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The Reciprocal Relationship of Thrombopoietin (c-Mpl Ligand) to Changes in the Platelet Mass During Busulfan-Induced Thrombocytopenia in the Rabbit

By David J. Kuter and Robert D. Rosenberg

Thrombopoietin (c-Mpl ligand) has recently been purified and is considered to be the humoral regulator of platelet production. To see whether this molecule possessed the physiologic characteristics necessary to mediate the feedback loop between blood platelets and the bone marrow megakaryocytes, we determined the relationship between blood levels of thrombopoietin and changes in the circulating platelet mass. We developed a model of nonimmune thrombocytopenia in rabbits by the subcutaneous administration of busulfan. Compared with pretreatment plasma, plasma taken from all thrombocytopenic rabbits at their platelet nadir contained increased amounts of thrombopoietin. All of this activity was neutralized by soluble c-Mpl receptor. We subsequently measured the level of thrombopoietin in the circulation over the entire time course after

the administration of busulfan. As the platelet mass declined, levels of thrombopoietin increased inversely and proportionally and peaked during the platelet nadir. With return of the platelet mass toward normal, thrombopoietin levels decreased accordingly. When platelets were transfused into thrombocytopenic rabbits near the time of their platelet count nadir, the elevated levels of thrombopoietin decreased. In addition, platelets were observed to remove thrombopoietin from thrombocytopenic plasma in vitro. **These results confirm that thrombopoietin is the humoral mediator of megakaryocytopoiesis and suggest that the platelet mass may directly play a role in regulating the circulating levels of this factor.**

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THE PHYSIOLOGIC mechanisms controlling megakaryocyte growth and differentiation and the formation of the megakaryocyte product, the platelet, are poorly understood. Most models for the regulation of megakaryocyte growth are derived from our understanding of red blood cell production, where an altered demand for red blood cells is detected by a renal sensing mechanism. This sensor regulates the production of erythropoietin, which then enters the circulation and alters the rate of red blood cell formation by the bone marrow.¹⁻⁵ Unfortunately, for the platelet there is little knowledge about the sensing mechanism, which platelet attribute is sensed, or the physiologic nature of the factor, thrombopoietin, whose release promotes megakaryocyte growth and platelet production.⁶

We have previously suggested⁶ that any putative thrombopoietin should have the following physiologic characteristics: (1) a low basal level, (2) circulating levels that vary reciprocally and proportionally to changes in the platelet mass, (3) a finite time interval (lag period) before the level of the circulating factor changes in response to alterations in the platelet mass, and (4) suppression of the levels of the factor after platelet transfusion.

A number of well-characterized recombinant cytokines [interleukin (IL)-1,⁷ IL-3,⁷ IL-6,⁸⁻¹⁰ IL-11,¹¹⁻¹³ and erythro-

poietin¹⁴] have thrombopoietic effects, but none has been shown to possess any of the physiologic properties described above. Until recently, none of the other candidate thrombopoietins (thrombopoietin,¹⁵⁻¹⁹ thrombopoietic stimulatory factor,²⁰⁻²³ megakaryocyte stimulatory factor,^{24,25} and megapoietin^{6,26,27}) had been obtained in a sufficiently purified state to allow any physiologic analysis. Although referred to by a variety of names (thrombopoietin,^{28,29} c-Mpl ligand,^{30,31} megakaryocyte growth and differentiation factor,³² and megapoietin²⁶), a protein with a unique amino acid sequence, hereafter referred to as thrombopoietin, has recently been purified and appears to have potent thrombopoietic activity. When purified²⁶ or recombinant thrombopoietins²⁸⁻³⁰ were injected into recipient animals, they increased the number, size, and ploidy of megakaryocytes and raised the platelet count.

This thrombopoietin was elevated in plasma obtained from thrombocytopenic animals,^{26,30-32} but its relationship to the platelet mass has not been fully demonstrated. To analyze these physiologic characteristics of thrombopoietin, we developed a model of prolonged, busulfan-induced thrombocytopenia in the rabbit. With this model system, we have found that thrombopoietin possesses all the physiologic characteristics to be expected of the molecule that regulates platelet production.

MATERIALS AND METHODS

Reagents and animals. Trisodium citrate was purchased from Mallinckrodt (Paris, KY). Propidium iodide, adenosine (free base), theophylline, busulfan, polyethylene glycol (average molecular weight 400), deoxyribonuclease I (type IV), and ribonuclease A (type I-A) were obtained from Sigma (St Louis, MO). Neutralizing rabbit antibodies to porcine transforming growth factor- β (TGF β ; lots J919 and J940) were obtained from R & D Systems (Minneapolis, MN). Male retired breeder (600 to 800 g) Sprague-Dawley-derived (CD) rats were obtained from Charles River Breeding Laboratories, Inc (Wilmington, MA), and New Zealand White rabbits (3 kg) were from Hazleton Research Products, Inc (Denver, PA). All animals were housed in single cages with free access to food and water for at least 1 week before use. All animal experiments were approved by the Committee on Animal Care at the Massachusetts

From the Department of Biology, Massachusetts Institute of Technology, Cambridge; the Hematology Unit, Massachusetts General Hospital, Boston; the Department of Medicine, Beth Israel Hospital, Boston; and Harvard Medical School, Boston, MA.

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Address reprint requests to David J. Kuter, MD, PhD, Hematology Unit, Cox 235, Massachusetts General Hospital, Fruit St, Boston, MA 02114.

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Institute of Technology (approval 90-041; Cambridge, MA). Antiserum reacting against rat platelets (APS) was prepared in rabbits as previously described.³³

Preparation of thrombocytopenic rabbits. Busulfan at a final concentration of 10 mg/mL in polyethylene glycol was prepared by making a slurry at approximately 100 mg/mL and stirring for 2 hours at room temperature. This suspension was then brought to the final volume in polyethylene glycol and heated at 74° to 80°C with stirring for 2 hours to dissolve residual crystals of busulfan. Rabbits were restrained, one ear was swabbed with 95% ethanol, and the lateral vein was cannulated with a 23-gauge scalp vein infusion set (Infusion Set, 23 gauge \times 0.75 in, 12-in tubing; Deseret Medical Inc, Sandy, UT). Between 0.75 and 1.25 mL of sodium pentobarbital (65 mg/mL; Anthony Products Co, Arcadia, CA) was infused over 1 to 2 minutes to produce anesthesia. After preparing the injection site by shaving off all the hair and scrubbing the site with 95% ethanol, busulfan (25 mg/kg per injection) was then administered by a single, deep subcutaneous injection into alternate sides of the lower abdomen on days 0 and 3. Animals were observed closely for 1 hour before being returned to their cages and were monitored daily for the next 15 to 100 days.

