The Effects of Acute Thrombocytopenia on Megakaryocyte-CFC and Granulocyte-Macrophage-CFC in Mice: Studies of Bone Marrow and Spleen

By Jack Levin, Francine C. Levin, and Donald Metcalf

The effects of acute thrombocytopenia, produced by platelet antiserum (PAS), on both megakaryocyte colony-forming cells (Meg-CFC) and granulocyte-macrophage colony-forming cells (GM-CFC) were studied. During the 1-hr to 14-day period following acute thrombocytopenia (platelet counts < 5% of normal), bone marrow and splenic cells of C57BL/6J mice were obtained and cultured for 7 days in 0.3% agar. Numbers of GM and Meg colonies were determined. At no times were alterations in frequency of GM-CFC and Meg-CFC detected in femoral bone marrow. In contrast, GM-CFC in spleen were increased from 3 to 7 days after PAS and from 4 to 7 days after normal serum (NS). Increase in Meg-CFC in the spleen occurred from 3 to 5 days after PAS with a lesser, not significant increase after NS. Alterations in white blood cells and hematocrit values were not detected. Similar responses were observed in germ-free mice and after rechallenge of animals that had received PAS or NS 14 days previously. The delayed increase in Meg-CFC indicates that they are unlikely to be responsible for the altered megakaryopoiesis previously reported in bone marrow after acute thrombocytopenia and was not due to inhibition by PAS. The increase in GM-CFC may reflect stimulation of the reticuloendothelial system by heterologous proteins.

The administration of heterologous platelet antiserum produces acute thrombocytopenia, which results in alterations in megakaryocytopoiesis. During the 60-hour period following acute thrombocytopenia, there is an increase in small acetylcholinesterase-positive cells (presumably immature megakaryocytes), the mitotic index of megakaryocytes, incorporation of thymidine by megakaryocytes, and in the rate of megakaryocyte maturation. There is also an increase in their number, size, and DNA content. Subsequently, an increase in the rate of platelet production can be demonstrated, which results in a period of rebound thrombocytosis before platelet levels again return to normal.

Recently, techniques have become available for the culture in vitro of cells that produce megakaryocyte colonies. However, few data are available concerning the effects of perturbation of thrombopoiesis in the donors on the characteristics of their subsequently cultured hematopoietic cells. Accordingly, we have studied the effects of acute thrombocytopenia on colony-forming cells (CFC) obtained from mice in which acute thrombocytopenia had been produced with platelet antiserum. Our results indicate important differences between alterations described previously in the bone marrows of acutely thrombocytopenic animals and changes observed in the frequency of various colony-forming cells from their bone marrow and spleen.

MATERIALS AND METHODS

Mice

C57BL/6J mice, approximately 8 wk of age, were used for these experiments. These mice were bred under specific pathogen-free conditions and then maintained under conventional conditions for approximately 1–3 wk before use. Germ-free animals were obtained from the Ramaciotti Laboratory of The Walter and Eliza Hall Institute and not removed from sterile-isolation until the time of sacrifice.

Preparation of Cell Suspensions

Femoral marrow cells were obtained from animals killed by cervical dislocation. Bone marrow from the femurs of mice was flushed into Eisen’s balanced salt solution (EBSS) with a 22-gauge needle. Single cell suspensions were prepared by gentle pipetting. Spleen cells were prepared by gentle disruption of spleen through a stainless steel, 100-mesh sieve. The resultant suspensions were allowed to stand for 5 min to let undispersed fragments sediment. The supernatants then were resuspended by gentle pipetting and viable cell counts performed using trypan blue.

Agar Cultures

All cultures were performed in 35-mm plastic Petri dishes (Kayline Plastics, Thebarton, South Australia, Australia) containing 1 ml of agar medium. The agar medium was an equal volume mixture of 0.6% Bacto agar and double strength Dulbecco’s modified Eagle’s medium. The composition of the double strength medium was: Dulbecco’s modified Eagle’s medium HGI6 Instant Tissue Culture Powder (Grand Island Biological Co., Grand Island, N. Y.) 10 g; 390 ml double glass-distilled water; 3 ml L-asparagine (6.7 mg/ml; final concentration in medium 20 μg/ml); 1.5 ml DEAE Dextran (50 mg/ml; final concentration in medium 75 μg/ml; mol wt
EFFECTS OF THROMBOCYTOPENIA OR Meg-CFC

500,000, Pharmacia, Sweden); 0.575 ml penicillin (2 × 10⁶ U/ml; Glaxo, Boronia, Victoria, Australia); 0.375 ml streptomycin (200 mg/ml; Glaxo); 4.9 g NaHCO₃ and 250 ml of fetal calf serum (Flow Laboratories, Stanmore, New South Wales, Australia).

