*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 11104–11108, November 1994
Physiology

# The purification of megapoietin: A physiological regulator of megakaryocyte growth and platelet production

(blood platelet/hematopoietic growth factor/thrombocytopenia/busulfan)

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*Communicated by Laszlo Lorand, July 8, 1994 (received for review June 3, 1994)*

ABSTRACT **The circulating blood platelet is produced by the bone marrow megakaryocyte. In response to a decrease in the platelet count, megakaryocytes increase in number and ploidy. Although this feedback loop has long been thought to be mediated by a circulating hematopoietic factor, no such factor has been purified. Using a model of thrombocytopenia in sheep, we have identified an active substance called megapoietin, which stimulated an increase in the number and ploidy of megakaryocytes in bone marrow culture. Circulating levels of this factor could be quantified with this assay and were found to be inversely proportional to the platelet count of the sheep. Levels increased from <0.26 pM in normal sheep to 25–40 pM in thrombocytopenic sheep. From large amounts of thrombocytopenic sheep plasma we have purified a 31,200-Da protein and found that it retained the ability to stimulate both megakaryocyte number and ploidy *in vitro*. Injection of partially purified megapoietin into rats stimulated a 24% increase in megakaryocyte number and a 60% increase in mean ploidy as well as a 77% increase in the platelet count. Sheep platelets bound megapoietin and the amount of platelets required to eliminate half the activity *in vitro* was close to the amount associated with this same level of activity *in vivo*. We believe that megapoietin is the physiologically relevant mediator of megakaryocyte growth and platelet production. Moreover, our data suggest that the level of megapoietin is directly determined by the ability of platelets to remove megapoietin from the circulation.**

Megakaryocytes are unusual bone marrow cells, which are responsible for producing circulating blood platelets. Although comprising <0.25% of the bone marrow cells in most species, they have >10 times the volume of typical marrow cells (1, 2). In addition, megakaryocytes undergo a process known as endomitosis whereby they replicate their nuclei but fail to undergo cell division and thereby give rise to polyploid cells. In response to a decreased platelet count, the endomitotic rate increases (3, 4), higher ploidy megakaryocytes are formed, and the number of megakaryocytes may increase up to 3-fold (1). In contrast, in response to an elevated platelet count, the endomitotic rate decreases (3, 4), lower ploidy megakaryocytes are formed, and the number of megakaryocytes may decrease by 50% (1).

The physiological feedback mechanism by which the mass of circulating platelets regulates the endomitotic rate and number of bone marrow megakaryocytes is not known. A circulating thrombopoietic factor has long been assumed to be involved but to date no such factor has been purified. Moreover, none of the recombinant cytokines with thrombopoietic activity, such as interleukin 6 (5), is known to be involved in mediating this physiological feedback loop.

To characterize this putative thrombopoietic factor, we have analyzed the physiological relationship between the bone marrow megakaryocytes and the circulating platelets. We have found that the magnitude of the changes in the number and ploidy of megakaryocytes was inversely and proportionally related to the circulating platelet mass (3) and that megakaryocyte number and ploidy were therefore markers of this feedback loop *in vivo*. We next developed a bone marrow assay in which increases in the number and ploidy of megakaryocytes *in vitro* (6) were used to identify an active substance in thrombocytopenic plasma that we have named megapoietin (3, 7). Levels of megapoietin could be quantified with this assay and were inversely proportional to the platelet mass (3, 7). Once elevated in thrombocytopenic animals, the megapoietin level could be reduced to normal by transfusion of platelets (3).

These results suggested that megapoietin was an ideal candidate to be the thrombopoietic factor mediating the feedback loop between the circulating platelets and the bone marrow megakaryocytes. We now report the development of a sheep model of thrombocytopenia, which has allowed us to purify megapoietin from thrombocytopenic plasma and to elucidate the characteristics of the purified protein.

