The Effects of Thrombopoietin on Megakaryocyte-CFC, Megakaryocytes, and Thrombopoiesis: With Studies of Ploidy and Platelet Size

By Jack Levin, Francine C. Levin, Donald F. Hull III, and David G. Penington

The effects of partially purified thrombopoietin (TPO), prepared from the plasma of thrombocytopenic rabbits, upon megakaryocyte colony-forming cells (Meg-CFC), megakaryocytes, and platelets were evaluated. The thrombopoiesis-stimulating activity of both types of preparations of TPO used was established by their ability to increase the levels of selenomethionine-75Se (75SeM) in the circulating platelets of mice, as described previously. TPO did not increase the frequency or total numbers of Meg-CFC or GM-CFC, although in some experiments, concentrations greater than required to stimulate thrombopoiesis in vivo were used. TPO did not enhance the ability of suboptimal concentrations of spleen conditioned medium to support colony growth. Human erythropoietin (EPO) at concentrations from 0.6 U/ml to 8 U/ml, in vitro, did not increase the frequency of bone marrow or splenic Meg-CFC or GM-CFC.

As previously reported, two types of megakaryocyte

THERE IS INCREASING EVIDENCE that a humoral factor, designated thrombopoietin, stimulates the production of platelets in animals and man. However, the mode of action of thrombopoietin remains unknown. It is unclear whether changes that occur after the induction of acute thrombocytopenia, i.e., an increase in the incorporation of tritiated thymidine by immature megakaryocytes and in endomitotic figures, followed by an increase in the rate of maturation, number, ploidy, and size of megakaryocytes, also occur after the administration of thrombopoietin. Since these alterations do not always occur concomitantly and since severe thrombocytopenia is required to produce marked changes in megakaryocytopenia, it seems likely that both quantitative and qualitative differences exist between the responses to thrombopoietin and an acute reduction in the circulating platelet mass.

The potential effects of thrombopoietin on colony-forming cells in vitro have not been established. Although it has been reported that addition to cultures of thrombopoiesis-stimulating activity derived from human embryonic kidney cells increased the frequency of megakaryocyte-CFC (Meg-CFC), these results have not been confirmed. High concentrations of erythropoietin also have been reported to increase the frequency of Meg-CFC.

A well established effect of thrombopoietin (TPO) is the ability to stimulate platelet production as measured by increased levels of selenomethionine-75Se or Na235SO4 in the platelets of recipient animals. However, thrombocytosis has not been regularly observed in association with increased levels of isotope. This has led to the suggestion that TPO may increase the specific activity of platelet proteins or increase platelet size. Since thrombopoietin may cause direct or indirect changes in cells ranging from megakaryocyte precursors to mature megakaryocytes, we have attempted to determine the effects of thrombopoietin on megakaryocyte colony-forming cells,
megakaryocytes, the rate of thrombopoiesis, and platelet volume. The results indicate a variety of changes occur in megakaryocytes, their precursors, and their progeny after the administration of thrombopoietin to mice.

MATERIALS AND METHODS

Mice

C57BL/6J mice, approximately 8 wk of age, were used for these experiments. The mice were bred under specific pathogen-free conditions and then maintained under conventional conditions for approximately 1-3 wk before use.

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 were then 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, as described previously. Cultures contained 50,000 or 100,000 bone marrow or 0.5 × 10^6 or 1 × 10^6 spleen cells/ml. These concentrations provided adequate numbers of colonies for evaluation but with a colony density low enough to allow accurate 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. Each set of cultures was prepared from the pooled cells of two experimental and two control mice.

Preparation of Spleen Conditioned Medium

C57BL/6J spleen cells were incubated for 7 days at a concentration of 2 × 10^6 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

After 7 days of incubation, cultures were scored for colony formation using an Olympus dissection microscope with semidirect 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. Further description of scoring and histochemical identification of megakaryocyte colonies with acetylcholinesterase stain, using the method of Karnovsky and Roots, are presented in detail in another paper.

For measurement of DNA, colonies were removed from cultures in which the colony density was too high to assure that no colony that was removed for examination had been contaminated by an adjacent colony, and that the entire colony had been removed. Individual data points were derived from at least 4 experiments. In each experiment, megakaryocyte colonies were removed from at least two culture dishes each of bone marrow and spleen from control and experimental animals.

After removal with a finely drawn Pasteur pipette, colonies were placed on egg-albumin-coated slides, air dried, and immersed in a solution of methanol, 40% formaldehyde (formalin), and glacial acetic acid in the proportions of 85:10:5 for a minimum of 1.5 hr. Other identical culture dishes were used for accurate scoring of the numbers of megakaryocyte colonies in cultures. In some experiments, smears of suspensions of bone marrow cells were prepared similarly.

Feulgen Staining

Feulgen-Schiff staining was performed as recommended by Kasten. Hydrolysis was carried out for 20 min in 1N HCl at 60°C.