At intervals, 1 to 5 mL of blood was removed aseptically from the lateral ear vein with a 23-gauge scalp vein infusion set for cell counts and for addition to bone marrow culture. Nine volumes of blood were drawn, with gentle mixing into syringes containing 1 vol of 3.8% sodium citrate. An aliquot of anticoagulated blood was then drawn into the Unopette Collection System (Becton-Dickinson, Rutherford, NJ) for platelet and white blood cell (WBC) count determinations using the hemacytometer (four chambers per sample), as originally described by Brecher and Cronkite.³⁴ A second aliquot was drawn into a microhematocrit capillary tube (Fisher Scientific Co, Pittsburgh, PA), and the hematocrit was determined after centrifugation. The hematocrit, WBC count, and platelet count were all corrected for dilution by the anticoagulant. The remaining blood was immediately centrifuged at 3,000g for 15 minutes at 4°C, and the platelet-poor plasma (PPP) was removed. After a second centrifugation at 3,000g for 15 minutes, the PPP was frozen at -80°C.

The conditions of centrifugation used here have been optimized to pellet all the platelets yet not damage those in the pellet. After counting the few platelets remaining in the supernatant, we can demonstrate the presence of no more than 0.01% of the starting number. At higher centrifugal forces, platelets in the pellet are damaged and release TGF β .

Platelet transfusion. Blood was obtained from donor rabbits that were anesthetized with pentobarbital and then exsanguinated by cardiac puncture. Nine parts of blood were collected into one part of disodium EDTA (1.66% in water), and the anticoagulated blood was immediately centrifuged for 8 minutes at 500g. The supernatant platelet-rich plasma (PRP) was then carefully removed. The remaining pelleted blood cells were twice resuspended to the original volume with Hanks' Balanced Salt Solution without calcium or magnesium (HBSS) and centrifuged as before. The supernatants were removed and added to the original PRP. The pooled platelet suspension was centrifuged for 15 minutes at 3,000g at 4°C, and the platelet pellet (containing 42×10^9 to 47×10^9 platelets) was resuspended in 4 mL of HBSS. From 250 mL of anticoagulated blood, about 65% of the total platelets and less than 1% of the WBC were recovered by this method.

On day 14 or 15, the lateral ear vein of thrombocytopenic, busulfan-treated rabbits was cannulated with a 23-gauge scalp vein infusion set. After demonstrating the free flow of blood, platelets were infused over 3 to 5 minutes, and the tubing was rinsed with a small volume of sterile normal saline. Animals were closely observed for 1 hour, and none demonstrated any complications after transfusion. The platelet count obtained 3 hours after infusion showed that over

90% of the infused platelets were circulating. We have previously shown that these platelets have a normal half-life in the circulation.²⁷

Thrombopoietin assay. Because direct addition of plasma to the culture caused coagulation of the bone marrow cells, we found it necessary either to heparinize our culture system or to defibrinogenate our plasma samples. The latter method proved more convenient, and, therefore, all rabbit plasma (PPP) samples were defibrinogenated by clotting them before addition to culture wells. Coagulation of rabbit PPP was performed by recalcification in glass tubes and incubation at 37°C for 2 hours. Clots were removed, and the resulting rabbit PPP-derived serum (PPADS) was heated at 56°C for 30 minutes and then filtered through a 0.45- μ m syringe filter (Millex-HA, Millipore Products Division, Bedford, MA). Potential TGF β contamination was neutralized by the addition of antibody to TGF β 1, as previously described.³⁵

Megakaryocyte-depleted rat bone marrow was prepared by the Percoll density-gradient centrifugation and filtration method.³³ The megakaryocyte-depleted bone marrow cells were resuspended to a density of 7×10^6 /mL (containing no more than 100 identifiable megakaryocytes per milliliter) in 3 mL of Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) containing penicillin (200 U/mL), streptomycin (200 μ g/mL), additional glutamine (0.592 mg/mL), and (unless otherwise indicated) 15% (vol/vol) rabbit PPADS. Cultures were routinely grown for 3 days at 37°C in a 5% CO₂ incubator. Subsequently, cells were harvested and stained for flow cytometry with APS and propidium iodide, as described previously.³⁵ For each culture, the number and ploidy of megakaryocytes were measured by flow cytometry, and the amount of thrombopoietin activity in the sample was determined as described below.

Each experiment was performed from a single bone marrow preparation and allowed us to assay up to 20 individual specimens under identical culture conditions. Between identical plasma specimens assayed in the same bone marrow preparation, there was routinely a coefficient of variation of less than 1% in the ploidy of megakaryocytes that grew. Between identical plasma specimens assayed in different bone marrow preparations, there was routinely a coefficient of variation of 5% to 8%. These differences reflect variations in the duration of culture and in the number of megakaryocyte precursors in the marrow.³⁵

The specificity of this thrombopoietin assay was evaluated by adding different cytokines at concentrations that ranged from 1/1,000 to 1,000 times the half-maximal effective dose. We determined that human and murine erythropoietin (Amgen, Thousand Oaks, CA) stimulated megakaryocyte growth³³ at high, nonphysiologic concentrations (1 to 2 U/mL), whereas interleukin (IL)-1, IL-2, IL-3, IL-6, IL-11, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), fibroblast growth factor, epidermal growth factor (EGF), M-CSF, tumor necrosis factor (TNF), leukemia inhibitory factor, stem cell factor, and the interferons demonstrated no stimulation at any level. TGF β 1 inhibited the cultures.³⁵ Only thrombocytopenic plasma and purified thrombopoietin (c-Mpl ligand)²⁶ stimulated this assay to any significant extent. As described in Results, all the activity in thrombocytopenic plasma in these experiments was neutralized by addition of soluble c-Mpl receptor.

Flow cytometry. Flow cytometry was performed as previously described³⁵ on a machine designed by H.M. Shapiro³⁶ and built by Y.G. Caine. A coefficient of variation of the 2N peak was maintained at 1.97% to 2.94% by careful alignment of the optical system. Cells were routinely run at 800 to 1,200 cells per second, and 1,000 megakaryocytes were analyzed.

Data analysis and determination of thrombopoietin level. Data from the flow cytometer were stored and analyzed on an Atari 130 XE computer (Sunnyvale, CA). The total number of nucleated cells and the total number of megakaryocytes in each assay were quanti-

fied as previously described.³⁵ Boundaries between each ploidy class were assigned from the DNA histogram, and the number of megakaryocytes in each ploidy group (4N to 32N) was counted and then expressed as a percentage of the total number of megakaryocytes $\geq 4N$. The mean ploidy for each distribution 4N to 32N (mean ploidy) was then determined as described by Arriaga et al.³⁷

Thrombopoietin stimulates both the number and ploidy of megakaryocytes that grow in culture.^{6,26} The rise in megakaryocyte number in culture is usually less pronounced than the increase in megakaryocyte ploidy and is seen at higher concentrations of thrombopoietin. In addition, the changes in megakaryocyte number are measured by flow cytometry with less sensitivity and less precision than the changes in megakaryocyte ploidy. Because of these differences between using change in the number of megakaryocytes versus change in the ploidy of megakaryocytes to quantify thrombopoietin, we routinely measured thrombopoietin activity for each sample tested by assessing its stimulatory effect only on ploidy (as expressed by the mean ploidy.)