Cultures contained 50,000 bone marrow or 10⁶ spleen cells/ml.

Preliminary experiments had indicated that these concentrations provided adequate numbers of colonies for evaluation but a colony density low enough to allow detection of megakaryocyte colonies. After addition of the appropriate number of cells to the agar medium, 1-ml volumes of the cell suspension in agar medium were pipetted into culture dishes that contained 0.2 ml of spleen conditioned medium. The culture dishes were mixed thoroughly, allowed to gel, and incubated for 7 days in a fully humidified atmosphere of 10% CO₂ in air.

Preparation of Spleen Conditioned Medium

C57BL/6J spleen cells were incubated for 7 days at a concentration of 2 × 10⁶ cells/ml in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), which contained 5% heat-inactivated human plasma and 0.05 ml of a 1:15 dilution of pokeweed mitogen/ml of culture medium (Grand Island Biological Co.). After incubation, the medium was centrifuged for 10 min at 3000 g. The supernatant fluid was removed, filtered (0.45-μ filter, Millipore Corp., Bedford, Mass.), and stored, ready for use, at −20°C.

Scoring of Cultures

Preliminary experiments indicated that megakaryocyte colonies reached their maximum numbers after 7 days of culture and that this characteristic was not altered by the experimental manipulations of the donor animals. Therefore, after 7 days of incubation, cultures were scored for colony formation using an Olympus dissection microscope with semi-indirect lighting. Magnification of 35–40× was used for identification of megakaryocyte colonies. Discrete aggregates of 50 or more cells were scored as colonies except megakaryocytes, for which 3 or more cells were considered a colony. Data in this article are based on triplicate cultures for each experimental point, which were scored by two independent observers. All colonies considered to be possibly megakaryocytic were removed from two of the three culture dishes, which constituted each triplicate set, with a finely drawn Pasteur pipette. They were then placed on egg albumin-coated slides, air dried, fixed in acetone for 15–20 min at room temperature, and then stained for acetylcholinesterase (see below). Culture dishes were deliberately “overpicked” to assure that all megakaryocyte colonies were removed and identified histochemically. Accordingly, the numbers of megakaryocyte colonies reported cannot be overestimates of their numbers in culture.

Acetylcholinesterase Stain

Since megakaryocytes are the only hematopoietic cells of the mouse that contain detectable acetylcholinesterase, the presence of this cytoplasmic enzyme was used to provide positive identification of megakaryocyte colonies. The colonies were stained according to the method of Karnovsky and Roots24 for 1.5 hr. After 1 day, nuclei were counterstained with methyl green (pH 4.2, 0.1 M sodium acetate buffer) for 1–2 sec (C.I. 42358, British Drug Houses Chemicals Ltd., Poole, England). No colony was scored as megakaryocytic unless it contained at least 3 cells that demonstrated the characteristic orange-brown color of Hatchett’s brown in their cytoplasm. Granulocytic and macrophage colonies were never positive.

Other experiments demonstrated that all cells in megakaryocyte colonies were benzidine negative, according to the method of Coo-

per,19 and none phagocytized carbon particles (provided by a dilute solution of India ink added directly to the culture at time of plating).

Platelet Antiserum

Platelet antiserum (PAS) was obtained from colored inbred rabbits (Commonwealth Serum Laboratories, Melbourne, Australia) that had been immunized with washed, frozen-thawed platelets from C57BL/6J mice. Immunization was induced by 3–4 serial subcutaneous and intramuscular injections of platelets suspended in incomplete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). Complete Freund’s adjuvant was used only for the first set of injections. After approximately 8–10 wk, potent antiserum was obtained. Normal serum (NS) was obtained prior to immunization. Both PAS and NS were adsorbed three times each with washed, platelet-free thymocytes, macrophages, granulocytes, and red blood cells from C57BL/6J mice. Granulocytes and macrophages were obtained following intraperitoneal injection of casein, as previously described.26 PAS and NS, thus adsorbed, did not produce alterations in hematocrit or white blood cell levels (see Results).

Preliminary experiments indicated that 0.05 ml of PAS was the lowest dose that produced reproducible, severe, acute thrombocytopenia (platelet counts less than 5% of normal), and this dose of PAS or NS was used in all experiments.

Blood Samples

Blood for performance of platelet counts and hematocrit values was obtained from the retroorbital plexus of unanesthetized mice, in capillary tubes that contained either EDTA and heparin for platelet counts (Drummond Scientific Co., Broomall, Pa.) or heparin for hematocrit determinations (Hawksley & Sons, Ltd., London, England).