Measurement of DNA

After staining of the colonies or bone marrow smears, immersion oil was placed on the slide and then a cover slip placed on top. Microdensitometric measurement of DNA in the individual cells of colonies or in recognizable megakaryocytes in bone marrow was carried out using an M-85 scanning microdensitometer (Vickers Instruments, England). Except for rare damaged cells or cells superimposed on each other, every cell in a colony was evaluated. At the same time, the total number of cells/colony was determined. Megakaryocytes were assigned to various ploidy classes as described by Paulus et al.

Each slide also contained two colonies of granulocytes obtained from the same set of cultures; the easily recognized granulocytes provided a diploid reference cell and an internal control to ensure that variations in staining did not account for apparent alterations in ploidy of megakaryocytes.

Thrombopoietin

Partially purified thrombopoietin was prepared from the plasma of thrombocytopenic rabbits, as described previously. Thrombopoietin (TPO), fraction WGA-II, was prepared by affinity chromatography at pH 7.4 and 25°C using wheat germ agglutinin (WGA) bound to agarose. Approximately 150-200 mg of TPO, fraction T-III, was used as the starting material. After application of the sample, the column was washed with phosphate-buffered NaCl until the effluent was free of protein. TPO was bound quantitatively by WGA and was eluted with 0.1 M N-acetylglucosamine. This resulted in a 4800-fold purification, as compared to initial plasma TPO activity, and a 700-fold purification, as compared to the protein fraction initially placed on the lectin-agarose column. To study in vivo effects on thrombopoiesis, TPO was administered to mice subcutaneously, in four equally divided doses during a 42-hr period. The total dose of protein for TPO fraction T-III was 10-40 mg/mouse and for fraction WGA-II was 40 μg/mouse. These doses had been previously shown to stimulate thrombopoiesis in mice.

Thrombopoietic activity in fractions of plasma was determined by measuring their effect on incorporation of selenomethionine-
EFFECTS OF THROMBOPOIETIN ON MEGAKARYOCYTE POIESIS

\(^{75}\text{Se}(^{75}\text{SeMe})^*\) into the newly forming platelets of CD-1 mice, as described previously.\(^{18,19}\) Effects of TPO on megakaryocyte colony-forming cells and megakaryocytes were also measured (vide infra).

For in vitro experiments, sterile TPO was added directly to cultures of bone marrow or spleen cells at concentrations ranging from 0.075 mg/ml to 18 mg/ml. Some of these concentrations exceed those attained in the blood following the administration of TPO to intact animals (vide supra).

**Erythropoietin**

Human erythropoietin (NIH pool no. H-23-Ta LSL or EG-3-15-LSL) was processed and assayed by the Hematology Research Laboratories, Children’s Hospital of Los Angeles. It was authorized for distribution by the Erythropoietin Committee of the National Heart, Lung, and Blood Institute. Sterile erythropoietin (EPO) was added directly to cultures of bone marrow or spleen cells at concentrations ranging from 0.6 U/ml to 8 U/ml. In some in vitro experiments, TPO and EPO were rendered essentially free of endotoxin prior to addition to the cultures (vide infra).

**Detection and Removal of Endotoxin**

Endotoxin was assayed in preparations of thrombopoietin or erythropoietin using the Limulus amebocyte lysate test, as described by Levin et al.\(^{20-22}\) 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 concentrations of endotoxin calculated. \(E.\) coli endotoxin (\(E.\) coli B lipopolysaccharide B, 055:B5, Difco Laboratories, Detroit, Mich.) was used as the standard.

Endotoxin was removed from samples of thrombopoietin (TPO) or erythropoietin (EPO) following incubation of these materials with amebocyte lysate as described previously.\(^{23}\) Following adsorption, the preparations of TPO and EPO, which contained the equivalent of <0.0001 \(\mu\)g/ml of \(E.\) coli endotoxin, were passed through a 0.45 \(\mu\) filter (Millipore Corp.).

**Platelet Sizing**

Animals were anesthetized with ether, and approximately 1 ml of blood was obtained by cardiac puncture with a plastic syringe that contained 0.02 ml of buffered EDTA. Blood samples were transfused to siliconized tubes, and blood for platelet counts and hematocrit values collected into capillary tubes from these specimens as described previously.\(^{18,19}\) Portions of the same samples were utilized to measure levels of \(^{75}\text{SeM}\) in circulating platelets. Two techniques were used to prepare platelets for sizing.