Given the nature of our assay, cultures that have undergone no stimulation of growth will still have a small number of low ploidy megakaryocytes present [persisting megakaryocytes remaining from the rat bone marrow placed into culture plus a modest amount of basal (non-thrombopoietin-dependent) growth from megakaryocyte precursor cells] and yield a baseline mean ploidy value of 8 to 9. As this is the mean ploidy value for control (nonthrombocytopenic) plasma samples as well as samples containing only buffer (HBSS), all control (nonthrombocytopenic) plasma samples contained undetectable amounts of thrombopoietin. When compared with the stimulatory effect of known amounts of purified thrombopoietin,²⁶ the control (nonthrombocytopenic) mean ploidy values of 8 to 9 reported here represent plasma thrombopoietin protein concentrations of less than 0.25 pmol/L, while the mean ploidy values of 12 to 16 indicate plasma thrombopoietin protein concentrations of 25 to 50 pmol/L. Therefore, the increase in mean ploidy over that of control (nonthrombocytopenic) plasma samples directly measures the amount of thrombopoietin in the sample being tested, and any decrease in mean ploidy below that of control plasma samples indicates the presence of inhibitory substances.

Except for ploidy distributions, statistical analysis of differences was performed with Student's *t* test.³⁸ Differences between ploidy distributions were tested for using the Mann-Whitney U test,³⁹ as previously described.³⁵

In vitro incubation of platelets with thrombocytopenic plasma. PRP was prepared from a normal donor rabbit as described above, and the platelet content was determined by hemacytometer. A volume of PRP that contained the same number of platelets (2.74×10^9 platelets) as that found in 6.67 mL of whole blood from a normal rabbit (platelet count, $0.41 \times 10^9/\text{mL}$) was centrifuged at 3,000g for 15 minutes, and the pellet was resuspended in 4 mL (the amount of PPP in 6.67 mL of whole blood) of thrombocytopenic rabbit PPP. After incubation for 1 hour at 4°C or at 20°C with occasional stirring, the platelets were removed by centrifuging twice at 3,000g for 15 minutes. After the second centrifugation, the supernatant PPP contained less than 0.01% of the platelets initially found in the blood sample, an amount that has inconsequential effect in our assay.³⁵ The supernatant PPP was then clotted as described above and assayed in bone marrow culture.

Preparation and use of soluble c-Mpl receptor. BaF3 cells were provided by Dr A. d'Andrea (Dana Farber Cancer Institute, Boston, MA). Murine c-Mpl was cloned from mouse bone marrow by polymerase chain reaction (PCR; Dr Y.C. Li, Department of Biology, Massachusetts Institute of Technology; manuscript in preparation) using primers designed according to the published murine sequence.⁴⁰ The 5' primer had the sequence GAAGATGCCCTCTTG-GCCCC and the 3' primer had the sequence GGCAGCAGCCCT-

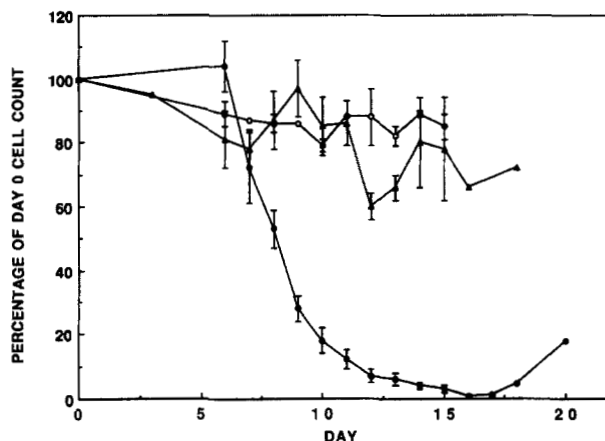


Fig 1. The effect of busulfan on rabbit peripheral blood cell counts. Seventeen rabbits were injected with busulfan on day 0 and day 3 as described in Materials and Methods. Blood samples were obtained at intervals over the next 20 days for measurement of the platelet count (filled circles), hematocrit (open circles), and WBC count (filled triangles). For each animal, cell counts were expressed as the percentage of the value on day 0, and then the average percentage (\pm SD) for all the animals was calculated for the platelet count (17 animals), hematocrit (14 animals), and the WBC count (13 animals).

GAAGGCAG. The truncated extracellular domain was then inserted into the *Bgl* II site of the LNL6-based EMC/NEO vector (gift of Dr J. Majors, Washington University, St Louis, MO) in both sense and antisense fashion. The constructs were electroporated into BaF3 cells, and stable transfectants were selected in the presence of G 418 (0.7 mg/mL). The expression of c-Mpl was confirmed by Northern blot analysis. The cell lines expressing sense (BaF3-Mpl) or antisense c-Mpl (BaF3-asMpl) were then expanded in culture in the presence of IL-3. The resulting cells were harvested, lysed by two cycles of freezing and thawing, and then centrifuged at 10,000g to remove particulate matter. A 40% to 60% ammonium sulfate cut was prepared from the supernatant, treated with 2-mercaptoethanol for 1 hour (to remove any contaminating TGF β 1), and dialyzed versus a 1,000-fold excess of HBSS.

The soluble Mpl-IgG fusion protein containing the extracellular domain of human Mpl³⁰ was provided to us by Dr Dan Eaton (Genentech, South San Francisco, CA).

RESULTS

Busulfan treatment produced severe thrombocytopenia but only modest leukopenia in rabbits. After the injection of a total of 50 mg/kg of busulfan in two equal doses on days 0 and 3, the decline in platelet count followed a very reproducible pattern. As shown in Fig 1, there was no decline from the average pretreatment platelet count of $456,250 \pm 78,646/\mu\text{L}$ ($n = 17$; range, 305,556 to 580,556) up to day 6, but then the count decreased rapidly over the next week to a nadir on day 14 ± 1 of $11,696 \pm 10,730/\mu\text{L}$ ($n = 17$; range, 1,500 to 40,000), an average decline to 2.6% of the pretreatment value. The platelet count usually remained below 10% of the pretreatment value from day 12 to day 19 and then returned to normal over the next 2 to 3 weeks without a rebound thrombocytosis (see Fig 4 for a typical response).

In those animals in whom phlebotomy was minimal, the

Table 1. Thrombocytopenic Plasma From Busulfan-Treated Rabbits Stimulated the Number and Ploidy of Megakaryocytes That Grew in Bone Marrow Culture

Rabbit No.	HCT	Platelet Count/ μ L	Total No. of Megakaryocytes/ Culture	4 N (%)	8 N (%)	16 N (%)	32 N (%)	Mean Ploidy
Normal pretreatment plasma								
117	39	556,944	11,674	15.1	50.4	31.7	2.8	9.3
35	39	470,833	9,384	22.3	46.6	27.5	3.6	8.7
112	33	461,111	8,497	17.2	46.0	32.4	4.4	9.4
113	39	305,556	6,628	21.2	48.4	25.5	4.9	8.8
37	37	538,889	10,037	22.9	43.7	29.2	4.3	8.9
36	40	404,167	9,654	15.0	51.1	30.7	3.2	9.3
Mean \pm SD	38 \pm 3	455,750 \pm 92,678	9,312 \pm 1,679	19.9 \pm 3.6	47.7 \pm 2.8	29.5 \pm 2.7	3.9 \pm 0.8	9.1 \pm 0.3
Thrombocytopenic plasma								
117	30	8,500	16,004	7.5	23.5	53.6	15.5	13.7
35	32	40,000	15,904	8.9	19.8	46.1	25.2	14.6
112	27	4,444	12,151	11.2	14.9	46.8	27.2	14.9
113	30	3,333	8,471	9.0	15.0	35.4	40.6	16.9
37	34	12,350	16,786	8.0	23.6	50.7	17.7	13.7
36	35	5,250	15,064	5.9	29.3	47.9	16.9	13.5
Mean \pm SD	31* \pm 3	12,313* \pm 13,954	14,063* \pm 3,178	8.4* \pm 1.8	21.0* \pm 5.6	46.7* \pm 6.2	23.8* \pm 9.5	14.6* \pm 1.3

