Mechanisms of Corticosteroid Action in Immune Thrombocytopenic Purpura (ITP): Experimental Studies Using ITP-Prone Mice, (NZW × BXSB) F₁

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To determine the mechanism by which platelet counts increase after corticosteroid therapy for human immune thrombocytopenic purpura (ITP), we studied the platelet kinetics using prednisolone (PDN)-treated ITP-prone mice, (NZW × BXSB) F₁ (W/B F₁). An increase in platelet counts was observed in W/B F₁ mice (n = 10, mean ± SD, 1,202 ± 202 ± 10²/µL) 4 weeks after treatment with PDN (2 mg/kg/d); no increase occurred in nontreated W/B F₁ mice (n = 5, 851 ± 126, P < .005). Prolonged platelet life-spans (PLSs) were observed in treated W/B F₁ mice (1.29 ± 0.40 days), but not in nontreated controls (0.80 ± 0.24 days, P < .01). No increase in platelet production (platelet turnover) was found in PDN-treated W/B F₁ mice, but significant decreases in platelet-associated antibodies (PAAs) and platelet-bindable serum antibodies (PBAs) were noted. Studies on organ localization of radiolabeled platelets showed that hepatic uptake significantly decreased in the treated W/B F₁ mice, but not in nontreated W/B F₁ mice. To elucidate the effect of PDN on the reticulo-endothelial phagocytic activity in W/B F₁ mice, we studied in vivo clearance of IgG-sensitized, ¹¹¹In-labeled autologous erythrocytes. W/B F₁ mice treated with PDN showed a marked impairment of their ability to clear these cells, although PDN had little effect on the number of splenic or hepatic macrophage Fcγ receptors. These results and our previous findings of splenectomy suggest that PDN improves platelet counts not only by suppressing systemic reticulo-endothelial phagocytic function but also by reducing antibody production.

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from circulation using multiple-hit model analyses, as recom-
manded by the International Committee for Standardization in Haematology. Extrapolation of the platelet survival curve to time zero was used in the determination of platelet recovery, which was calculated as:

\[
\text{Platelet Radioactivity at Time Zero (cpm) \times Blood Volume (mL)} \]

\[
\text{Platelet Radioactivity Totally Injected (cpm)} \]

where blood volume (mL) = body weight (g) \times 0.078. Platelet turnover was calculated using the following formula:

\[
\text{Turnover} = \frac{\text{Platelet Count} \times 10^7/\mu L \times 90}{\text{Platelet Lifespan (days) \times Platelet Recovery (\%)}},
\]

For stable platelet counts, it is assumed that platelet turnover equals platelet production. Mice were killed 72 hours after injection to determine the organ localization of the radiolabeled platelets. The liver, spleen, lungs, and bone marrow (femurs) were removed to determine the level of radioactivity in the organs.

PAAs and PBAs. We used a modified method of platelet suspension immunofluorescence test for PAAs and PBAs, as previously described. Briefly, platelet suspension was obtained from EDTA blood (50 μL) by differential centrifugation. After washing, the platelets were fixed with 1% paraformaldehyde. The platelets were resuspended in 100 μL of EDTA-phosphate-buffered saline (PBS) to a platelet count of 3 × 10^10/μL. The platelets were resuspended with 5 μL of fluorescein isothiocyanate (FITC)-conjugated goat antimouse Ig (Becton Dickinson) for 30 minutes to determine PAAs. For the detection of PBAs, 100 μL of normal BALB/c platelets (3 × 10^10/μL) were incubated with 100 μL of serum (1:4 dilution) for 30 minutes at room temperature. FITC-conjugated goat antimouse Ig was then added. After final washing, samples were analyzed on a FACScan (Becton Dickinson). For the quantification of PAAs and PBAs, we used modified methods of Lazarchik et al. The intensity of fluorescence, expressed as the mean channel of the histogram, was calculated in each sample, and then compared with that of the control. A ratio of relative fluorescence (FR) was determined, where FR = (mean channel of sample)/(mean channel of control). The autofluorescence of the platelets (for PAAs) and the second antibody alone (for PBAs) were used as controls.