## MATERIALS AND METHODS

**Reagents, Animals, and Thrombocytopenic Plasma.** Busulfan was purchased from Sigma. Male Sprague–Dawley rats (200–250 g) were obtained from Charles River Breeding Laboratories. Male sheep (60–100 kg) were purchased from and maintained at East Acres Biologicals (Southbridge, MA).

Sheep were rendered thrombocytopenic by injecting subcutaneously a total of 900 mg per $m^2$ of body surface area of busulfan in two doses on days 0 and 3 as outlined by Kitchens and Weiss (8). Platelet and leukocyte (WBC) counts were monitored at intervals thereafter (3). In six sheep, 45 ml of blood was collected at various times into 5 ml of 3.8% citrate and prepared for megapoietin assay (9). During their platelet nadir, sheep were subjected to plasmapheresis of 2–3 units daily for 2–3 days and then sacrificed. The blood obtained during plasmapheresis and at sacrifice was anticoagulated with sodium citrate (final concentration, 0.38%), centrifuged twice at 3000 × *g* for 15 min to remove platelets and cell debris, and frozen at −20°C.

**Measurement of Megapoietin Activity.** The *in vitro* assay of megapoietin has been outlined in previous publications (3, 6, 7, 9). In brief, rat bone marrow cells were cultured for 3 days with defibrinogenated plasma or various dilutions of purified protein samples and then the number and ploidy of megakaryocytes were determined by flow cytometry. The mega-

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Abbreviation: WBC, leukocyte.

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poietin level of a sample was determined by the magnitude of the increased number or mean ploidy of megakaryocytes produced.

The *in vivo* assay for megapoietin was as follows: partially purified protein from normal or thrombocytopenic sheep plasma as well as control buffer (20 mM Tris·HCl, pH 8.6/150 mM NaCl) was filtered through a 0.22-$\mu$m filter and then injected subcutaneously twice daily into rats for 2 or 4 days. The injected material contained no endotoxin as judged by the *Limulus* assay (endotoxin kit, Sigma). We then obtained blood for cell counts as well as bone marrow for determination of the number and ploidy of megakaryocytes as described (3, 6, 7, 9).

**Megapoietin Purification.** All procedures were carried out at 4°C. The sheep plasma was thawed and fibrinogen was precipitated at a final concentration of 7.5% polyethylene glycol ($M_r$ 3350; J. T. Baker, Jackson, TN). The supernatant was then loaded onto a CM Sepharose (Pharmacia) column (35 × 30 cm) and the adsorbed protein was step-eluted with 300 mM NaCl/50 mM $NaH_2PO_4$, pH 6. The harvested protein was then reapplied to a CM Sepharose column (50 mm × 100 cm) at 50 mM NaCl/50 mM $NaH_2PO_4$, pH 6, and eluted with a linear NaCl gradient in the same buffer system. The fractions containing megapoietin activity were pooled, charged to a DEAE-Sepharose column (50 mm × 100 cm; Pharmacia), and eluted with a linear NaCl gradient in 20 mM Tris·HCl (pH 8.6). The fractions possessing megapoietin activity were pooled, concentrated, and applied to a ceramic hydroxylapatite column (19 mm × 10 cm; Bio-Rad) at 100 mM NaCl/10 mM $KH_2PO_4$, pH 6.8. The unadsorbed protein was reapplied to a ceramic hydroxylapatite column (7.8 mm × 10 cm; Bio-Rad) in 20 mM NaCl/10 mM $KH_2PO_4$, pH 6.8, and bound protein was step-eluted with 0.18 M $KH_2PO_4$ (pH 6.8). The fractions exhibiting megapoietin activity were concentrated with a 1-ml Mono S column (Pharmacia), charged to a 1-ml wheat germ agglutinin-Sepharose column (Pharmacia), and step-eluted with chitodiose (4 mg/ml in 0.125 M NaCl/50 mM $NaH_2PO_4$, pH 7.5). The material with megapoietin activity was bound to a 1-ml heparin Sepharose column (Pharmacia) and eluted with a linear NaCl gradient in 25 mM $NaH_2PO_4$ (pH 7.5). The fractions with megapoietin activity were pooled and applied to a Tosohaas TMS-250 column (7.8 mm × 7.5 cm; The Nest Group, Southborough, MA), and bound proteins were eluted with a gradient (10%/90% to 70%/30%) of acetonitrile/isopropanol (3:1)/trifluoroacetic acid (0.1%). The material containing megapoietin activity was pooled and then filtered on a Tosohaas TSK G-3000 column (7.5 mm × 60 cm; The Nest Group, Southborough, MA) in 50% trifluoroacetic acid (0.1%)/50% acetonitrile/isopropanol (3:1).