Normal and thrombocytopenic plasmas were obtained from each rabbit on day 0 (before busulfan injection) and on day 13, respectively, and added to rat bone marrow cultures as described in Materials and Methods. After 3 days of growth, the cells were harvested and stained for flow cytometry. The total number of megakaryocytes and the relative megakaryocyte ploidy distribution were determined for each culture. Each pair of normal and thrombocytopenic plasmas was assayed in a different bone marrow culture.

Abbreviation: HCT, hematocrit.

* $P < .01$ when compared with normal (pretreatment) plasma.

hematocrit declined gradually over 2 weeks (Fig 1) from an average of 37.4 ± 2.5 ($n = 14$; range, 33.9 to 41.1) to a nadir on day 13 ± 2 of 30.9 ± 1.9 ($n = 14$; range, 27.8 to 34.1), an average drop of 17%.

The response of the WBC count was more variable. In 13 of the 17 animals analyzed, the average pretreatment WBC count of $8,826 \pm 2,052/\mu$ L (range, 4,938 to 12,531) decreased gradually over 2 weeks (Fig 1) to a nadir on day 13 ± 1 of $5,849 \pm 1,889/\mu$ L (range, 2,531 to 9,670), an average drop of 34%. Four animals experienced severe leukopenia (WBC count less than $2,000/\mu$ L) that occurred on day 13 and necessitated their being killed (a prestudy parameter established to insure optimal animal care). All four were found to have woody edema and necrosis at the injection sites that were related to the inadvertent intradermal injection of the busulfan. In 52 subsequent animals where meticulous attention has been given to using deep, subcutaneous injections, this problem has not recurred.

Except for this complication, all animals appeared healthy with no weight loss, decline in appetite or stool production, or change in coat texture. There was no overt or occult bleeding detected in any of the animals. After resolution of the thrombocytopenia, all the peripheral blood cell counts returned to normal. Several animals have been observed for over 3 years without any evidence of long-term hematologic abnormality.

Thrombopoietin was present in the plasma from thrombocytopenic busulfan-treated rabbits. In a total of six rabbits, we have compared the effect in bone marrow culture of thrombocytopenic plasma obtained at the platelet nadir to that of plasma obtained before treatment (Table 1). The average normal (pretreatment) and thrombocytopenic hemato-

crits and platelet counts (Table 1) of these rabbits were identical to those of the larger group of animals described in Fig 1. For all six animals, plasma obtained at the platelet nadir markedly stimulated the number, size, and ploidy of megakaryocytes that grew in bone marrow culture (Table 1). This effect was visually apparent by inspection of the cultures using phase contrast microscopy (Fig 2) and was readily quantified by flow cytometry. Compared with pretreatment plasma samples, all thrombocytopenic plasmas stimulated an increase in the total number of megakaryocytes per culture from $9,312 \pm 1,679$ to $14,063 \pm 3,178$. Likewise, all thrombocytopenic plasmas stimulated a shift in the modal megakaryocyte ploidy class from 8N to 16N and an increase in the mean ploidy from 9.1 ± 0.3 to 14.6 ± 1.3 . The percentage of 32N megakaryocytes increased sixfold.

These six animals varied somewhat in the extent of their thrombopoietin response (mean ploidy). This variation did not appear related to the small differences in the platelet counts and probably represented variation in the responsiveness of the different assay preparations to thrombopoietin.⁶

These increases in megakaryocyte number and ploidy were solely due to the presence of thrombopoietin (c-Mpl ligand) in the thrombocytopenic samples. After addition of soluble c-Mpl receptor (Fig 3), all stimulatory activity was eliminated, and the mean ploidy of the thrombocytopenic samples was identical to the control (nonthrombocytopenic) samples. Therefore, it is the extent of the increase in mean ploidy above the control (nonthrombocytopenic) values that measures the amount of thrombopoietin present in the sample being tested. These experiments have also been performed using a different soluble c-Mpl receptor, Mpl-IgG,³⁰ and identical results were obtained.