Blood for measurement of colony-stimulating factor was obtained by cardiac puncture of mice that had been anesthetized with ether. Blood was either obtained without anticoagulation for serum or placed into a plastic tube that contained sodium citrate (9 parts blood:1 part sodium citrate, final concentration 0.38% sodium citrate) for preparation of plasma.

Blood for determination of white blood cell counts also was obtained by cardiac puncture and was drawn into 1-ml syringes that contained 0.02 ml of buffered EDTA (0.143 M, 5.3 g/100 ml distilled H₂O, pH 7.0, British Drug Houses Ltd., Poole, England).

Blood Cell Counts

Microhematocrit values were determined by the method of Strumia, Sample, and Hart21 using a microhematocrit centrifuge (Hawksley & Sons, Ltd.). Platelets were counted by the method of Bull, Schneideman, and Brecher,22 using a Coulter electronic particle counter, model B (Coulter Electronics, Hialeah, Fla.). Total white blood cell counts were obtained with a Coulter electronic particle counter, Model S, after determination that counts thus obtained approximated those obtained using a standard counting chamber. Differential white blood cell counts were performed on smears of heart blood that had been stained with May-Grünwald-Giemsa stains.

Detection of Endotoxin

Endotoxin was assayed using the Limulus amebocyte lysate test, as described by Levin et al.34 A quantity of 0.05 ml of the material to be tested was incubated with an equal volume of amebocyte lysate for 4 hr at 37°C and then at room temperature until the next morning. Serial observations of the gelation reaction were made visually, and the concentration of endotoxin calculated. E. coli
endotoxin (E. coli B, lipopolysaccharide B, 055:B5, Difco Laboratories, Detroit, Mich.) was used as the standard. Inhibitors were removed from samples of plasma by dilution and boiling, as previously described.

Miscellaneous

Guinea pig complement was provided by Beverley Pike. It had been adsorbed initially with agarose (Calbiochem, San Diego, Calif.) and then with mouse bone marrow and spleen cells (each at a concentration of 10^8 cells/ml) for 60 min at 4°C. Activity of the adsorbed complement was confirmed using antibody directed against mouse cortisone-resistant lymphocytes (generously provided by Dr. John Schrader).

For one series of experiments, serum was obtained from C57BL/6J mice 3 hr after they had received 5 μg E. coli endotoxin intravenously (E. coli W, lipopolysaccharide W, 0111:B4, Difco) and used as a source of colony-stimulating factor. For another series of experiments, serum was obtained from mice 3 hr after they had received 5 μg E. coli endotoxin intravenously (E. coli W, lipopolysaccharide W, 0111:B4, Difco) and used as a source of colony-stimulating factor.

Statistical analyses were carried out using the Mann-Whitney rank sum test, from which were derived the two-tailed p values.

RESULTS

Effects of Platelet Antiserum on Platelet Counts, Hematocrit Values, and White Blood Cell Counts

In order to evaluate the effect of acute thrombocytopenia on colony-forming cells (CFC), 0.05 ml of platelet antiserum (PAS) was administered intravenously, via the tail vein, to mice. Preliminary experiments had demonstrated that 0.05 ml produced severe acute thrombocytopenia that was, however, not sustained. Rebound thrombocytosis occurred 5–7 days later (Fig. 1). No change in white blood cell counts or hematocrit values occurred at 1, 4.5, and 24 hr or at 5, 7, and 10 days after administration of PAS (Table 1). However, daily and group variability in white blood cell and hematocrit levels may have obscured slight effects.

<table>
<thead>
<tr>
<th>Time After Injection</th>
<th>Hct (%)</th>
<th>WBC/cu mm</th>
<th>Hct (%)</th>
<th>WBC/cu mm</th>
<th>Hct (%)</th>
<th>WBC/cu mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34.1 ± 0.6</td>
<td>4,729 ± 540</td>
</tr>
<tr>
<td>4 hr</td>
<td>38.8 ± 0.8</td>
<td>5,000 ± 800</td>
<td>35.0 ± 0.9</td>
<td>3,400 ± 600</td>
<td>34.7 ± 1.1</td>
<td>3,200 ± 300</td>
</tr>
<tr>
<td>24 hr</td>
<td>33.4 ± 1.6</td>
<td>3,967 ± 900</td>
<td>36.0 ± 0.9</td>
<td>3,514 ± 300</td>
<td>34.1 ± 1.4</td>
<td>6,814 ± 800</td>
</tr>
<tr>
<td>5 days</td>
<td>32.4 ± 1.4</td>
<td>5,042 ± 620</td>
<td>33.4 ± 1.2</td>
<td>4,543 ± 270</td>
<td>36.9 ± 0.9</td>
<td>4,829 ± 600</td>
</tr>
<tr>
<td>7 days</td>
<td>32.8 ± 0.9</td>
<td>3,700 ± 290</td>
<td>33.2 ± 1.1</td>
<td>3,933 ± 640</td>
<td>33.2 ± 2.0</td>
<td>3,800 ± 520</td>
</tr>
<tr>
<td>10 days</td>
<td>31.0 ± 1.8</td>
<td>6,033 ± 700</td>
<td>34.7 ± 1.8</td>
<td>6,967 ± 860</td>
<td>33.0 ± 2.1</td>
<td>6,450 ± 150</td>
</tr>
</tbody>
</table>