**Examination of Megapoietin Preparations at Different Stages of Purification.** Protein preparations were labeled with $^{125}I$ and then analyzed by SDS/PAGE under reducing and nonreducing conditions as described by Laemmli (10). The levels of megapoietin activity present in the various electrophoretic bands were assessed by slicing the nonreduced SDS gels, eluting the protein into Hanks' balanced salt solution containing 15% fetal bovine serum, and assaying for megapoietin *in vitro* as outlined above. The overall recovery of megapoietin activity from nonreduced SDS/PAGE was 13–22%.

TSK purified protein was digested with endoproteinase Lys-C (Boehringer Mannheim) at 37°C for 24 h, the peptides were then separated on a $C_4$ column (Vydac, no. 214TP52; Hesperia, CA), and the amino acid sequence of isolated peptides was obtained with the model 477A protein sequencer with on-line model 120 phenylthiohydantoin amino acid analyzer (Applied Biosystems).

## RESULTS

**Megapoietin Appeared in the Circulation of Thrombocytopenic Busulfan-Treated Sheep.** After administration of busulfan to 106 sheep, the normal platelet count (508,000 ± 86,000 platelets per $\mu$l) increased slightly by day 3 but by day 7 began to decline to its nadir (111,000 ± 63,000 platelets per $\mu$l) on days 16 and 17, a decrease to 23% ± 3% of normal (Fig. 1). The WBC count also began to decline from normal (5919 ± 1179 WBC per $\mu$l) after day 7 and reached its nadir (2007 ± 1157 WBC per $\mu$l) on days 12–16, a drop to 34% ± 4% of normal. The hematocrit did not decline from normal (47 ± 6) until day 10, when it began to fall to its nadir (41 ± 9) on day 17, a decline to 90% ± 9% of normal.

In six sheep, plasma samples were taken at frequent intervals for determination of megapoietin activity. Not until after day 7 did plasma samples stimulate the number and mean ploidy of megakaryocytes that grew in bone marrow culture (Fig. 1). For all six sheep, the magnitude of this stimulation was inversely proportional to the platelet count and peaked at the time the platelet count was at its nadir. Comparing the effect of plasma samples taken prior to busulfan treatment to those taken at the platelet nadir for all six sheep, the number of megakaryocytes produced in culture increased from 3203 ± 1656 to 6430 ± 1955, an average increase to 202% ± 97%. The mean ploidy increased from 10.8 ± 1.9 to 16.9 ± 2.8, an average increase to 158% ± 13%.

**The Purification of Megapoietin from Thrombocytopenic Plasma.** Using the bone marrow culture assay, we developed an 11-step isolation procedure to obtain megapoietin in a virtually homogenous state from thrombocytopenic sheep plasma. From 20 liters of plasma, we isolated ≈1.5 $\mu$g of megapoietin with a final purification of ≈9 millionfold and a final recovery of ≈1.6%.