**Rapid micromethod**

Three double-oxalated capillary tubes (Drummond Scientific Co., Broomall, Pa.) were filled approximately two-thirds full and then centrifuged for only 5 sec in a Drummond Microhematocrit centrifuge (Drummond Scientific Co.). The centrifuge head was allowed to coast to a halt without braking. The tubes then were removed from the centrifuge and immediately placed in an upright position to preserve the interface between the platelet-rich plasma (PRP) and the loosely packed red cells. Each tube then was scored at the interface with a fine saw and broken using a thumbnail as a fulcrum. The PRP from the three tubes was combined in the tiny central well of the lid from a no. 2063 polypyylene tube (Falcon, Oxnard, Calif.) and covered with a no. 4 cork to prevent evaporation. PRP was transferred with a 3 lambda Unopette (Coulter Electronics, Hialeah, Fla.) into an accuvette (Coulter Electronics) that contained Isoton II (Coulter Electronics) that had been twice filtered through a 0.22 \(\mu\) Millipore filter (Millipore Corp.). Dilution of PRP was adjusted to maintain the concentration index of the particle counter between 3\% and 4.5\% (machine counts between 3000 and 5000). Approximately 50-60 min elapsed between collection of the blood samples and determination of mean platelet volume.

**Slow centrifugation method**

Five milliliters of Tris \(N\) buffer was added to each blood sample after removal of blood for hematocrit, platelet count, and previously described method for determination of platelet size. The diluted sample of whole blood was centrifuged at 160 \(g\) for 20 min at room temperature (International Model U Centrifuge, International Equipment Co., Boston, Mass.). The PRP was transferred with a siliconized Pasteur pipette into a siliconized glass tube. The PRP was mixed by repeated aspiration into the pipette and then approximately 0.2 ml was placed in a polypyylene tube (no. 2063, Falcon, Oxnard, Calif.) until platelet sizing was performed approximately 2-3 hr later.

Platelet sizing was performed with a Coulter Electronic Particle Counter, Model \(Z_{40}\), employing a 50 \(\mu\) long tunnel aperture tube (Model No. 5060, Coulter Electronics) and a 100 lambda manometer, in conjunction with a Coulter Channelizer II, a Coulter Experimental Log Amplifier, and a Coulter X-Y plotter. Settings on the Coulter \(Z_{40}\) included: matching switch, 20K; gain, 6; I/I (amplitude current), 1/2; bandwidth, normal; lower threshold, 4; upper threshold, 100; high resolution, In/50 \(\mu\); and exclusion, 4. The Channelizer was set at base channel threshold (BCT), 4; window width (WW), 100; edit, off; and maximum count/channel, 1000. The log amplifier was operated in the log mode.

Mean channel number of a peak was determined by averaging the channel number for the two points at 70\% of the maximum peak height. The geometric mean volume of a peak then could be calculated by comparison with a latex particle (PVT, mean diameter 2.020 \(\mu\), Dow Diagnostics, Indianapolis, Ind.) of known volume as follows:

\[
V = \text{antilog } (\log V' + (N - N')/25.77)
\]

where \(V\) is volume, \(N\) the mean channel number of the platelet sample, and the prime notation the values for the latex standard. (The value 25.77 is the log constant derived from the calibration procedure for the log amplifier.)

**Calibration**

Careful calibration, much finer than the procedure provided at installation, was necessary for accurate particle sizing. Additional measures to improve reliability included use of: (1) a Sola Constant Voltage Transformer, Harmonic Neutralized, Type CVS (Shape Electronics, Elk Grove Village, Ill.) to eliminate apparent changes in particle size due to alterations in line voltage and to minimize background noise created by electronic interference; (2) a 1000 ml vacuum trap for the \(Z_{40}\) to reduce mechanical interference and to insure steady flow of solute through the aperture tube while sizing; (3) an aperture tube suited for the particles to be studied, such that particle diameters were within 2\%–20\% of the diameter of the aperture tube; (4) high resolution circuitry, available on the \(Z_{40}\), to correct for distortion created by particles traversing the aperture tube with a curved trajectory and thereby produce a better fit of the log amplifier.

\*Selenomethionine-\(^{75}\text{Se},\) Sethotope, E.R. Squibb and Sons, New Brunswick, N.J. Specific Activity, 200–250 mCi/mg.

†THAM (72.8 g); 18 g NaCl: 240 ml 0.2 \(N\) HCl; and 40 ml 4.8\% EDTA in 4 liters of distilled \(H_2O\).
Table 1. The Effects of Administration of Thrombopoietin (Fraction T-III) on Granulocyte-Macrophage-CFC and Megakaryocyte-CFC

