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 202 ± 202 ± 10⁹/L) 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 (0.60 ± 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, ¹²⁵I-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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MATERIALS AND METHODS

**Mice.** BALB/c, BXSB, and NZW mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). W/B F₁ mice (hybrids of NZW females and BXSB males) were raised under specific pathogen-free conditions in the animal facility of Kiwa Experimental Animal Laboratories (Wakayama, Japan).

**Platelet counts.** Blood (20 μL) was diluted in a buffer containing ammonium oxalate (Unopette Kits, Becton Dickinson, Sunnyvale, CA). Platelets were counted using a hemocytometer under a phase-contrast microscope.

**Prednisolone treatment.** Each mouse was intraperitoneally (IP) injected daily for 4 weeks with PDN (Predonine; Shionogi Pharmaceutical Co., Osaka, Japan) (2 mg/kg in 0.1 mL of isotonic saline). Control mice received the same volume of saline. No mice died during treatment, and all mice survived for more than 3 weeks after the end of treatment.

**Platelet labeling and kinetics studies.** ¹²⁵I-In-tropolone was used for the labeling of platelets. Details of the methods are described elsewhere.¹⁴ Recipient mice were injected intravenously with the radiolabeled platelets (approximately 185 KBq in 0.2 mL). Blood (15 μL) was then taken 1 minute, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 48 hours after injection, and the radioactivity counted using a well-typed scintillation counter. The mean PLS was estimated by calculating the rate of platelet disappearance.
from circulation using multiple-hit model analyses, as recom-
mended by the International Committee for Standardization in 
Hematology. Extrapolation of the platelet survival curve to time 
zero was used in the determination of platelet recovery, which was 
calculated as

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\text{Platelet Radioactivity at Time Zero (cpm) \times Blood Volume (mL)}
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\text{Platelet Radioactivity Totally Injected (cpm)}
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where blood volume (mL) = body weight (g) \times 0.078. Platelet 
turnover was calculated using the following formula:

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\text{Turnover} = \frac{\text{Platelet Count} \times (10^9/\mu L) \times 90}{\text{Platelet Lifespan (days) \times Platelet Recovery (\%)}}
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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 \mu L) by differential centrifugation. After 
washing, the platelets were fixed with 1% paraformaldehyde. The 
platelets were resuspended in 100 \mu L of EDTA-phosphate-
buffered saline (PBS) to a platelet count of \(3 \times 10^9/\mu L\). They were 
then incubated with 5 \mu L of fluorescein isothiocyanate (FITC)-
conjugated goat antimouse Ig (Becton Dickinson) for 30 minutes to 
determine PAAs. For the detection of PBAs, 100 \mu L of normal 
BALB/c platelets (3 \times 10^9/\mu L) were incubated with 100 \mu L 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 

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\text{FR} = \frac{\text{mean channel of sample}}{\text{mean channel of control}}
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The autofluorescence of the platelets (for PAAs) and the second antibody alone (for PBAs) 
were used as controls.

Clearance studies. \(Na^+_9\text{CrO}_4\) (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 \mu L) obtained from 
untreated W/B F\(_m\) mice in acid-citrate-dextrose (ACD) was 
washed three times in ACD-saline (pH 6.8). Three milliliters of 
washed erythrocytes (RBCs) (5.0 \times 10^6/\mu L) 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 10^6/mL of isotonic saline. Equal volumes of 
purified antimouse RBC IgG (rabbit) (Inter-Cell Technologies, 
Inc, Hopewell, NJ; 1:750 dilution) and \(^{99}\text{Cr}\)-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 \times 10^9/ 
\mu L). Recipient mice were injected intravenously with treated 
RBCs (0.2 \mu L) and blood (15 \mu L) 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.

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\text{Macrophage Fc\gamma receptor (Fc\gamma R) assay. Macrophase Fc\gamma R ex-}
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expression was assessed by the binding of 2.4G2 (rat monoclonal 
antibody against antimouse Fc\gamma II 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 
mixed and washed twice with Hanks' balanced salt solution 
(HBSS), then incubated at 37°C for 60 minutes with 5 \mu L 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 \times 10^9/100 \mu L) 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 \mu L of FITC-conjugated rabbit F(ab')

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directed against rat Ig (Serotec 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 \times 10^6 cells within the 
gate were analyzed using a FACScan. Data were expressed as FR 
(as described above).