*In this paper, the term granulocyte refers to the combination of neutrophil and eosinophil colonies or their collective CFC.

Table 1. Effects of Platelet Antiserum (PAS), Normal Serum (NS), or Phosphate-Buffered Saline (PBS) on Hematocrit Values and White Blood Cell Counts*
EFFECTS OF THROMBOCYTOPENIA OR Meg-CFC

Fig 2. Effect of platelet antiserum (PAS) or normal serum (NS) on neutrophil-macrophage CFC (NM-CFC), eosinophil CFC (EO-CFC), and megakaryocyte CFC (Meg-CFC) in bone marrow. Mean total number of NM-CFC plus EO-CFC, which collectively constitute GM-CFC in these cultures (top panel), and Meg-CFC (bottom panel) at various times after administration of 0.05 ml of PAS or NS is shown. Normal controls received phosphate-buffered saline. 100,000 nucleated bone marrow cells were cultured. Four to six experiments were performed on each of days 1, 2, 3, 4, 5, and 7; 2–3 experiments were performed at each of the other times shown (only 1 experiment at 16 hr.).

Fig 3. Effect of platelet antiserum (PAS) or normal serum (NS) on neutrophil-macrophage CFC (NM-CFC) and eosinophil CFC (EO-CFC) in spleen. Mean total number of NM-CFC plus EO-CFC at various times after administration of 0.05 ml of PAS or NS is shown. Normal controls received phosphate-buffered saline. 50,000 nucleated bone marrow cells were cultured. Four to six experiments were performed on each of days 1, 2, 3, 4, 5, and 7; 2–3 experiments were performed at each of the other times shown (only 1 experiment at 16 hr.). Two spleens were pooled for each individual experiment. One standard error is indicated only where statistically significant differences occurred.

megakaryocyte colonies detected when femoral bone marrow cells were cultured at 50,000 cells/ml (Fig. 2) or 100,000 cells/ml (data not shown). In marked contrast, from 3 to 7 days following the administration of PAS and from 4 to 7 days following the administration of NS, there was an increase in granulocyte and macrophage CFC in the spleen (Fig. 3). Maximum increase in CFC occurred 5 days after administration of PAS (eightfold increase) or NS (fivefold increase). However, there was a statistically significant increase from days 3 through 7 only following PAS ($p < 0.05$ at all time points).

Similar responses were noted in the number of megakaryocyte-CFC in the spleen (Fig. 4). From days 3 to 5 after administration of PAS, there was a significant increase in megakaryocyte-CFC ($p < 0.05$), with the maximum sixfold increase occurring 4 days after administration of PAS. A lesser increase was observed following administration of NS, with a maximum increase of only threefold occurring on day 4. However, at no time were the number of Meg-CFC statistically significantly increased above control levels in animals that had received NS. In all groups of animals, the number of Meg-CFC was significantly increased only following PAS administration.

Fig 5. Effect of platelet antiserum (PAS) or normal serum (NS) on spleen weight and number of nucleated cells in the spleen. Mean spleen weight and total nucleated cells per spleen at various times after administration of PAS or NS are shown. Four to six experiments were performed on each of days 1, 2, 3, 4, 5, and 7; 2–3 experiments were performed at each of the other times shown (only 1 experiment at 16 hr.). Two spleens were pooled for each individual experiment. Control values ± 1 S.D. are based on 35 experiments (70 spleens).
animals, normal levels of CFC were observed 14 days after administration of PAS or NS (data not shown).

In one experiment, an attempt was made to determine if splenectomized mice would demonstrate an increase in bone marrow GM-CFC and Meg-CFC following induction of acute thrombocytopenia. Fourteen days after splenectomy, at which time the mean platelet count was 2.16 × 10^12/liter, mice received PAS, and their bone marrow cultured 5 days later. GM-CFC and Meg-CFC in bone marrow were not increased 5 days after administration of PAS to splenectomized animals. However, their mean platelet count at that time was 3.20 × 10^12/liter, higher than observed at any time following recovery from PAS in normal animals.