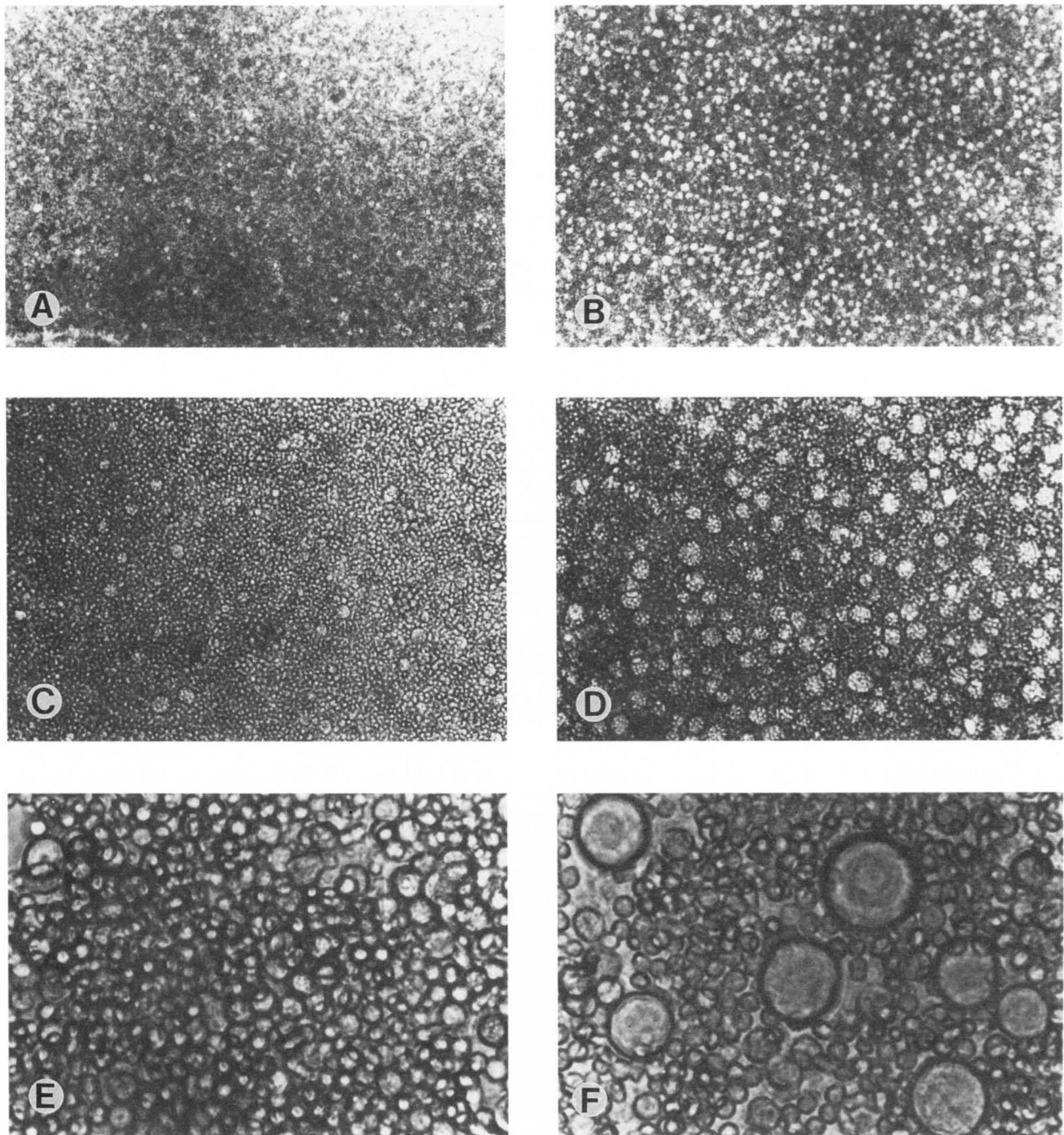


Fig 2. Phase contrast microscopy of rat bone marrow cultures after the addition of pretreatment (A, C, E) and thrombocytopenic (B, D, F) rabbit plasma. Plasma was obtained from rabbit 35 just before busulfan injection on day 0 (platelet count, 470,833/ μ L) and during thrombocytopenia on day 13 (platelet count, 40,000/ μ L) and added to rat bone marrow cultures as described in Materials and Methods. After 3 days of growth, the cultures were examined by phase contrast microscopy at an original magnification of $\times 5$ (A, B), $\times 12.5$ (C, D), and $\times 50$ (E, F). The large cells are all megakaryocytes. The flow cytometry analysis of these cells is presented in Table 1.

Urine from several thrombocytopenic rabbits was collected, and the protein was concentrated by 80% ammonium sulfate precipitation and then dialyzed against culture medium. Unlike simultaneous serum specimens, none of the urine specimens showed any stimulatory activity when added to bone marrow culture (data not shown).

Thrombopoietin levels in the circulation were inversely proportional to the platelet count of the rabbit. To determine whether the plasma thrombopoietin concentration was related to the degree of thrombocytopenia, five rabbits were treated with busulfan, and samples were taken at variable intervals from day 0 to day 100. As shown for a representa-

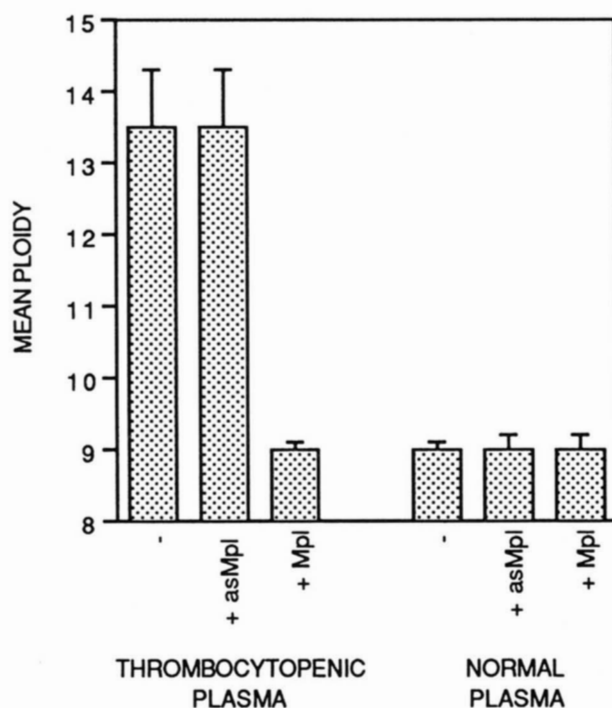


Fig 3. Soluble c-Mpl receptor completely removed all thrombopoietin from thrombocytopenic plasma. A pool of equal amounts of normal (pretreatment) plasma and a second pool of equal amounts of thrombocytopenic plasma from the six rabbits described in Table 1 were prepared. Equal amounts of soluble c-Mpl receptor (+Mpl), soluble anti-sense c-Mpl receptor (+asMpl), or HBSS (-) were added to the normal plasma pool or thrombocytopenic plasma pool. After incubation at 20°C for 1 hour, samples were added to bone marrow culture in triplicate, and the effect (\pm SD) on the mean ploidy (thrombopoietin content) was determined.

tive rabbit in Table 2 and Fig 4, the extent of stimulation of both the number and ploidy of megakaryocytes for all the animals was inversely proportional to the platelet count on the day the plasma sample was obtained. The stimulatory

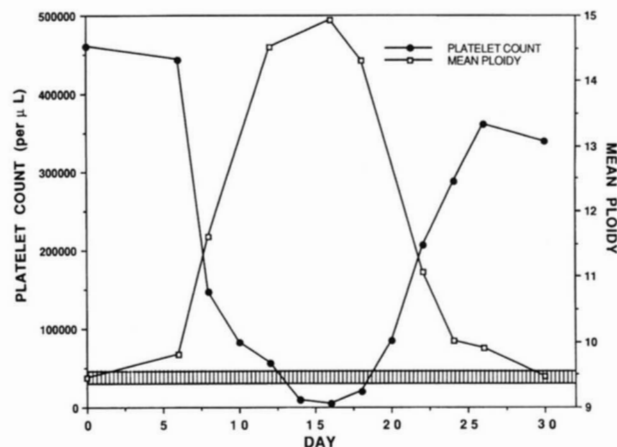


Fig 4. Levels of thrombopoietin were inversely proportional to the platelet count. As described in Table 2, blood samples were obtained from rabbit 112 from day 0 to day 100 after the first injection of busulfan. The level of thrombopoietin was quantified by measuring the stimulation of megakaryocyte mean ploidy (\square) and is shown for the first 30 days as a function of the platelet count (\bullet). The effect of pretreatment (day 0) plasma (\pm 2 SD) is shown for the megakaryocyte mean ploidy (\blacksquare). All values outside of this region show a statistically significant differences ($P < .05$) from their respective day 0 values.

activity first appeared on day 8 ± 2 ($n = 5$) at a time when the platelet count was usually about 50% of normal and the level of stimulatory activity peaked during the platelet nadir. With an increase in the platelet count toward normal, the stimulatory effect rapidly declined. At a platelet count of about 50% of normal, the stimulatory effect was again near to the baseline (day 0) value. Although summarized best by the changes in mean ploidy (Fig 4), the extent of stimulation by the thrombocytopenic plasma was also readily apparent by the changes seen in each individual ploidy class (Table 2) and by the change in the number of megakaryocytes that grew in culture (Table 2).