<table>
<thead>
<tr>
<th>Bone Marrow (50,000 Cells/Culture)</th>
<th>Number of Colonies</th>
<th>Spleen (10^4 cells/culture)</th>
<th>Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombopoietin Gran.-Mac.</td>
<td>Control Gran.-Mac.</td>
<td>Thrombopoietin Gran.-Mac.</td>
<td>Control Gran.-Mac.</td>
</tr>
<tr>
<td>44 ± 5.1</td>
<td>1 ± 0.4</td>
<td>17 ± 1.6</td>
<td>4 ± 0.9</td>
</tr>
<tr>
<td>(11)</td>
<td>(11)</td>
<td>(11)</td>
<td>(11)</td>
</tr>
<tr>
<td>Thrombopoietin Bone Marrow</td>
<td>Control Spleen</td>
<td>Thrombopoietin Bone Marrow</td>
<td>Control Spleen</td>
</tr>
<tr>
<td>Gran.-Mac.</td>
<td>(10)</td>
<td>Gran.-Mac.</td>
<td>(10)</td>
</tr>
<tr>
<td>Meg.</td>
<td>16,252 ± 2,029</td>
<td>Meg.</td>
<td>1,669 ± 292</td>
</tr>
<tr>
<td>(10)</td>
<td>(10)</td>
<td>(11)</td>
<td>(10)</td>
</tr>
<tr>
<td>Total Colony-Forming Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gran.-Mac.</td>
<td>Thrombopoietin</td>
<td>Control</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>16,426 ± 2,187</td>
<td>456 ± 95</td>
<td>284 ± 73</td>
<td>292 ± 71</td>
</tr>
<tr>
<td>(11)</td>
<td>(11)</td>
<td>(11)</td>
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* TPO was administered in 4 equally divided doses (total dose was 20–40 mg/mouse). Bone marrow and splenic cells were obtained for culture 2 or 4 days after the initial injection of TPO. Since the results were similar, they were pooled.

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

The Effects of Thrombopoietin In Vivo

Thrombopoietin (TPO), fraction III, prepared from the plasma of thrombocytopenic rabbits, as described previously, was administered to mice. Ten to twenty mg of this preparation of TPO had been shown previously and in additional current experiments (vide infra) to stimulate thrombopoiesis in vivo. Bone marrow and splenic cells were obtained for culture either 2 or 4 days after the initial injection of TPO. The 2-day period corresponds to the time at which the administration of selenomethionine-Se to mice subsequently demonstrates that TPO has stimulated thrombopoiesis. The 4-day period was chosen because the latter interval elapses before numbers of Meg-CFC increase following stimulation of thrombopoiesis by acute thrombocytopenia.

The administration of TPO (fraction T-III) did not increase the frequency of GM-CFC or Meg-CFC in either the bone marrow or spleen of recipient mice at either interval studied (Table 1). Similarly, the total number of CFC in the femoral bone marrow and spleen did not increase (Table 1). In two other experiments, the administration of a more purified preparation of TPO (fraction WGA-II) did not increase the frequency or total number of GM-CFC or Meg-CFC. There was no alteration of splenic weight in the recipients of TPO.

The Effects of Thrombopoietin In Vitro

The addition of thrombopoietin (TPO) to cultures of splenic cells, at final concentrations that ranged from 18 mg/ml to 0.075 mg/ml, did not alter the frequency of splenic GM-CFC or Meg-CFC (Table 2). The upper range of these concentrations exceeded the total dose of TPO (fraction T-III) required to stimulate thrombopoiesis in an intact mouse (10 mg/mouse). Furthermore, 0.75–18 mg/ml of TPO failed to increase the frequency of CFC when added to cultures that contained suboptimal levels of spleen conditioned medium. TPO did not inhibit the ability of spleen conditioned medium to support colony growth (Table 2). In one experiment, the addition of TPO (18 mg/ml) on the fourth day of culture did not increase the frequency of CFC. WEHI or mouse lung conditioned medium did not support the growth of Meg-CFC, either alone or in the presence of TPO.

In seven additional experiments, at concentrations that ranged from 0.75 mg/ml to 9 mg/ml, TPO did not increase the frequency of GM-CFC or Meg-CFC in cultures of bone marrow cells. Removal of endotoxin from preparations of TPO by adsorption with Limulus amebocyte lysate (see Materials and Methods) did not increase the frequency of colony formation by spleen cells at TPO concentrations from 0.037 mg/ml to 0.6 mg/ml (GM-CFC, 10 ±0.5; Meg-CFC, 2 ± 0.3; 10^6 spleen cells cultured; 12 experiments).

Statistics

Statistical analyses were carried out using the Student's t test or Chi-square test, from which 2-tailed p values were derived.

RESULTS

A series of experiments were performed to determine whether thrombopoietin or erythropoietin produce detectable effects on megakaryocyte and granulocyte-macrophage colony-forming cells.
The TPO fraction designated WGA-II is more purified than fraction T-III; 28 20–40 μg/mouse can stimulate thrombopoiesis. In 5 experiments, WGA-II at concentrations that ranged from 0.9 μg/ml to 19 μg/ml failed to increase the frequency of either GM-CFC or Meg-CFC. WGA-II did not enhance the ability of either suboptimal concentrations of spleen conditioned medium or WEHI conditioned medium to support colony growth. WGA-II and T-III alone did not support colony growth.