Clearance studies. Na_2^10CrO_4 (37 MBq/mL) in sterile isotonic saline was obtained from Daiichi Radioisotope Co (Tokyo, Japan). Labeling and clearance studies were performed according to the methods of Shear et al. Briefly, blood (0.5 mL) obtained from nontreated W/B F, mice in acid-citrate-dextrose (ACD) was washed three times in ACD-saline (pH 6.8). Three milliliters of washed erythrocytes (RBCs) (5.0 × 10^11/mL) was incubated with 3.7 MBq sodium chromium in a 37°C water bath for 45 minutes. The labeled RBCs were then washed three times in ACD-saline and resuspended in 10^7/mL of isotonic saline. Equal volumes of purified antimouse RBC IgG (rabbit) (Inter-Cell Technologies, Inc, Hopewell, NJ; 1:750 dilution) and Cr-labeled RBC suspension were incubated under constant agitation for 30 minutes at 37°C. After washing three times in ACD-saline, radiolabeled, IgG-coated RBCs were resuspended in isotonic saline (2 × 10^11/mL). Recipient mice were injected intravenously with treated RBCs (0.2 mL) and blood (15 μL) was taken at 1, 5, 10, 30, 60, and 120 minutes. The samples were suspended in 1.0 mL of distilled water, and radioactivity counted as mentioned above. The number of cpm at 5 minutes after injection was taken as 100%, and the rate of disappearance of labeled RBCs from circulation calculated in each mouse.

Macrophage Fcγ receptor (FcγR) assay. Macrophage FcγR expression was assessed by the binding of 2.4G2 (rat monoclonal antibody against antimouse FcγII receptor) according to the methods of Petroni et al. Briefly, spleen mononuclear cell suspensions isolated from the density gradient were washed three times in 5 mmol/L EDTA-PBS with 0.1% sodium azide. Splenic macrophage-rich fractions were then obtained by the plastic dish method. For the isolation of hepatic macrophages, liver was minced and washed twice with Hanks' balanced salt solution (HBSS), then incubated at 37°C for 60 minutes with 5 mL of HBSS containing 2.5 mg of collagenase (type II; Sigma Chemical Co, St Louis, MO) and 1.0 mg of trypsin inhibitor (type 1; Sigma). The resulting cell suspension was filtered by passing it through sterile cotton gauze and centrifuged three times at 60g for 1 minute to remove hepatocytes. The supernatant was washed three times with HBSS and resuspended with EDTA-PBS. This hepatic or splenic macrophage-rich fraction (more than 50% were morphologically identified as macrophage, 1 × 10^7/100 μL) was incubated with saturating concentrations of purified 2.4G2 or rat IgG at 4°C for 30 minutes. After washing three times in EDTA-PBS, the cells were then incubated with 50 μL of FITC-conjugated rabbit F(ab)₂, directed against rat Ig (Seronet Co, Oxford, UK; 1:100 dilution) for 30 minutes. After final washing, macrophages were sectioned with the scatter-gating method, and a total of 1 × 10⁴ cells within the gate were analyzed using a FACScan. Data were expressed as FR (as described above).

Statistical analyses. The means of platelet counts, PLSs, and PAAs were compared using a paired or unpaired Student's t-test at the .05 significance level.

RESULTS

Effect of PDN treatment on the platelet counts in W/B F, mice. Figure 1 illustrates the changes in platelet counts after PDN treatment. Platelet counts of 1-month-old W/B F, mice (male) already showed thrombocytopenia (n = 5, shaded area). Three-month-old W/B F, mice (male) already showed thrombocytopenia (n = 15, 702 ± 105 × 10^9/μL). PDN led to an increase in platelet counts in W/B F, mice (n = 10, 854 ± 128 × 10^9/μL) 2 weeks after PDN treatment. Three or 4 weeks after PDN treatment, the increase in W/B F, mice (3 weeks, 1,022 ± 125 × 10^9/μL; 4 weeks, 1,202 ± 202 × 10^9/μL) became statistically significant, in comparison with controls (n = 5, 642 ± 82 × 10^9/μL; P < .001).

Fig 1. The effect of PDN on platelet counts in W/B F, mice. Mice were treated with PDN (2 mg/kg/d) for 4 weeks (n = 10, □). Control W/B F, mice were treated with the same volume of isotonic saline (n = 5, □). Bars represent mean ± SD. Shading shows the normal range of platelet counts (1-month-old W/B F,). (*) P < .001.
The control W/B F, mice, which were treated with isotonic saline alone, showed a slight decrease in platelet counts with age (Fig 1), which was similar to the platelet counts of nontreated W/B F, mice (data not shown); this decrease with age was considered to be a natural course of the disease.