Statistical analyses. The means of platelet counts, PLTs, 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\(_m\) mice. Figure 1 illustrates the changes in platelet counts 
after PDN treatment. Platelet counts of 1-month-old W/B F\(_m\) mice were 1,105 \pm 134 \times 10^9/\mu L (n = 5, shaded area). 
Three-month-old W/B F\(_m\) mice (male) already showed 
therbocytopenia (n = 15, 702 \pm 105 \times 10^9/\mu L). PDN led 
to an increase in platelet counts in W/B F\(_m\) mice (n = 10, 
854 \pm 128 \times 10^9/\mu L). PDN led to an increase in platelet 
counts in W/B F\(_m\) mice (n = 10, 1,022 \pm 125 \times 10^9/\mu L; 4 weeks after PDN treatment. Three 
or 4 weeks after PDN treatment, the increase in W/B F\(_m\) 
mice (3 weeks, 1,022 \pm 125 \times 10^9/\mu L; 4 weeks, 1,202 
\pm 202 \times 10^9/\mu L) became statistically significant, in compar-
is with controls (n = 5, 642 \pm 82 \times 10^9/\mu L; P < .001).

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\text{Fig 1. The effect of PDN on platelet counts in W/B F\(_m\) mice. Mice were treated with PDN (2 mg/kg/dq)}
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\text{for 4 weeks (n = 10, \bullet). Control W/B F\(_m\) mice were treated with the same volume of isotonic saline}
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\text{(n = 5, \squares). Bars represent mean \pm SD. Shading shows the normal}
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\text{range of platelet counts (1-month-old W/B F\(_m\). (*) P < .001.}
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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 × 10⁹ platelets/μL/d), in contrast to nontreated W/B F, mice (2,297 ± 364; P < .05). Table 2 summarizes the uptake of ¹¹¹In-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 platelet recovery and platelet lifespan, and a significant decrease both in PAA (FR, mean ± SD) (before, 4.9 ± 2.8; after, 2.4 ± 0.8; n = 5, P < .05) and PBA values (before, 4.4 ± 0.9; after, 2.2 ± 0.8; n = 5, P < .01) was observed. The peak channel of fluorescence intensity of PAAs and PBAs on FACS analyses shifted to the left after PDN treatment (Fig 2C and D), whereas that of the control W/B F, mice shifted to the right, suggesting that both PAA and PBA values increased (Fig 2A and B) 4 weeks after PDN treatment. No significant decrease in PAA or PBA values was found 2 weeks after initiation of PDN treatment (PAA, 4.4 ± 2.3; PBA, 3.9 ± 1.1).

Clearance of IgG-coated RBCs. ⁵²Cr-labeled RBCs coated with IgG antibody were injected into 3-month-old W/B F, mice, and the fate of the cells then determined (Fig 3). Nontreated W/B F, mice (n = 4) showed a rapid clearance of these cells, whereas W/B F, mice treated with PDN for 4 weeks (n = 4) showed a much lower clearance rate. At 2 hours, 35% ± 12% (mean ± SD) of the IgG-coated RBCs remained in the circulation in the treated group, compared with 11% ± 6% in the controls (P < .05). ⁵²Cr-labeled, nonsensitized RBCs were not removed from the circulation (Fig 3).

Effect of PDN treatment on macrophage FcγRI expression. To evaluate the effect of PDN on the FcγR number of splenic or hepatic macrophages, we analyzed the macrophage FcγRI expression using the rat monoclonal antibody (2.4G2) that specifically binds to FcyRII on mouse macrophages. Table 3 shows the FcγRI expression of W/B F, mice after 4 weeks of PDN treatment. Although the expression of FcγRI on hepatic or splenic macrophages from PDN-treated mice slightly decreased, no statistically significant change was found by FACS analyses, suggesting that PDN has little effect on the number of FcγRs.

**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 erythematosus-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...
were examined platelet destruction rather than by increasing platelet production. These findings suggest that PDN may act by either mean uptake. These findings are consistent with our previous findings present findings are consistent with our previous findings presence of the systemic RES other than the spleen (probably the liver).

are accelerated, and that PDN may act by suppressing platelet destruction rather than by increasing platelet production.

The exact mechanism of PDN-induced suppression of the RES has not been fully defined. One interesting finding of our study on organ distribution is that PDN induces a relative decrease in hepatic uptake compared with non-treated controls, without a significant change in splenic uptake. These findings suggest that PDN may act by either directly suppressing the phagocytic activity of Kupffer cells or by modulating the hepatic blood flow. In either case, the present findings are consistent with our previous findings that the limited effect of splenectomy may be due to the presence of the systemic RES other than the spleen (probably the liver).

Cells (macrophages, etc) in the RES such as the liver and spleen that bear receptors for the Fc portion of IgG (FcyR) can specifically ingest and destroy particles coated with IgG in vivo, and this Fc-mediated clearance is thought to be important in the pathogenesis of ITP. In fact, an increased number of FcyR on these cells has been reported in patients with ITP, and some new treatments using FcyR blockade have been developed. 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 FcyR number. Our data failed to show a significant decrease in splenic or hepatic macrophage FcyR 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 FcyR expression. Alternatively, PDN may not influence the FcyR 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 FcγRII) 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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