**Effects of Platelet Antiserum or Normal Serum on Total Nucleated Cells in Bone Marrow and Spleen Weight**

Although total nucleated cells in the spleen did not significantly increase above normal levels at any time during these studies, a statistically significant difference was observed between the 24-hr period following administration of PAS and days 2 and 3 (p < 0.05) (Fig. 5). A similar difference existed following administration of NS (initial 24-hr period versus day 2, p < 0.05). Although some of these differences may be questionable, since all 1-hr, 5-hr, and 16-hr groups were studied during a 2-wk period, the 6 groups of animals that constituted the basis for the day-1 data were studied during a period of 6 mo.

Spleen weights did not significantly vary from normal; however, there was a statistically significant difference between spleen weights during the 24-hr period following administration of PAS and days 2 and 3 (p < 0.01). Therefore, it appears that during the 24-hr period following administration of PAS or NS, there was a relative reduction in total number of nucleated cells in the spleen and spleen weight, both of which subsequently rose during the following 2–3-day period. Numbers of nucleated cells and spleen weights remained relatively high in both groups of animals until there was a decrease of total nucleated cells on day 10 and of spleen weight on day 14 (not shown). Total numbers of nucleated cells in the femur remained within normal limits throughout the duration of the experiments in both groups of animals, although there was a gradual modest relative increase in animals that received PAS, with maximum numbers of cells on days 3, 4, and 5.

As a result of increased numbers of nucleated cells in the spleen in association with the increase in frequency of splenic CFC, total splenic GM-CFC increased fivefold, 4–5 days after NS and 8–10-fold 3–5 days after PAS. At the time of maximum increase, total GM-CFC/spleen was approximately 15,000 and 27,000, following NS and PAS, respectively. Similarly, total splenic Meg-CFC increased threefold 4 days following NS and 6–7-fold 4–6 days following PAS. At times of maximum increase, total Meg-CFC/spleen was approximately 1600 and 3600, respectively. Total GM-CFC and Meg-CFC did not significantly increase in the bone marrow (approximately 21,000 GM-CFC and 1000 Meg-CFC/femoral shaft).

**Effects of Multiple Injections of Platelet Antiserum or Normal Serum**

Because of data that indicate that the response of the bone marrow to sustained thrombocytopenia may differ from that observed following acute thrombocytopenia, experiments were performed in which 3 injections of PAS or NS were given on alternate days for a total of 3, 0.05-ml doses. During this period, platelet counts of animals that had received PAS repeatedly fell to a mean of 0.069 × 10^12/liter after each injection and remained below 0.2 × 10^12/liter for 5 days. Platelet counts then rose and reached a mean of 1.084 × 10^12/liter on the third day and 1.40 × 10^12/liter on the fifth day after the last of the 3 injections of PAS. Bone marrow and spleen cells were obtained for culture either 5 days after the initial injection of PAS or NS (i.e., 1 day after the last of the 3 injections) or 5 days after the last injection (i.e., 9 days after the first injection). These time points were chosen because of our initial observations that maximum or near maximum levels of GM- and Meg-CFC occurred 5 days after a single injection of PAS or NS. The data obtained from these two time points were similar and therefore were pooled (Table 2). As previously observed following a single injection of PAS, no significant increase in GM-CFC or Meg-CFC was noted in the bone marrow, but statistically significant increases in both were noted when cells from the spleen were cultured (p < 0.02).

**Effects of Platelet Antiserum or Normal Serum in Germ-Free Mice and on Levels of Colony-Stimulating factor**

In an attempt to evaluate the mechanism by which PAS (and to a lesser extent NS) produced an increase in splenic CFC, the role of bacterial endotoxin was considered. Accordingly, experiments were performed in germ-free mice to evaluate the potential role of endotoxin from the gastrointestinal tract in causing some of the observed changes. A similar pattern of response was noted (Table 3). Four to five days after PAS, a 5–10-fold increase was noted in GM-CFC and
EFFECTS OF THROMBOCYTOPENIA OR Meg-CFC

Table 2. Effects of Multiple Injections of Platelet Antiserum (PAS), Normal Serum (NS), or Phosphate-Buffered Saline (PBS) on CFC*

<table>
<thead>
<tr>
<th></th>
<th>PAS</th>
<th>NS</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>70 ± 2.3</td>
<td>3 ± 0.3</td>
<td>53 ± 7.9</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>Spleen</td>
<td>103 ± 22.6</td>
<td>18 ± 1.5</td>
<td>30 ± 4.6</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
</tbody>
</table>

*0.05 ml of PAS, NS, or PBS were injected serially on alternate days into separate groups of animals. Each group received a total of 3 injections of PAS, NS, or PBS. Bone marrow and spleen cells were cultured either 1 day after the last injection (equivalent to 5 days after the first injection) or 5 days after the last injection. The results were identical and therefore were pooled.