Because megapoietin activity was destroyed by reducing agents, two aliquots of the final TSK product, one iodinated, were run unreduced in adjacent lanes on an SDS gel. The lane containing the labeled protein was subjected to autoradiography. The lane containing unlabeled protein was sliced and the eluted protein was tested for megapoietin activity. As shown in Fig. 2, four labeled protein bands were seen. The 52,100-Da minor species contained no megapoietin activity, was absent when labeled by the chloramine-T method, and was not present in most other preparations. Megapoietin activity (Fig. 2) was associated with three proteins of 41,600, 35,700, and 27,800 Da (unreduced). These three proteins contained 7%, 20%, and 73%, respectively, of the total protein radioactivity and 5%, 16%, and 79%, respectively, of the megapoietin activity. In more recent preparations, the

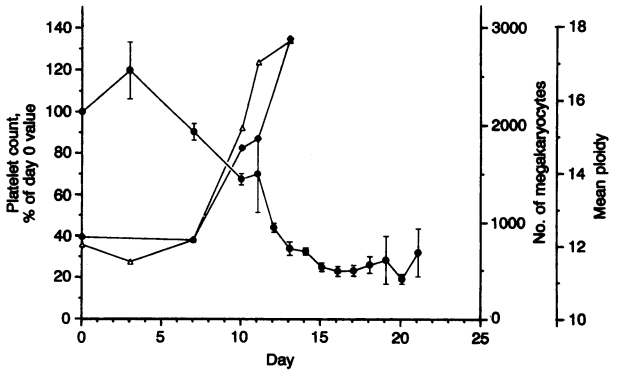


FIG. 1. After administration of busulfan to 106 sheep, platelet counts (±SD) (●) as well as the stimulatory effect of plasma samples on the number (◆) and mean ploidy (△) of megakaryocytes were determined at frequent intervals. All values for mean ploidy and number of megakaryocytes after day 7 differ from pretreatment values ($P < 0.05$).

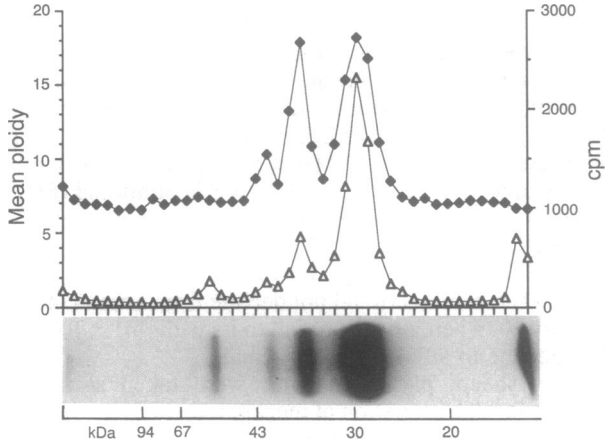


FIG. 2. Two aliquots of the final TSK purified protein, one iodinated, were electrophoresed (unreduced) on SDS gels. The iodinated lane was subjected to autoradiography and also quantified with the PhosphorImager (△). The other lane was eluted and megapoietin activity was measured by assessing the effect on mean ploidy (◆).

41,600-Da protein has not been seen and when the other two proteins were electrophoresed under reduced conditions they ran as a 36,700-Da minor protein and a 31,200-Da major protein.

The TSK purified protein was digested with endoproteinase Lys-C and the resulting peptides were sequenced. Three peptides with unique sequences were obtained: KDPSAIFLNFQQLLRGK, KRAPPAXAVPGSISPLLTLNK, and KLPXRTSGLLETXSSVSARTTGFGLP.

***In Vitro* Characteristics of Purified Megapoietin.** The TSK purified protein stimulated a visually apparent (Fig. 3*A*) increase in the number (from 10,076 ± 1121 to 15,505 ± 1577) and mean ploidy (from 10.4 ± 0.5 to 18.8 ± 1.6) of megakaryocytes. Individual ploidy classes showed commensurate changes with the 64*N* megakaryocytes, for example, increasing from 0.21% ± 0.08% to 4.34% ± 1.00%.

