequelae of ITP may involve immune dysfunction and autoimmunity. The identification of platelet-reactive T lymphocytes in children with ITP suggests that immune-mediated destruction of platelets may play a significant role in the pathogenesis of this disorder. Further studies are needed to determine the role of these T lymphocytes in the development and resolution of ITP in children.
T LYMPHOCYTES IN CHILDHOOD ITP

in children presumably involves platelet destruction mediated by autoantibodies produced in the spleen, where the relatively slow blood flow and high density of macrophages allows efficient Fc-receptor-mediated clearance. However, despite these differences, it is currently impossible to identify accurately at the time of diagnosis which child will develop an acute or chronic disorder.

Our study showed no major differences in T-cell immunophenotype among children with acute versus chronic ITP. Although children with acute ITP had higher numbers of CD45RA+ (naive) and lower numbers of CD45RO+ (memory) T lymphocytes (Table 1 and Fig 1), these differences may simply reflect the younger age of children with acute ITP. Similarly, there were no substantial differences in the TCR variable β gene usage among our patient groups, although 2 boys with chronic ITP had an elevated number of circulating Vβ8 T lymphocytes. It is interesting that one patient had predominantly Vβ8, CD4+ cells and the other had Vβ6, CD8+ cells, suggesting a selective expansion. Increased usage of TCR variable gene segments have been previously reported in patients with abnormal clones of B cells; the role of Vβ8+ T cells in the pathophysiology of immune-mediated platelet destruction will require additional investigation.

Autoantigen-reactive T-cell clones have been previously established from patients with Graves' disease and in multiple sclerosis. Moreover, T-cell clones in patients with aplastic anemia showed differences in phenotype and cytokine production compared with clones from normal volunteers. We established T-cell clones in our patients with ITP using limiting dilution and nonspecific stimulation (PHA, IL-2, irradiated feeder cells), but in the absence of specific platelet antigen stimulation, for several reasons. First, the platelet proteins and epitopes that could serve as T-lymphocyte autoantigens in ITP are numerous. In addition, we wanted to investigate the baseline frequency of platelet-reactive T cells in patients with ITP, without selecting for clones that expanded in vitro in the presence of platelet antigens. Finally, we wished to measure the reactivity of T-cell clones during initial in vitro exposure to platelets, rather than following continuous stimulation. However, in part because the T-cell clones were not established with specific antigen stimulation, we found long-term propagation to be difficult; in general, clones could be expanded for only 3 months. Consequently, experiments using platelet proteins to generate antigen-specific T-cell clones are currently underway, and should allow long-term stimulation and propagation. The establishment of long-term platelet-reactive T-lymphocyte clones will allow investigation of platelet antigen specificity, major histocompatibility complex (MHC)-restriction, and mechanisms of platelet destruction.

Allogeneic platelets were used as a source of antigen to allow standardization of the platelet reactivity assay because most patients had few circulating autologous platelets. However, we recognized that allogeneic platelets might stimulate T lymphocytes via their surface class I MHC molecules. Semple and Friedman attempted to eliminate this potential platelet MHC antigenicity using acid-treated platelets, but surface MHC expression is regenerated during the first 10 hours using this method. Because no platelet-reactive clones were established from lymphocytes of normal controls, we do not believe that MHC alloantigenicity was the source of the observed platelet reactivity.

We report for the first time that platelet-reactive T-cell clones can be identified from the peripheral blood of children with ITP, particularly from those with chronic disease. Six clones were isolated from four patients with chronic ITP, out of a total of 113 clones tested in this group. This relatively high frequency of platelet-reactive clones is not surprising because Semple and Freedman found platelet reactivity in bulk PBMC from adult patients with chronic ITP. Interestingly, these four children with chronic ITP and platelet-reactive clones also had PBMC platelet reactivity significantly higher than controls, acute ITP patients, and the other children with chronic ITP (Fig 2). Therefore, it is likely that a subset of patients with chronic ITP has T-cell reactivity against platelets, which is measurable at both the bulk polyclonal lymphocyte level as well as at the clonal level. Although no platelet-reactive clones were established from normal controls, it is possible that autoreactive clones could have been identified if more normal clones had been tested.

The immunologic mechanisms by which autoreactive T lymphocytes could mediate platelet clearance and destruction are speculative. Each of the 6 CD4+ platelet-reactive T-cell clones also expressed the surface CD29 (4B4) antigen, thus characterizing them as helper-inducer cells. Therefore, autoreactive CD4+, 4B4+ cells may recognize platelet antigens and induce autoreactive B lymphocytes to produce antiplatelet antibodies. The measurement of IL-2 secretion by 4 of 7 clones suggests that platelet stimulation may induce proliferation of other T cells (paracrine growth); our assay would not have detected the minute amounts of IL-2 secretion capable of autocrine T-cell stimulation. Two clones (CD4+ SH11B7 and CD8+ CH10B3) did not have additional platelet reactivity in the presence of APC. Both of these clones expressed NK marker CD56, raising the possibility that direct platelet interaction and cytotoxicity might occur by platelet-reactive T lymphocytes. Although these potential mechanisms are only speculative, the establishment of autoreactive T-cell clones from patients with ITP should allow better understanding of the pathophysiology of immune-mediated platelet destruction.

REFERENCES
43. Sugawara S, Abe T, Kumagai K: A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3. J Immunol Methods 100:83, 1987
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RE Ware and TA Howard