Table 2. Extent of Stimulation of Megakaryocyte Number and Ploidy in Bone Marrow Culture by Thrombocytopenic Plasma Was Inversely Proportional to the Platelet Count

Day	Platelet Count/ μ L	Total No. of Megakaryocytes/Culture	4 N (%)	8 N (%)	16 N (%)	32 N (%)	Mean Ploidy
0	461,111	8,497 \pm 117	17.2 \pm 0.2	46.0 \pm 0.4	32.4 \pm 0.5	4.4 \pm 0.7	9.5 \pm 0.1
6	443,056	8,282	18.3	40.0	35.8	5.9	9.8
8	146,292	10,230	11.0	35.1	43.2	10.7	11.6
12	56,667	9,554	10.2	18.8	45.8	25.1	14.5
16	4,444	12,151	11.2	14.9	46.8	27.2	14.9
18	20,500	13,139	8.1	22.1	47.6	22.2	14.3
22	206,667	9,451	10.3	40.2	41.8	7.7	11.1
24	287,500	8,800	13.9	45.8	34.2	6.1	10.0
26	361,111	9,281	15.7	43.5	35.3	5.5	9.9
30	338,750	8,400	13.3	53.0	29.7	4.0	9.5

Rabbit 112 was treated with busulfan on days 0 and 3, and blood samples were taken at frequent intervals over the next 100 days for the determination of cell counts and for addition to bone marrow culture. Cultured cells were harvested after 3 days of growth for analysis by flow cytometry. The data for day 0 are the average of duplicate specimens, and the remaining data are single determinations. Samples taken from days 30 to 100 were also assayed and were the same as the day 0 values. In a separate experiment, plasma from day 14 was found to stimulate the mean ploidy to the same extent as plasma from day 16. The megakaryocyte mean ploidy and total number of megakaryocytes per culture are also plotted in Fig 4.

Table 3. Platelet Transfusion Reduced the Elevated Levels of Thrombopoietin in a Thrombocytopenic, Busulfan-Treated Rabbit

Day	Platelet Count/ μ L	4 N (%)	8 N (%)	16 N (%)	32 N (%)	Mean Ploidy
0	509,722 (100%)	25.2 \pm 0.2	50.1 \pm 1.6	22.7 \pm 1.3	2.1 \pm 0.1	8.1 \pm 0.1 (0%)
12	93,889	17.4	46.3	32.9	3.4	9.3
14	64,444	11.0	39.9	42.7	6.5	10.9
15	31,667 (6.2%)	8.8* \pm 0.1	39.1* \pm 1.4	46.1* \pm 0.0	6.0* \pm 1.4	11.3* \pm 0.2 (100%)
15.1	207,778 (41%)	16.9 \pm 1.2	52.9 \pm 0.6	28.2 \pm 0.2	2.1 \pm 0.7	8.9 \pm 0.2 (11% \pm 18%)†
16	130,000	12.1	45.5	37.9	4.6	10.2
17	105,000	10.7	38.6	43.4	7.3	11.1
18	97,778	9.7	34.5	47.2	8.5	11.7
22	425,000	rc19.9	52.5	25.5	2.1	8.6

Rabbit 105 was treated with busulfan on days 0 and 3, and the decline in platelet count was monitored. On day 15, 42.25×10^9 rabbit platelets in 5 mL of HBSS were infused. Blood samples were obtained on the days indicated for cell counts and for addition to bone marrow culture. After 3 days of growth, cells were harvested, and the megakaryocyte ploidy distributions were determined by flow cytometry. Data for days 0, 15, and 15.1 are presented \pm SD. Data in parentheses are the average of three separate assays of these samples, with the megakaryocyte mean ploidy on day 0 normalized to 0% and that on day 15 normalized to 100%. The mean ploidy values are also plotted in Fig 5.

* $P < .05$ when compared with either day 0 or day 15.1 values.

† $P < .05$ when compared with day 15 value.

Platelet transfusion reduced the level of thrombopoietin in thrombocytopenic, busulfan-treated rabbits. To determine whether the elevated level of thrombopoietin found in the circulation of thrombocytopenic, busulfan-treated rabbits was really due to the low platelet count and not due to the means by which the platelet count had been lowered, platelets were transfused into thrombocytopenic rabbits as they approached their platelet nadir, and the effect on the circulating level of thrombopoietin was measured. As shown in Table 3 and Fig 5, 3 hours after increasing the platelet count from 6.2% to 41% of normal, the thrombopoietin level (mean ploidy) decreased from 11.3 ± 0.2 to 8.9 ± 0.2 , an $89\% \pm 18\%$ decline. As the transfused platelets subsequently decreased in the circulation, the level of thrombopoietin again increased to maximum, only to decrease again as the rabbit's own platelet production recovered.

This experiment has been repeated using a second animal

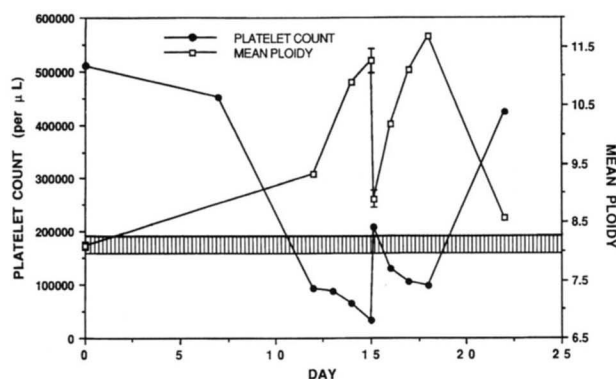


Fig 5. Transfusion of platelets (●) reduced the elevated level of thrombopoietin (□) in a thrombocytopenic, busulfan-treated rabbit. As described in Table 3, platelets were transfused into rabbit 105 on day 15, and the effect on circulating levels of thrombopoietin was quantified. The hatched bar indicates the mean ploidy (thrombopoietin level) of pretreatment (normal) rabbit plasma (± 2 SD). All megakaryocyte mean ploidy (thrombopoietin) values outside this range differ from normal, with a statistical significance of $P < .05$.

with similar results. On day 14, rabbit 104 received a platelet transfusion of 47×10^9 rabbit platelets, which raised the platelet count from 3% of the pretreatment value to 37%. Plasma taken 3 hours after the transfusion showed an $81\% \pm 8\%$ decrease in the level of thrombopoietin present in the pretransfusion (thrombocytopenic) plasma.

As platelet transfusion did not restore the platelet count entirely to its pretreatment level, the decline in the level of thrombopoietin was only partial, but its level was identical to that which would have been predicted for this degree of thrombocytopenia. However, by day 18, thrombopoietin levels were again maximally elevated despite a platelet count of $97,778/\mu$ L (at least 75% of which would be expected to be transfused platelets, as suggested by the data in Fig 4). This apparent discrepancy probably reflects diminished ability of the transfused platelets to metabolize thrombopoietin due to their older age or saturated clearance capacity (see Discussion).

If the pretransfusion and posttransfusion thrombopoietin levels represent steady-state values, the data suggest that thrombopoietin has a circulating half-life under 45 minutes. This rough estimate has been based on the finding that the levels drop from one presumed steady state to the next over 3 hours, ie, over four half-lives. As levels in these animals were not measured earlier than 3 hours after transfusion, levels could have decreased faster and would, therefore, have predicted an even shorter circulating half-life.