The linear increase in mean ploidy in our bioassay was related to a logarithmic increase in megapoietin concentration (Fig. 3*B*) with a limit of detection of 1.67 pM. Over the same megapoietin concentrations, the number of megakaryocytes in culture showed an identical dose–response relationship and increased from 9245 ± 205 to 13,319 ± 1470. When thrombocytopenic plasma was subsequently compared in culture with purified megapoietin, we estimated that it contained megapoietin at ≈25–40 pM and that normal plasma contained <0.25 pM.

***In Vivo* Effects of Megapoietin.** When partially purified (310-fold through the DEAE-Sepharose step) megapoietin was injected into recipient rats (Fig. 4*A*), the mean ploidy increased over time to a level comparable to that seen in rats made severely thrombocytopenic by injection of anti-platelet antibody (3). Changes in individual ploidy classes were apparent after 2 days and by 4 days (Fig. 4*B*) were comparable to those seen in severely thrombocytopenic rats. After 4 days, but not after 2 days, the percentage of megakaryocytes in the bone marrow increased from 0.128% ± 0.005% to 0.159% ± 0.023% ($P < 0.05$).

Although unchanged after 2 days of injection, after 4 days the platelet count (Fig. 4*C*) had increased from 1.295 ± 0.127 × $10^6$ to 2.295 ± 0.265 × $10^6$ platelets per μl, an increase of 77%. There was no change in the WBC count (9.9 ± 5.9 × $10^3$ vs. 9.6 ± 4.2 × $10^3$ WBC per μl) or hematocrit (40 ± 1 vs. 40 ± 3) in these animals. Rats simultaneously injected with buffer showed no differences from simultaneously harvested, uninjected rats or from 356 normal rats previously analyzed. In addition, we performed an identical purification from normal sheep plasma (which contained <1% of the megapoietin activity seen with thrombocytopenic plasma at the same stage of purification) and injected twice daily the same amount of protein or buffer into rats for 4 days. There was no effect on megakaryocyte ploidy (19.6 ± 1.3 vs. 18.5 ± 1.9), individual ploidy classes, or the platelet count (1.401 ± 0.101 × $10^6$ vs. 1.383 ± 0.064 × $10^6$ platelets per μl).

Another group of rats was injected for 4 days with partially purified megapoietin (Fig. 4*D*). The platelet count began to increase on day 5, peaked at day 7, and then returned to normal. Again, there was no effect on the WBC count or hematocrit in the animals.

**Sheep Platelets Bind to and Remove Megapoietin.** When thrombocytopenic sheep plasma or plasma reconstituted with purified megapoietin was incubated with an amount of sheep platelets equal to that which would have been present in these plasmas *in vivo* in the absence of thrombocytopenia, all of the megapoietin was removed (Fig. 5*A*). Because incubation of platelets with normal sheep plasma did not produce inhibition of the cultures (Fig. 5*A*), this effect was not simply due to the release of some inhibitory substance from platelets. In other experiments, equivalent amounts of