The Effects of Erythropoietin In Vitro

The addition of human erythropoietin (EPO) to cultures of spleen cells at final concentrations that ranged from 8 U/ml to 0.6 U/ml did not alter the frequency of splenic GM-CFC or Meg-CFC (Table 2). Furthermore, 8 U/ml of EPO did not increase the frequency of CFC when incubated in the presence of suboptimal levels of spleen conditioned medium.

In 4 additional experiments, EPO (2 each at 4 U/ml and 2 U/ml) failed to increase the frequency of CFC in cultures of bone marrow cells. Removal of endotoxin from a preparation of EPO by adsorption with Limulus amebocyte lysate (see Materials and Methods) did not increase the frequency of colony formation at EPO concentrations of 4 U, 2 U, 1 U, or 0.6 U/ml. EPO alone did not support colony growth.

The Effects of Thrombopoietin on Ploidy Distribution of Megakaryocyte-CFC and Megakaryocytes

As previously reported, two types of megakaryocyte colonies were present in soft agar cultures. 23,32,33 We have designated these as big cell or heterogeneous colonies, reflecting their morphological appearance. 23 Furthermore, our previous studies have demonstrated that each colony type has a distinctive pattern of ploidy distribution. 23 The addition of TPO directly to cultures of bone marrow or splenic cells at final concentrations as high as 9–18 mg/ml (i.e., adequate to stimulate thrombopoiesis in vivo in intact mice) produced a slight but not statistically significant increase in the proportion of 16N, 32N, and 64N megakaryocytes in big cell colonies (176 cells/12 colonies studied). There was a statistically significant increase from 37% to 44% (p < 0.05) in the proportion of 4N cells and a decrease in the proportion of 32N cells from 3% to 0.3% (p < 0.005) in heterogeneous colonies (283 cells/9 colonies studied).

Furthermore, the proportion of 16N cells was significantly increased and the proportion of 2N and 4N cells decreased in big cell colonies derived from mice that had received TPO (Fig. 1). The increase in the proportion of 16N cells following the administration of TPO was observed in the progeny of Meg-CFC obtained either 2 days after the initial injection of TPO (at the time 75SeM is given for performance of a bioassay for thrombopoiesis) or 4 days after the initial injection of TPO. There was no detectable change in the ploidy of heterogeneous colonies. There was no increase in the number of cells per colony either after administration of TPO to mice or after the addition of TPO to cultures in vitro.

As previously reported with our methods, 5,8 we found that 16N megakaryocytes constituted the most common ploidy group in recognizable megakaryocytes in the bone marrow. Although they remained the most common in mice that had received TPO, 2 days after the initial injection of TPO there was an increase in 32 N megakaryocytes from 22.5% to 28.0% (Chi-square, 2.69; p < 0.09), an increase in 64N cells from 1.3% to 5.7% (Chi-square, 11.7; p < 0.001), and a decrease in 8N megakaryocytes from 23.3% to 14.6% (Chi-square, 11.48; p < 0.001) (Table 3). Therefore, changes in ploidy distribution were observed in both

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</thead>
<tbody>
<tr>
<td>Control</td>
<td>9–18 mg/ml</td>
<td>2.2–4.5 mg/ml</td>
<td>0.1–0.9 mg/ml</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>14 ± 1.9</td>
<td>4 ± 0.7</td>
<td>14 ± 3.7</td>
<td>3 ± 1.2</td>
<td>11 ± 4.2</td>
<td>4 ± 1.7</td>
<td>17 ± 1.2</td>
</tr>
<tr>
<td>(11)</td>
<td>(11)</td>
<td>(4)</td>
<td>(4)</td>
<td>(5)</td>
<td>(5)</td>
<td>(17)</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>4 U/ml</td>
<td>2 U/ml</td>
<td>1 U/ml</td>
<td>18 ± 2.2</td>
<td>4 ± 1.3</td>
<td>17 ± 1.9</td>
</tr>
<tr>
<td>14 ± 1.0</td>
<td>5 ± 1.5</td>
<td>15 ± 1.3</td>
<td>5 ± 0.3</td>
<td>(5)</td>
<td>(5)</td>
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</tr>
<tr>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

* TPO or EPO was added to the culture dishes immediately prior to 1 ml of cell suspension in agar medium. Final concentrations are indicated. Similar results were obtained at TPO concentrations of 0.075–0.18 mg/ml and EPO concentrations of 8 U/ml and 0.6 U/ml (data not shown).

The mean ± 1 SE is shown. Numbers in parentheses indicate number of experiments.
platelets for sizing (Table 4). However, this effect was observed only with the higher dose (20 mg/mouse) of T-III. Platelet counts did not significantly increase. Similar results were observed following the administration of fraction WGA-II, prepared from the plasma of thrombocytopenic donors (40 μg/mouse) (Table 5). Fractions N-III or WGA-II, prepared from the plasma of normal donors, did not stimulate thrombopoiesis or increase mean platelet volume (Tables 4 and 5).