The mean ± 1 SE is shown. Numbers in parentheses indicate number of experiments.

Table 3. Effects of Platelet Antiserum (PAS), Normal Serum (NS), or Phosphate-Buffered Saline (PBS) on CFC in Germ-Free Mice*

<table>
<thead>
<tr>
<th></th>
<th>PAS</th>
<th>NS</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>51</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>(1)</td>
<td></td>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td>Spleen</td>
<td>53 ± 3.1</td>
<td>13 ± 5.2</td>
<td>5 ± 0.7</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
</tbody>
</table>

*PAS, NS, or PBS (0.05 ml) was administered to germ-free animals that were maintained in sterile isolation until sacrifice 4 or 5 days later. The mean ± 1 SE is shown. Numbers in parentheses indicate number of experiments.

a 4–13-fold increase in Meg-CFC (p < 0.05). Baseline levels of CFC were lower in the spleens of germ-free animals than in conventional animals.

Levels of colony-stimulating factor (CSF) in both conventional and germ-free animals were measured. Increased levels of CSF were observed at 1, 4, and 17 hr after administration of either PAS or NS to conventional animals but only following administration of PAS to germ-free animals (Table 4). CSF was not detected in serum obtained from animals 1–5 days following administration of PAS or NS. Interestingly, although animals that had received PAS were severely thrombocytopenic at 1, 4, and 17 hr (see Fig. 1), their serum (or plasma) did not support the growth of megakaryocyte colonies.

Other observations that indicated that endotoxin was unlikely to be the cause of the changes observed were the lack of leukopenia at 1 and 4 hr after administration of PAS or NS (Table 1) and normal differential white blood cell counts as those times (data not shown). Although both PAS and NS had become contaminated with endotoxin, probably as a result of multiple adsorptions with various types of blood cells, the concentration of endotoxin was such that the total dose received by a mouse would have been less than 0.005 μg. In addition, rechallenge of animals that had received PAS or NS 14 days previously resulted in the predicted responses 5 days after the second injection of PAS or NS, respectively.

Effect of Platelet Antiserum or Normal Serum on CFC In Vitro

We determined if PAS or NS had a direct effect on CFC either by adding PAS or NS directly to cultures or by preincubating PAS or NS with bone marrow or splenic cells before culture. Preincubation of PAS or NS with bone marrow or spleen, in the presence of complement, did not result in reduction of numbers of GM- or Meg-CFC when the cells were cultured subsequently (Table 5). Similar results were obtained following preincubation with buffer (PBS) and complement (data not shown). Direct addition of PAS or NS to cultures of bone marrow or spleen resulted in reduction of detectable CFC (p < 0.005) only when 0.05 ml (the dose administered in vivo) was added to cultures (Table 6). However, smaller volumes, which more closely corresponded to calculated concentrations of PAS in the blood of the recipient animals (0.01 and 0.0075 ml) produced no effect.

DISCUSSION

We have demonstrated an increase in megakaryocyte-CFC (Meg-CFC) among the hematopoietic cells obtained from mice in which acute thrombocytopenia had been produced. However, an increase in Meg-CFC was detected only in the spleen, not bone marrow, during a period from 1 hr to 14 days following induction of acute thrombocytopenia. Further-
more, an increase in frequency of splenic Meg-CFC occurred only after a lag of 2 days and did not reach maximum levels until 4–5 days following acute thrombocytopenia, in contrast to the time of maximum increase in recognizable megakaryocytes in the bone marrow, which has been reported to occur within 60 hr. This indicates that Meg-CFC are unlikely to be the cells immediately responsible for changes noted in bone marrow shortly after induction of acute thrombocytopenia. In this regard, it was surprising that Meg-CFC were not detected in soft agar cultures at times later than 4 days or with cells obtained from the spleen.

The delay in increase of Meg-CFC is apparently not due to their inhibition or suppression by the antiserum used, since in vitro studies did not demonstrate such an effect with doses that would have been effectively present in our intact animals. Additionally, the pattern of response of platelet levels indicates that the platelet antiserum did not suppress the bone marrow and splenic cells responsible for recovery from thrombocytopenia. Therefore, Meg-CFC detected in soft agar cultures may represent cells more immature or primitive than those immediately responsive to acute thrombocytopenia (and presumably, at least in part, to thrombopoietin). Their increased number 4–5 days after acute thrombocytopenia, at a time when the donor animals had normal or slightly increased platelet levels, do not prevent the occurrence of rebound thrombocytosis. The data of Radley et al. also suggest that rebound thrombocytosis after platelet antiserum is derived from more mature precursor cells than the class of primitive stem