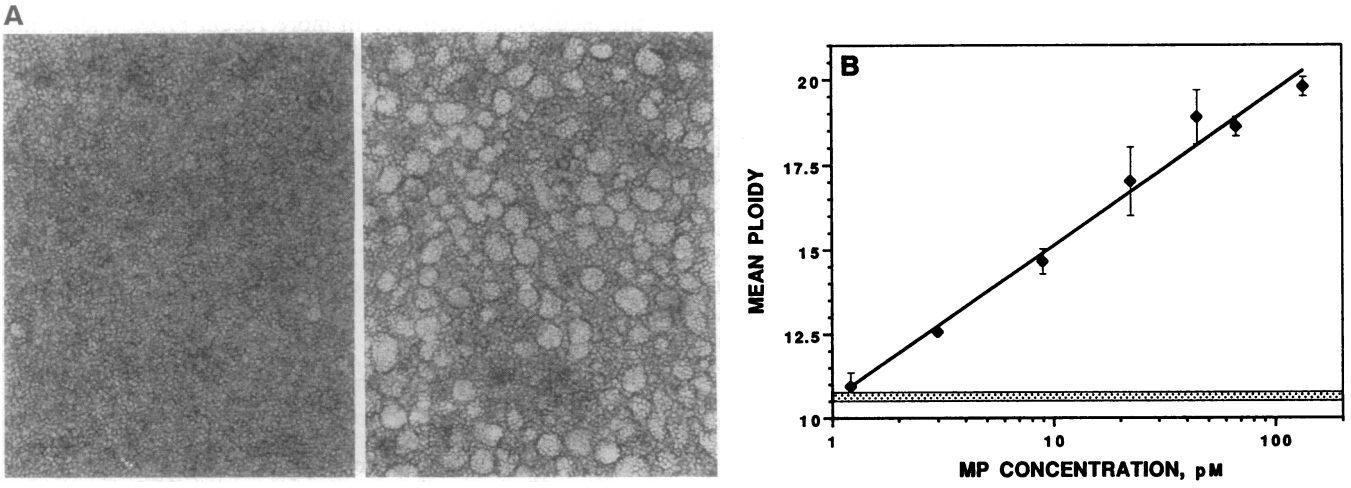


FIG. 3. Purified megapoietin stimulated megakaryocyte growth. (*A*) Phase-contrast photomicrograph of unstimulated (*Left*) and megapoietin-stimulated (50 pM) (*Right*) bone marrow cultures. (×6.63.) (*B*) Specimens (0.3 ml) containing the indicated amount of megapoietin (MP) were added to culture and the effect on mean ploidy (±SD) was determined. Stippled bar indicates mean ploidy (±SD) of unstimulated bone marrow cultures. All mean ploidy values >11.50 differ from unstimulated cultures by $P < 0.05$.