**DISCUSSION**

The administration of thrombopoietin, obtained from the plasma of thrombocytopenic rabbits, caused a detectable alteration in the frequency of megakaryocyte-CFC (Meg-CFC) or granulocyte-macrophage-CFC (GM-CFC) when bone marrow or splenic cells obtained from the recipient mice were cultured in soft agar. A lack of effect was observed when 2–4 times the minimum dose detectable in a well-established bioassay (vide infra) was administered. Furthermore, the total number of CFC was unaltered.

Similarly, the addition of thrombopoietin (TPO) directly to cultures of bone marrow or splenic cells, even at concentrations that exceeded the total dose required to stimulate thrombopoiesis in vivo, failed to increase the frequency of Meg-CFC or GM-CFC. A lack of effect also was observed when TPO was incubated in the presence of suboptimal concentrations of spleen conditioned medium or with WEHI or mouse lung conditioned medium. Nakeff reported that serum from thrombocytopenic mice increased the frequency of Meg-CFC in plasma clot cultures of bone marrow. However, in our previous studies, plasma or serum obtained from mice at 1, 4, or 17 hr after the induction of acute thrombocytopenia (at which times levels of TPO have been shown to be elevated) also failed to support the growth of Meg-CFC in cultures. Paradoxically, only GM-CSF was detected in the blood of these animals. Petursson also has indicated that serum from thrombocytopenic mice did not support megakaryocyte colony formation in methylcellulose. These observations provide additional although indirect proof that TPO does not increase the frequency of Meg-CFC. At the current time, a direct effect of thrombopoietin, derived from the plasma of thrombopoietin.

**Table 3. The Effect of Thrombopoietin on the Ploidy Distribution of Bone Marrow Megakaryocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cells Measured</th>
<th>Mean Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2N</td>
</tr>
<tr>
<td>Control (3)</td>
<td>462</td>
<td>25.3 ± 4.1</td>
</tr>
<tr>
<td>TPO (3)</td>
<td>261</td>
<td>14.6 ± 1.0†</td>
</tr>
</tbody>
</table>

*Bone marrow was obtained from mice 2 days after the administration of the first of 4 equally divided doses of thrombopoietin (Fraction T-III) (see Materials and Methods). The mean ± 1 SE is shown. Numbers in parentheses indicate number of experiments.

†p Values, experimental versus control: 8N, <0.001; 32N, <0.09; 64N, <0.001.
cytopenic animals, on the frequency of Meg-CFC has not been observed. Similarly, presumably immunologically mediated thrombocytopenia in humans was not associated with increased levels of the factor that increased Meg-CFC in plasma clot cultures of human bone marrow.36

Our results differ from those reported by Williams et al.,12 who observed an increase in Meg-CFC after addition of thrombopoiesis-stimulating factor (TSF)‡ derived from supernatants of a human embryonic kidney cell line. The increase in Meg-CFC was detected only when WEHI-conditioned medium also was present in the cultures; TSF alone did not support colony growth.12 Although the same source of thrombopoiesis-stimulating factor (i.e., kidney cell supernatant) was reported to increase the frequency of Meg-CFC when added to bone marrow cells cultured in plasma clots, the effects of control culture medium were not described.13 Furthermore, other workers have been unable to confirm this observation using either plasma clots15, Vainchenzer, unpublished observations or methylcellulose, or support colony growth. TSF, alone or in combination with leukocyte conditioned medium, did not support the growth of human Meg-CFC in agar cultures.14 Therefore, the ability of supernatants of a human embryonic kidney cell line to increase the frequency of Meg-CFC in vitro has not been confirmed.

Importantly, the preparation used by Williams et al. also contained GM-CSF.12 Aye also reported that the conditioned medium obtained from the same human

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### Table 4. The Effects of Thrombopoietin (Fraction T-III) on Thrombopoiesis and Platelet Volume*

<table>
<thead>
<tr>
<th>Fraction Tested</th>
<th>No. Experiments</th>
<th>Percent Incorporation (SeM x 10⁹)</th>
<th>Rapid Micromethod (μL)</th>
<th>Slow Centrifugation (μL)</th>
<th>Platelet Count (x 10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NaCl)</td>
<td>8</td>
<td>2.96 ± 0.12</td>
<td>3.76 ± 0.05</td>
<td>3.66 ± 0.03</td>
<td>1.167 ± 0.033</td>
</tr>
<tr>
<td>N-III, 20 mg</td>
<td>4</td>
<td>3.23 ± 0.28</td>
<td>3.71 ± 0.06</td>
<td>3.60 ± 0.04</td>
<td>1.359 ± 0.042</td>
</tr>
<tr>
<td>N-III, 10 mg</td>
<td>4</td>
<td>3.08 ± 0.17</td>
<td>3.79 ± 0.07</td>
<td>3.62 ± 0.04</td>
<td>1.234 ± 0.047</td>
</tr>
<tr>
<td>T-III, 20 mg</td>
<td>8</td>
<td>4.03 ± 0.12†</td>
<td>4.01 ± 0.06†</td>
<td>3.82 ± 0.03†</td>
<td>1.190 ± 0.037</td>
</tr>
<tr>
<td>T-III, 10 mg</td>
<td>6</td>
<td>3.78 ± 0.16†</td>
<td>3.92 ± 0.06</td>
<td>3.60 ± 0.03</td>
<td>1.229 ± 0.059</td>
</tr>
</tbody>
</table>