### Table 4. Detection of Colony-Stimulating Factor (CSF) in Serum After Administration of Platelet Antiserum (PAS), Normal Serum (NS), or Phosphate-Buffered Saline (PBS)*

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>PAS</th>
<th>NS</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Serum was obtained by cardiac puncture from anesthetized animals at the times indicated and tested for CSF activity in cultures that contained 50,000 bone marrow cells. 0.1 ml of a 1:6 dilution was used as the stimulus for 1-ml cultures. The mean number of granulocyte-macrophage colonies ± 1 SE is shown. Numbers in parentheses indicate number of experiments.

No inhibitors were detected by comparing the above results with cultures in which 1:18 dilutions of serum were used. Sera obtained 1, 2, 3, 4, and 5 days following administration of PAS did not demonstrate CSF (data not shown). Serum from mice that had received endotoxin (see Materials and Methods) produced 54 ± 15 colonies.

### Table 5. Effect of Preincubation With Platelet Antiserum (PAS) or Normal Serum (NS) and Complement on CFC*

<table>
<thead>
<tr>
<th>Control</th>
<th>Number of Colonies</th>
<th>PAS</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow (50,000 cells/culture)</td>
<td>34 ± 6.8</td>
<td>0.4 ± 0.2</td>
<td>26 ± 0.9</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>Spleen (10⁶ cells/culture)</td>
<td>30 ± 6.7</td>
<td>8 ± 3.5</td>
<td>31 ± 9.5</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td>(3)</td>
<td></td>
</tr>
</tbody>
</table>

*PAS or NS (0.05 ml) was incubated with 0.5 ml of a suspension of bone marrow (6 × 10⁶/ml) or spleen (60 × 10⁶/ml) cells at 4°C for 30 min. Complement (0.2 ml) then was added and the incubation continued at 37°C for an additional 30 min. The cells were then washed twice, resuspended, and cultured. Control suspensions were not exposed to complement or serum. The mean ± 1 SE is shown. Numbers in parentheses indicate number of experiments performed. The frequency of granulocyte-macrophage or megakaryocyte colonies after 7 days of culture was not reduced.
cells perturbed by 5-FU. Therefore, two different stages of the maturation sequence of stem cells and different feedback mechanisms apparently are involved in producing the effects previously reported in bone marrow and the results observed by Radley et al. and ourselves.

This conclusion is supported by the observations of Goldberg et al. that megakaryopoiesis, in irradiated recipients of normal bone marrow, was suppressed only if transfusion-induced thrombocytosis was produced approximately 1 wk following bone marrow transplantation. Those authors concluded that only committed megakaryocyte precursors were responsive to this physiologic feedback mechanism, whereas pluripotent stem cells (and presumably Meg-CFC) were not. The time of appearance of increased Meg-CFC in our mice support this hypothesis, as do the data of Williams et al., who failed to suppress Meg-CFC in bone marrow by hypertransfusion of platelets.

The significant increase in granulocyte-macrophage CFC (GM-CFC) in the spleen from 3 to 7 days after acute thrombocytopenia and to a lesser degree in mice that received normal rabbit serum was unanticipated. Interestingly, Ebbe et al. obtained increased numbers of CFU-S from spleens of mice 4–6 days after they had received platelet antiserum (PAS). As we observed, their donor mice that had received PAS demonstrated increased numbers of nucleated spleen cells. Also comparable to our data were lack of similar increases of CFU-S in bone marrow and concomitant proportional increases of both hematopoietic colonies and numbers of megakaryocytes in the spleens of irradiated recipients of spleen cells from PAS-treated donors.

Because an established effect of bacterial endotoxin is the production of increased GM-CFC, we considered this possibility. Endotoxin could have caused an increase in colony-stimulating factor (CSF) and a subsequent increase in GM-CFC either by being administered as a contaminant of platelet antiserum or normal serum or, as a result of our treatment, entering the systemic circulation from the gastrointestinal tract of the mice. Introduction of an amount of endotoxin adequate to produce the observed increase in GM-CFC seems unlikely, since the sera injected contained a concentration of endotoxin that would have resulted in a maximum total dose of only 0.005 μg/mouse, a dose too low to produce the results we observed. When PAS was injected into germ-free mice, an increase in splenic GM-CFC and Meg-CFC also was observed. However, injection of normal rabbit serum did not increase either GM-CFC or Meg-CFC. This suggests that PAS produced an effect not related to the entrance of endotoxin from the gastrointestinal tract into the circulation. It has been shown previously that production of increased levels of CSF by irradiation, as a result of the entrance of endotoxin from the gastrointestinal tract into the circulation, can be prevented by using germ-free animals.