Rabbit platelets removed thrombopoietin from thrombocytopenic rabbit plasma *in vitro*. The reduction in thrombopoietin levels seen after platelet transfusion *in vivo* may be an indirect effect due to the interruption of a feedback loop that stimulates thrombopoietin production or it may be due to the effect of platelets directly removing thrombopoietin from the circulation. To see if the latter mechanism was possible, rabbit platelets were added back to thrombocytopenic rabbit plasma *in vitro* in an amount identical to that which would be found in PRP from a nonthrombocytopenic rabbit. When incubated at 4°C , platelets removed 42% of the thrombopoietin (Table 4). However, at 20°C , platelets removed 95% of the thrombopoietin.

Table 4. Rabbit Platelets Removed Thrombopoietin From Thrombocytopenic Rabbit Plasma In Vitro

	4 N (%)	8 N (%)	16 N (%)	32 N (%)	Mean Ploidy
I. Thrombocytopenic plasma incubated with platelets					
Pretreatment (normal) plasma	20.0 ± 2.9	46.6 ± 2.2	29.0 ± 0.9	4.4 ± 1.1	9.1 ± 0.3 (0%)
Thrombocytopenic plasma	12.4* ± 1.3	38.7* ± 1.9	39.2* ± 0.4	9.7* ± 0.9	11.0* ± 0.1 (100%)
Thrombocytopenic plasma + platelets at 4°C	13.8* ± 0.7	43.4 ± 0.8	36.9* ± 1.5	5.9* ± 1.6	10.2* ± 0.2 (58%)
Thrombocytopenic plasma + platelets at 20°C	18.3 ± 2.3	47.6 ± 4.8	30.4 ± 1.6	3.7 ± 1.4	9.2 ± 0.2 (5%)
II. Normal plasma incubated with platelets					
Pretreatment (normal) plasma	14.4 ± 1.2	50.9 ± 1.8	31.8 ± 1.6	3.0 ± 1.2	9.4 ± 0.3 (0%)
Thrombocytopenic plasma	10.0* ± 2.1	44.3* ± 3.0	38.5* ± 2.5	7.2* ± 1.3	10.8* ± 0.5 (100%)
Normal plasma + platelets at 20°C	14.1 ± 2.5	51.9 ± 0.8	30.9 ± 0.9	3.2 ± 1.0	9.4 ± 0.3 (0%)

Pools of pretreatment (normal) and thrombocytopenic (day 13) PPP were prepared from several busulfan-treated rabbits. Rabbit platelets (2.74×10^9) were then added to some 4-mL aliquots, incubated for 1.5 hours at either 4°C or 20°C and removed by centrifugation as described in Materials and Methods. Plasma was added in quadruplicate to rat bone marrow cultures, and the cells were harvested after 3 days for flow cytometry. All data are presented \pm SD. The numbers in parentheses are the relative percentage increases in the megakaryocyte mean ploidy, where 100% is defined as the value of thrombocytopenic plasma and 0% the value of pretreatment (normal) plasma. Although the platelet and plasma samples were identical, I and II were assayed in different bone marrow preparations.

* $P < .05$, when compared with pretreatment plasma.

To exclude the possibility that platelets were releasing some inhibitory substance, the following three experiments were performed. First, these experiments were repeated in the presence of neutralizing antibody to TGF β 1, and identical results were obtained. Next, when pretreatment (normal) plasma (Table 4) was incubated with rabbit platelets, there was no effect of the platelet addition on the ploidy of megakaryocytes that grew in culture. Had the platelets released any inhibitory material, the mean ploidy would have declined below that of the pretreatment (normal) value, as we have previously shown.³⁵ Finally, to test directly for the possible presence of inhibitors released into thrombocytopenic plasma after platelet addition (and subsequent removal of platelets by centrifugation), a 1:1 mix was performed between it and thrombocytopenic plasma that had not been exposed to platelets. As shown in Fig 6, this 1:1 mix produced the same number (Fig 6A) and mean ploidy (Fig 6B) as that obtained when normal and thrombocytopenic plasmas were mixed, thus demonstrating the absence of any inhibitor.

DISCUSSION

Most efforts to stimulate the production of thrombopoietin *in vivo* have used animals in which thrombocytopenia was generated by exchange transfusion,⁴¹⁻⁴³ irradiation,^{44,45} or anti-platelet antibody.^{27,46,47} Unfortunately, each of these methods has a number of problems that frustrate attempts to perform physiology experiments. Exchange transfusion is arduous, may not produce extreme degrees of thrombocytopenia, and can itself cause changes in megakaryocyte maturation rate.⁴⁸ Irradiation produces not only thrombocytopenia but also severe neutropenia and anemia⁴⁹ and has been shown to injure stromal cells, thereby releasing some hematopoietic factors.⁵⁰ Finally, the infusion of anti-platelet antibody does not usually produce prolonged thrombocytopenia and may also generate cytokines after the formation of antigen-antibody complexes.⁵¹ Given this situation, we were surprised to find how little attention had been given to another model system of thrombocytopenia, namely, that produced by the administration of the chemotherapeutic agent busulfan.⁵²⁻⁶¹

As suggested by the work of Spector,⁵⁴ we modified the protocol of Evensen and Jeremic⁵³ by injecting busulfan subcutaneously instead of intraperitoneally into rabbits. The resulting decline in platelet count was identical to that described by Evensen and Jeremic,⁵³ who found that the platelet count started to decrease after day 6 and reached a nadir of 3.1% on day 13. The hematocrit in their studies decreased from 36.4 to 32.6, which is comparable to that described by us. The only real difference seen between their study and ours was that the WBC count decreased much more in their animals, from 9,100 to 2,800, presumably due to the different route of injection. When we paid meticulous attention to avoiding intradermal injection of the busulfan, neutropenia was minimal, and all animals developed profound thrombocytopenia which lasted for 1 week. After recovery, there was no evidence of long-term bone marrow failure, a problem that has been reported for mice injected with busulfan.⁵⁸⁻⁶¹ Although this model of thrombocytopenia has been useful in our studies on megakaryocytopoiesis, it may also be helpful in other areas of investigation directed at identifying the role of platelets in pathologic processes, such as tumor metastases, pulmonary hypertension, atherosclerosis, or endothelial injury.