*The data were derived from samples of blood obtained 64 hr after the administration of the first of 4 equally divided doses of thrombopoietin (see Materials and Methods). The total dose of Fraction T-III or N-III was 20 mg or 10 mg/mouse. The mean ± 1 SE is shown. Numbers in parentheses indicate number of mice.

†p < 0.005 as compared to controls or normal donors.

### Table 5. The Effects of Thrombopoietin (Fraction WGA-II) on Thrombopoiesis and Platelet Volume*

<table>
<thead>
<tr>
<th>Fraction Tested</th>
<th>No. Experiments</th>
<th>Percent Incorporation (SeM x 10⁹)</th>
<th>Rapid Micromethod (μL)</th>
<th>Slow Centrifugation (μL)</th>
<th>Platelet Count (x 10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NaCl or Tris)</td>
<td>6</td>
<td>2.55 ± 0.11</td>
<td>3.74 ± 0.03</td>
<td>3.55 ± 0.03</td>
<td>1.038 ± 0.032</td>
</tr>
<tr>
<td>WGA-II, 40 μg</td>
<td>3</td>
<td>2.93 ± 0.21</td>
<td>3.78 ± 0.06</td>
<td>3.58 ± 0.03</td>
<td>1.049 ± 0.064</td>
</tr>
<tr>
<td>WGA-II, 40 μg</td>
<td>3</td>
<td>3.57 ± 0.12†</td>
<td>4.03 ± 0.06†</td>
<td>3.80 ± 0.04†</td>
<td>1.064 ± 0.050</td>
</tr>
</tbody>
</table>

*The data were derived from samples of blood obtained 64 hr after the administration of the first of 4 equally divided doses of thrombopoietin (see Materials and Methods). The total dose of Fraction WGA-II was 40 μg/mouse. The mean ± 1 SE is shown. Numbers in parentheses indicate number of mice.

†p < 0.005 as compared to controls or normal donors.

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‡In this article, the term thrombopoiesis-stimulating factor (TSF) refers to the activity present in culture medium conditioned by a line of human embryonic kidney cells, described by McDonald et al. (McDonald TP, Clift R, Lange RD, Nolan D, Tribby IIE, Barlow GH: Thrombopoietin production by human embryonic kidney cells in culture. J Lab Clin Med 85:59-66, 1975). The term thrombopoietin (TPO) has been restricted to thrombopoiesis-stimulating activity derived from blood.
embryonic kidney cell line contained not only GM-CSF but also a factor that increased the frequency of erythroid bursts (BFU-E) in the presence of thrombopoietin. Both of these observations have been confirmed recently by Vainchenker (unpublished observations). This suggests that some effects produced by the factor(s) described by McDonald et al. are not specific for megakaryocytes and their precursors. However, TSF may play a role in promoting megakaryocyte maturation rather than having a direct effect on the frequency or proliferative state of the population of megakaryocyte precursors that are currently designated as colony-forming cells, as was previously suggested.

Previous investigators have reported that high concentrations (1.5–3 U/ml) of erythropoietin (EPO) increase the frequency of Meg-CFC in plasma clot cultures, a finding not confirmed by Mazur et al. Unfortunately, none of these studies indicated the effects of EPO on the frequency of GM-CFC, and therefore, the specificity of this effect is unclear. Furthermore, at high concentrations, a hormone may bind to receptor sites other than its own, thereby mimicking the activity of another hormone byactivating the latter's receptor sites. This is particularly likely if erythropoietin and thrombopoietin have similar basic structures, as has been suggested previously.

In contrast, we were unable to increase the frequency of either Meg-CFC or GM-CFC by the addition of 0.6–8.0 U/ml of human EPO to cultures of bone marrow or splenic cells. Our failure to detect an increase in Meg-CFC after addition of either EPO or TPO to agar cultures was not due to inhibition by the presence of endotoxin, as has been described for some preparations of EPO, because no stimulation was observed after removal of endotoxin from our preparations by adsorption with Limulus amebocyte lysate. The disparities between the results from different laboratories and the different observations following the use of plasma clot and agar systems further suggest possible nonspecificity of the ability of EPO to support the growth of megakaryocyte colonies.