Other observations that mitigate against endotoxin being the mediator of increased levels of GM-CFC are the lack of acute leukopenia during the 4-hr period following administration of serum and the absence of subsequent granulocytosis. Furthermore, Metcalf and Wilson reported that a dose of endotoxin, sufficient to produce an increase in splenic GM-CFC also produced an increase in bone marrow CFC, and we did not observe the latter. Although the temporal pattern of increased CSF following administration of endotoxin has not been established fully, it appears to differ from our observations. In addition, Metcalf and Wilson observed that prior exposure to endotoxin prevented the subsequent rise in CFC usually observed after 1 μg of endotoxin, compatible with the development of tolerance to endotoxin. When we rechallenged animals 14 days after administration of platelet antiserum, the same increase in splenic GM-CFC and Meg-CFC was observed.

What other mechanisms might account for the increase in CSF observed in the plasma of animals following administration of platelet antiserum or normal serum? Both types of sera were extensively adsorbed to remove antibodies against myeloid, lymphoid, and erythroid cells. Perhaps the presence of

### Table 6. Effect of Platelet Antiserum (PAS), Normal Serum (NS), or Phosphate-Buffered Saline (PBS) on CFC In Vitro

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PAS</th>
<th>NS</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow (50,000 cells/culture)</td>
<td>43 ± 12.8</td>
<td>1.5 ± 0.3</td>
<td>16 ± 10.8</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>(4)</td>
<td>(4)</td>
<td></td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>Spleen (10⁴ cells/culture)</td>
<td>16 ± 3.7</td>
<td>4.2 ± 1.0</td>
<td>5 ± 2.7</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>(5)</td>
<td>(5)</td>
<td></td>
<td>(5)</td>
<td></td>
</tr>
</tbody>
</table>

*PAS, NS, or PBS (0.05 ml) was added to culture dishes at the time of plating. The mean ± 1 SE is shown. Numbers in parentheses indicate number of experiments performed. Lower volumes of PAS (0.01 and 0.0075 ml) did not reduce the numbers of colonies.
antigen-antibody complexes, particulate material, or heterologous antigens in the rabbit sera produced increased levels of CSF, which subsequently resulted in increased GM-CFC, as reported previously.\(^3,34,37,38\) It appears that as a result of experimental manipulation, stimulation of the reticuloendothelial system occurred (both splenic nucleated cells and weight were increased, see Fig. 5), which resulted in an increase in GM-CFC. Massive platelet destruction following administration of PAS, with subsequent phagocytosis, presumably played a major role in producing this response.\(^37\) It is noteworthy that NS produced a lesser response. McNeill has suggested that CFC can be directly stimulated by antigen-α-macroglobulin complexes or indirectly following release of CSF.\(^34\) Even the administration of mouse red blood cells to other mice produced increased numbers of CFC\(^34\) and both our platelet antiserum and normal rabbit serum were extensively adsorbed with red blood cells.

The absence of alteration in bone marrow CFC at a time when marked increases in both GM-CFC and Meg-CFC were observed in the spleen provides another example of the important role of the spleen of the mouse in the responses to various stimuli of hematopoietic cells, and the need to concomitantly study both bone marrow and spleen under such circumstances in order to obtain a valid physiologic overview. Evidence that the stem cell pool of the spleen contains a greater proportion of cells from which megakaryocytes are derived than the bone marrow\(^9,39\) is reflected, in vivo, by greater increases in Meg-CFC in the spleen following administration of platelet antiserum.\(^30\) and current observations. The necessity to continue observations of cells from bone marrow and spleen, after peripheral blood counts have returned to normal, is also indicated by our results. Finally, our data indicate the importance of evaluating all colonies in culture, in experimental models of this type, to determine if a presumably specific stimulus is in fact perturbing more than one group of hematopoietic cells and their precursors.

**ACKNOWLEDGMENT**

Extensive discussions with Dr. David Penington contributed greatly to these studies. Dr. Gregory Johnson offered much advice and assistance and Dr. Justin McCarthy valuable comments. Rod Mitchell provided a variety of important technical assistance. Steven White provided extensive statistical consultations and assistance. We wish to thank Dr. D.C. Cowling of the Hematology Department of the Royal Melbourne Hospital for permission to use the facilities of his laboratory and the members of his staff for their generous help. The National Science Foundation provided travel funds for Dr. Jack Levin.

**REFERENCES**

The effects of acute thrombocytopenia on megakaryocyte-CFC and granulocyte-macrophage-CFC in mice: studies of bone marrow and spleen

J Levin, FC Levin and D Metcalf