Platelet kinetic studies. Table 1 shows the PLSs of each mouse before and after treatment. The PLSs of male BALB/c (3 months) and female W/B F, (3 months) mice were 2.11 ± 0.28 and 1.44 ± 0.21 days, respectively, whereas those of male W/B F, (3 months) mice were significantly shortened (0.60 ± 0.24 days). Four weeks after PDN treatment, a significant prolongation of PLSs was observed (1.29 ± 0.40 days), in contrast to controls (0.47 ± 0.32 days; P < .01); values were also significantly prolonged in contrast to those before PDN treatment (0.60 ± 0.24 days; P < .01). The effect of PDN treatment on platelet recovery and platelet turnover is also shown in Table 1. The male W/B F, mice showed a significantly increased platelet turnover, in contrast to the male BALB/c and female W/B F, mice (P < .01), which showed no such increase. Four weeks after PDN treatment, a decreased turnover was observed in W/B F, mice (1,579 ± 252 × 10^9 platelets/μL/d), in contrast to nontreated W/B F, mice (2,297 ± 364; P < .05). Table 2 summarizes the uptake of 311In-labeled platelets by major organs 3 days after injection. The spleen and liver of male W/B F, mice were the main accumulation sites of labeled platelets; the mice showed mild hepato-splenomegaly. A slight but significant decrease in turnover was observed. The peak channel of fluorescence intensity of “I-labeled platelets by major organs

| Abbreviation: ND, not determined. |
| Table 1. Effect of PDN Treatment on Platelet Kinetics in W/B F, Mice |

<table>
<thead>
<tr>
<th>Mice</th>
<th>Age (mo)</th>
<th>Sex</th>
<th>No.</th>
<th>PDN Treatment</th>
<th>Platelet Recovery (%)</th>
<th>Platelet Lifespan (d)</th>
<th>Platelet Turnover (× 10^9 platelets/μL·d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>3</td>
<td>M</td>
<td>6</td>
<td>–</td>
<td>77.3 ± 7.2</td>
<td>ND</td>
<td>667 ± 63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72.4 ± 4.9</td>
<td>ND</td>
<td>863 ± 79</td>
</tr>
<tr>
<td>W/B F</td>
<td>3</td>
<td>M</td>
<td>5</td>
<td>–</td>
<td>58.2 ± 8.4</td>
<td>56.2 ± 4.9</td>
<td>2,020 ± 334†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>56.6 ± 7.5</td>
<td>59.2 ± 9.4</td>
<td>2,297 ± 364</td>
</tr>
</tbody>
</table>

Platelet recovery, platelet lifespan, and platelet turnover were calculated as described in Materials and Methods. Mice were examined 4 weeks after PDN treatment. Values represent mean ± SD.

DISCUSSION

A number of investigators have studied the effects of corticosteroids on experimentally induced hemolytic anemia or thrombocytopenia in animals. In most experiments, heterologous antisera were injected directly in animals, and kinetics studies were then performed. These studies differed from ours in that they were not able to systematically examine the in vivo effects of PDN on autoantibody production, etc, because these animals do not spontaneously produce antiplatelet antibodies. In contrast, W/B F, mice spontaneously produce antiplatelet antibodies resulting in thrombocytopenia. We therefore consider the W/B F, mouse to be a useful animal model for the investigation of the mode of action of corticosteroids in ITP.

W/B F, mice are known to develop systemic lupus erythematous-like diseases and show high levels of circulating immune complexes (CICs). It is therefore conceiv-
able that CICs are involved in thrombocytopenia in these mice. However, we think that the increased PAA levels are not due to the absorption of CICs because (1) murine platelets have no Fc receptor; (2) other typical SLE-prone mice such as BXSB, MRL/lpr, and NZB/NZW F1, which show high CIC levels, have normal platelet counts; (3) the sera from these mice do not bind to platelets of normal mice; and (4) purified F(ab')2 fragments of W/B F1 IgG can also bind to normal platelets (unpublished data).