Previous studies from our laboratory have indicated that stimulation of thrombopoiesis by acute thrombocytopenia increases the mean ploidy level of megakaryocyte colonies of the big cell type. Our current investigations demonstrated that megakaryocyte colonies derived from either the bone marrow or spleen of mice that had previously received TPO contained a significantly increased proportion of 16N cells and a significantly decreased proportion of 2N and 4N cells. As observed following induction of acute thrombocytopenia, there was no detectable change in heterogeneous colonies and no change in the number of cells/colony in either colony type. Changes in ploidy of the progeny of Meg-CFC following administration of TPO were associated with alterations in ploidy of megakaryocytes in the bone marrows of identically treated animals, in which increases in the proportions of 32N and 64N megakaryocytes were observed.

Therefore, it appears that TPO has an effect on Meg-CFC that results in the production of progeny with increased amounts of DNA and that corresponding changes occur in megakaryocytes in the bone marrow. Whether the increased ploidy observed in recognizable bone marrow megakaryocytes represents a direct effect of TPO on these cells or is the consequence of the above described alterations of Meg-CFC cannot be determined from our currently available data. In this regard, although the addition of high concentrations of TPO directly to cultures failed to produce a significant increase in ploidy of big cell colonies, there was a slight increase in the proportion of 16N, 32N, and 64N cells. Furthermore, Kellar et al. have recently reported that TPO increased the incorporation of 3H-thymidine (3H-TdR) into preparations of guinea pig megakaryocytes and also that megakaryocytes cultured in the presence of TPO maintained higher ploidy levels than controls.

The thrombopoiesis-stimulating activity of both of the preparations of TPO used in these experiments was confirmed by their effect on levels of selenomethionine-75Se (75SeM) in the circulating platelets of recipient mice. Interestingly, although there was no change in platelet counts in these animals, after a 48-hr period of administration of TPO, there was an increase in the mean platelet volume. Most previous studies have failed to demonstrate an increase in mean platelet volume after administration of thrombopoiesis-stimulating factor or plasma from thrombocytopenic donors, although a recent report detected an increase in the mean platelet volume of mice that had received TSF.

Other studies have suggested that stimulation of thrombopoiesis by thrombopoietin results in a subpopulation of large platelets. Odell et al. observed a burst of large platelets 18 hr after the induction of acute thrombocytopenia. However, the relationship between stimulation of megakaryocyte proliferation, increased ploidy, and platelet size is complex and poorly understood. Some data indicate a potentially inverse relationship between ploidy of megakaryocytes and the size of the platelets they produce, at least during normal thrombopoiesis. However, this relationship may no longer exist when thrombopoiesis has been stimulated. Increased megakaryocyte size and ploidy following acute thrombocytopenia suggest that in this circumstance, increased ploidy is asso-
associated with an increase in platelet size. Our results suggest that under the conditions of our experiments, megakaryocytes with increased ploidy (and presumably increased in size) produced large platelets, as previously suggested. The increase in mean platelet volume may correspond to the increase in mean corpuscular volume of circulating red blood cells that occurs after administration of EPO.

Neither following the induction of acute thrombocytopenia nor after administration of TPO did we detect increased ploidy in heterogeneous colonies, although increased ploidy did develop in the more mature, big cell colonies. Erythroid bursts (BFU-E), which are believed to be the progeny of a cell more primitive than CFU-E, are relatively unresponsive to EPO in vivo, whereas CFU-E are EPO dependent. Perhaps the relationship between the heterogeneous megakaryocyte colony, which contains many immature cells and has a lower mean ploidy level, and the more mature appearing big cell colony is similar to that which exists between BFU-E and CFU-E.

We have demonstrated that TPO has direct or indirect effects on megakaryocytopoiesis that are manifested by the earliest detectable megakaryocyte precursors (i.e., the production of megakaryocyte colonies with increased ploidy levels), by an increase in ploidy of recognizable megakaryocytes, and lastly by the production of large platelets. Whether the last event is independent of the effects on megakaryocyte precursors and immature megakaryocytes remains to be elucidated. Erythropoietin also has been shown to produce a variety of effects on erythropoiesis, including stimulation of DNA synthesis, which depend at least in part on the state of differentiation of the target cells.

Our data suggest similarities between the effects of acute thrombocytopenia and thrombopoietin and are compatible with at least some of the consequences of acute thrombocytopenia being mediated through the humoral regulator, thrombopoietin. Although changes following the administration of TPO were less marked, perhaps larger doses of TPO for a longer period of time would have produced more striking alterations in thrombopoiesis. Detailed studies of the exact temporal relationships of the changes we have described are necessary in order to more completely characterize the response to TPO.

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The effects of thrombopoietin on megakaryocyte-cfc, megakaryocytes, and thrombopoiesis: with studies of ploidy and platelet size

J Levin, FC Levin, DF 3d Hull and DG Penington