Plasma obtained from all thrombocytopenic, busulfan-treated rabbits at their platelet nadir markedly stimulated both the number and ploidy of megakaryocytes that grew in our rat bone marrow cultures. The extent of stimulation of megakaryocyte growth was identical to that we have previously shown to occur with plasma from both rats²⁷ and rabbits⁶ made thrombocytopenic by the injection of anti-platelet antibody. In our assay system, these stimulatory effects are due solely to the presence of thrombopoietin. We have extensively investigated this assay system^{6,26,27,33,35} and have demonstrated its specificity for thrombopoietin in three ways. First, except for thrombocytopenic plasma^{6,26,27,33} and very high levels of erythropoietin,³³ no other recombinant or purified cytokine demonstrated activity in this assay. The thrombocytopenic plasma samples used here did not contain elevated amounts of erythropoietin. Second, the active mole-

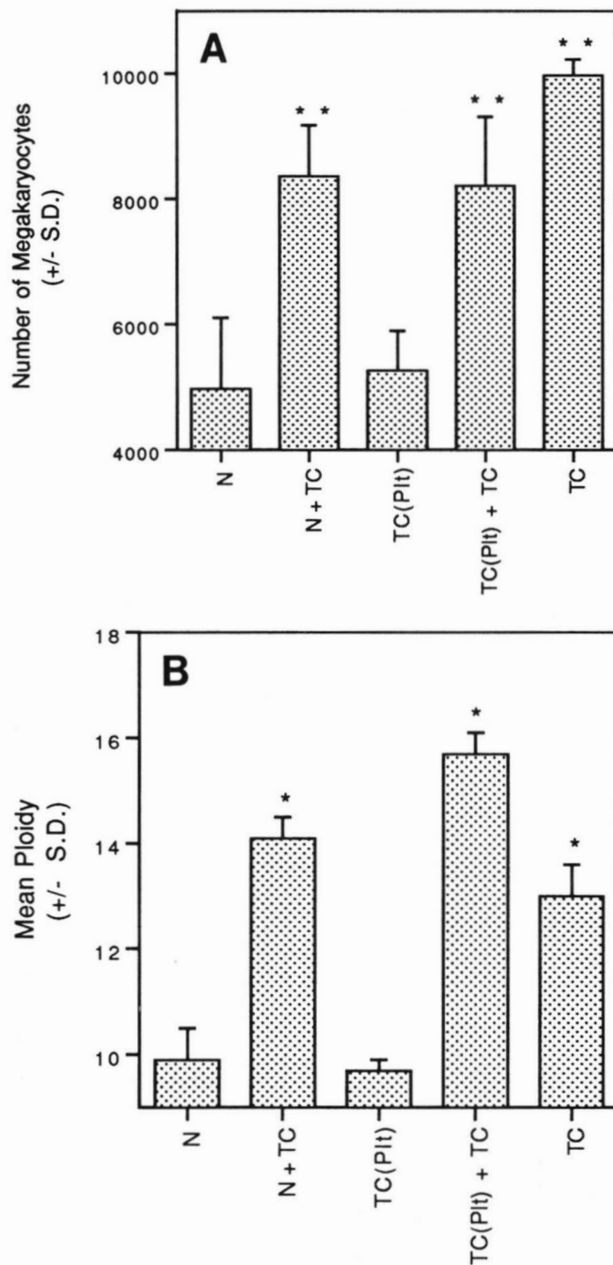


Fig 6. Platelets do not release any inhibitory substances during incubation with thrombocytopenic (TC) plasma. TC rabbit plasma was incubated with platelets at 20°C exactly as described in Table 4, and the platelets were removed by centrifugation to produce TC(Plt) plasma. Normal (N), TC, and TC(Plt) plasma were then added to bone marrow cultures at a final concentration of 15% and compared with cultures grown in the presence of a 1:1 mix of N and TC plasmas (N + TC) or of a 1:1 mix of TC(Plt) and TC plasmas (TC(Plt) + TC), at a final concentration of 30% (15% each). The number of megakaryocytes per culture (A) and the mean ploidy (B) were determined by flow cytometry. * $P < .05$; ** $P < .02$ compared with N or TC(Plt) plasmas.

cule in thrombocytopenic plasma has been purified to homogeneity by us and has an amino acid sequence identical to that of the c-Mpl ligand.²⁶ Third, after the addition of soluble c-Mpl receptor to thrombocytopenic samples (Fig 3), all of the stimulatory activity was eliminated.

We have previously demonstrated that platelet transfusion into rats prevented the expected increase in megakaryocyte ploidy if given within 3 hours of the onset of acute thrombocytopenia,²⁷ and Mazur et al⁶² have shown that platelet transfusion into thrombocytopenic humans rapidly decreased the elevated levels of megakaryocyte colony-stimulating activity. Here we have shown that transfusion of platelets into thrombocytopenic rabbits decreased the levels of thrombopoietin in vivo by 89%, and restoration of the platelet count to normal in vitro in samples of thrombocytopenic plasma decreased thrombopoietin levels by 95%. Both of these effects were due to the removal of thrombopoietin from plasma by the platelet [presumably by the thrombopoietin (c-Mpl) receptor] and were not due to the release of inhibitory substances from platelets. Three lines of evidence demonstrated the absence of any significant release of inhibitors from platelets. First, we have previously shown that TGF β 1 is the only platelet substance that inhibits our assay.³⁵ Even if platelets have released all their granule contents, all of their inhibitory effect is neutralized by the addition of antibody to TGF β .³⁵ Addition of this antibody in the experiments performed here was without effect. Second, if platelets had released any inhibitory substance, then incubation of platelets with normal plasma should have suppressed mean ploidy values below those seen with normal plasma alone. Such suppression was not observed in the experiments described in Table 4. Finally, if platelets had released any inhibitor into thrombocytopenic plasma, then this inhibitor should have been detected in the 1:1 mixing experiment. No evidence for any inhibitor was found in this experiment (Fig 6).

Thrombopoietin (c-Mpl ligand) has all the characteristics to be expected of the physiologically relevant mediator of megakaryocyte growth and platelet production. As shown here, thrombopoietin has low basal levels that change inversely and proportionally to changes in the platelet mass. Furthermore, our experiments demonstrate that the infusion of platelets into thrombocytopenic rabbits decreases the level of thrombopoietin with a time dependency that is also inversely proportional to the mass of the transfused platelets. These results extend and confirm our previous work with rabbits made thrombocytopenic by the injection of anti-platelet antibody. In those experiments, we also showed an inverse and proportional relationship between thrombopoietin levels and the platelet mass, a low basal level, and at least a 3-hour lag time before thrombopoietin levels responded to an acute change in platelet mass.⁶ Collectively, these results support the contention that thrombopoietin is the physiologically relevant mediator of the feedback loop between the circulating platelet mass and the bone marrow megakaryocytes.

The results of our current studies also suggest that we need to reconsider our concept of how elevated levels of thrombopoietin are produced. As shown in Table 4, when platelets were added to thrombocytopenic plasma in vitro, they removed the thrombopoietin by a temperature-dependent process. This effect is comparable with the way the terminally differentiated macrophage^{63,64} and neutrophil^{65,66} metabolize their respective regulatory factors, M-CSF and

G-CSF. We have long considered that any putative thrombopoietin would be regulated in the same intricate fashion as erythropoietin,¹⁻⁶ which does not directly interact with its terminally differentiated cell, the erythrocyte. It now appears to us more likely that the thrombopoietin mechanism may be more like that of M-CSF and G-CSF, in which the mass of the terminally differentiated cell regulates the circulating level of its respective regulatory factor with possibly less stringent control over the production rate of these factors. Indeed, it is conceivable that variations in platelet age or function may also alter the ability of the platelet to clear thrombopoietin from the circulation and, thus, modulate thrombopoietin levels and, ultimately, platelet production. Evidence supporting this model for the platelet has previously been suggested by the work of de Gabriele and Penington.⁶⁷ With purified thrombopoietin,²⁶ we hope to be better able to understand this regulatory mechanism.

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