In this study, we have shown that PDN leads to increased platelet counts and prolonged PLSs in W/B F1 mice. Clearance studies using IgG-coated RBCs also showed a significant suppression of the ability to clear these cells. These observations suggest that one of the mechanisms of action of PDN is the suppression of the reticulo-endothelial phagocytic function. Consequently, PDN may modulate PLSs and increase platelet counts. Furthermore, marked decreases in PAA and PBA values were noted after PDN treatment in W/B F1 mice, suggesting that another mechanism of PDN is the reduction of autoantibody production. It remains uncertain which mechanism is the most important with regard to the increased platelet counts in these mice, although we think that for at least 2 weeks after the treatment, PDN may act by suppressing reticulo-endothelial phagocytic activity, rather than by reducing antibody production, as platelet counts increased extremely rapidly (within 2 weeks) without any significant changes in the PAA or PBA values.

Another possible effect of PDN on PAA and PBA values is impairment of the interaction between antibodies and antigens, resulting in decreased binding of antibodies to platelets. Although this is conceivable, it seems unlikely, because PDN cannot interfere with the binding of serum antiplatelet antibodies to platelets for long periods without any significant reduction in antibody production.

We have recently examined the effect of splenectomy on platelet kinetics in W/B F1 mice, and found that platelet counts increased and PLSs were prolonged, but normal levels were not reached even after splenectomy; splenectomy had little effect on either PAA or PBA values. From these findings, we consider that the limited effect of splenectomy may be explained by the presence of the systemic RES other than the spleen. In the present study, PDN showed a greater therapeutic effect on platelet counts than splenectomy. It is therefore likely that PDN improves platelet counts not only by suppressing antibody production but also by blocking the systemic RES (including the spleen).

Recently, Gernsheimer et al showed that platelet production was impaired in some ITP patients. If this is the case, PDN may improve the platelet count by increasing platelet production. However, in nontreated W/B F1 mice, increased platelet turnover (platelet production) was observed, and a relatively reduced platelet turnover was noted in PDN-treated W/B F1 mice. These findings indicate that, in W/B F1 mice, both platelet production and destruction

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### Table 2. Organ Distribution of 111In-Labeled Autologous Platelets in W/B F1, Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age (mo)</th>
<th>Sex</th>
<th>No.</th>
<th>PDN Treatment</th>
<th>Weight (mg)</th>
<th>% Distribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Liver</td>
<td>Spleen</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>W/B F1</td>
<td>3</td>
<td>F</td>
<td>5</td>
<td>-</td>
<td>1,190 ± 120</td>
<td>120 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32 ± 7</td>
<td></td>
</tr>
<tr>
<td>W/B F1</td>
<td>3</td>
<td>M</td>
<td>5</td>
<td>-</td>
<td>1,800 ± 310</td>
<td>396 ± 60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29 ± 5</td>
<td></td>
</tr>
<tr>
<td>W/B F1</td>
<td>3</td>
<td>M</td>
<td>5</td>
<td>+</td>
<td>1,720 ± 250</td>
<td>330 ± 72</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21 ± 44</td>
<td></td>
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</table>

*Mice were killed 72 hours after injection of labeled platelets, and percentage of radioactivity totally injected then determined. Values represent mean ± SD.
†Mice were treated with isotonic saline for 4 weeks.
‡P < .05 versus data in nontreated male W/B F1 mice.
Cells (macrophages, etc) in the RES such as the liver and spleen that bear receptors for the Fc portion of IgG (FcγR) can specifically ingest and destroy particles coated with IgG in vivo,29,30 and this Fc-mediated clearance is thought to be important in the pathogenesis of ITP.31,32 In fact, an increased number of FcγR on these cells has been reported in patients with ITP,33,34 and some new treatments using FcγR blockade have been developed.35,36 We have shown a decreased clearance of IgG-coated RBCs in PDN-treated W/B F, mice, and have examined whether these results correlate with the alterations in macrophage FcγR number. Our data failed to show a significant decrease in splenic or hepatic macrophage FcγR numbers in PDN-treated W/B F, mice. One reason is that our FACS analyses may not be sensitive enough to detect minimal changes in FcγR expression. Alternatively, PDN may not influence the FcγR number, but rather the affinity of the FcγR in the macrophages. A further and more detailed examination of FcγR activity (including analyses of other FcγR types, such as FcγRI and FcγRIIa, and in vitro experiments for platelet phagocytosis) is needed to clarify the PDN-induced FcγR modulation.

We conclude that corticosteroids improve platelet counts in W/B F, mice not only by suppressing systemic reticuloendothelial phagocytic function but also by reducing autoantibody production, and that they have little effect on platelet production.

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Mechanisms of corticosteroid action in immune thrombocytopenic purpura (ITP): experimental studies using ITP-prone mice, (NZW x BXSB) F1

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