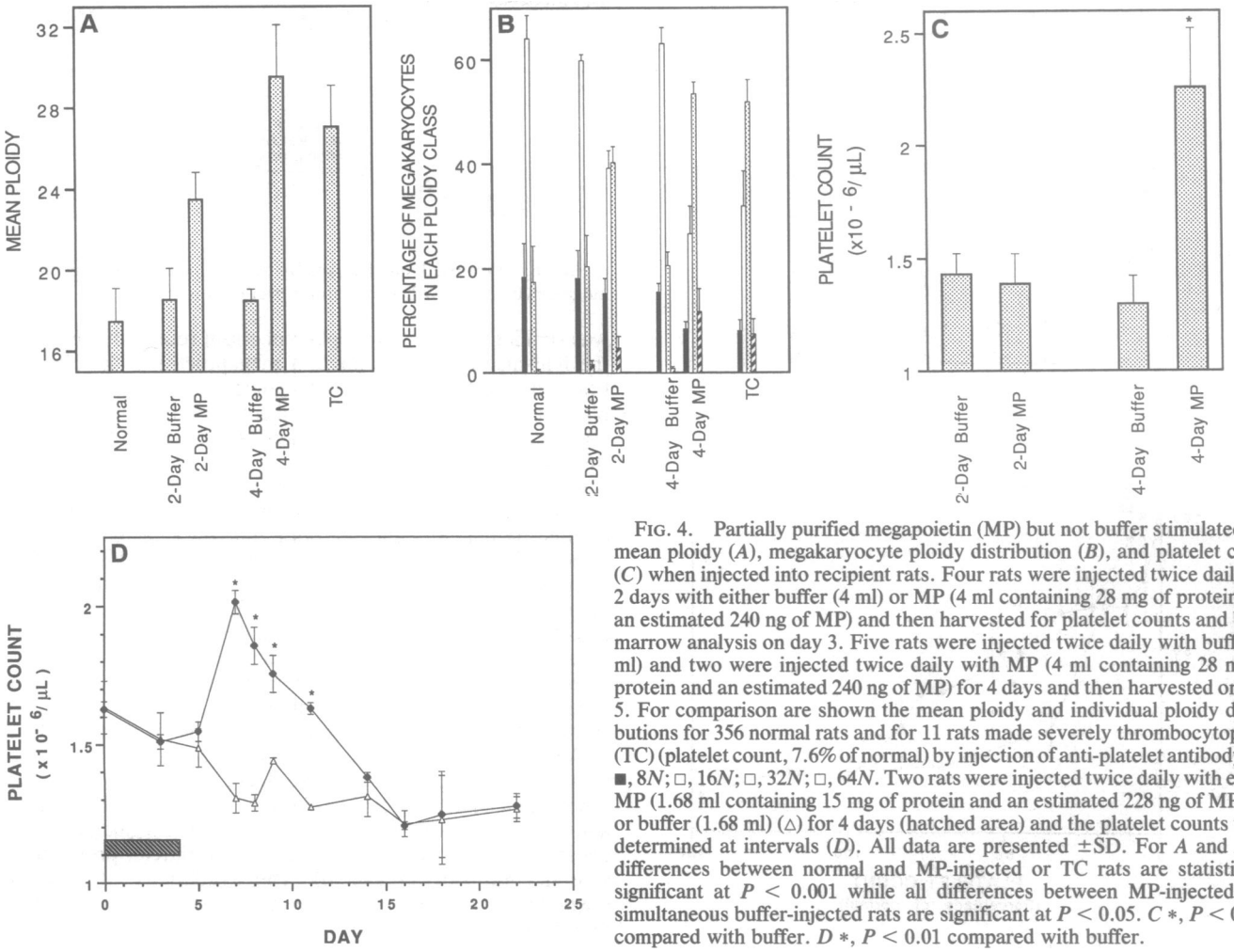


FIG. 4. Partially purified megapoietin (MP) but not buffer stimulated the mean ploidy (*A*), megakaryocyte ploidy distribution (*B*), and platelet count (*C*) when injected into recipient rats. Four rats were injected twice daily for 2 days with either buffer (4 ml) or MP (4 ml containing 28 mg of protein and an estimated 240 ng of MP) and then harvested for platelet counts and bone marrow analysis on day 3. Five rats were injected twice daily with buffer (4 ml) and two were injected twice daily with MP (4 ml containing 28 mg of protein and an estimated 240 ng of MP) for 4 days and then harvested on day 5. For comparison are shown the mean ploidy and individual ploidy distributions for 356 normal rats and for 11 rats made severely thrombocytopenic (TC) (platelet count, 7.6% of normal) by injection of anti-platelet antibody (3). ■, 8*N*; □, 16*N*; □, 32*N*; □, 64*N*. Two rats were injected twice daily with either MP (1.68 ml containing 15 mg of protein and an estimated 228 ng of MP) (◆) or buffer (1.68 ml) (△) for 4 days (hatched area) and the platelet counts were determined at intervals (*D*). All data are presented ±SD. For *A* and *B* all differences between normal and MP-injected or TC rats are statistically significant at $P < 0.001$ while all differences between MP-injected and simultaneous buffer-injected rats are significant at $P < 0.05$. *C* *, $P < 0.001$ compared with buffer. *D* *, $P < 0.01$ compared with buffer.

rat platelets, but not purified rat erythrocytes or lymphocytes, also removed all the megapoietin.

When normal sheep plasma reconstituted with purified megapoietin was exposed to various amounts of sheep platelets so as to approximate *in vitro* different levels of thrombocytopenia, an increasing extent of megapoietin removal was observed as a function of platelet addition (Fig. 5*B*). At a platelet count of 24% of normal, half of the apparent megapoietin activity was removed as judged by mean ploidy. This relationship should be compared with the six thrombocytopenic sheep described in Fig. 1 in which the relative levels of megapoietin increased *in vivo* over time as the platelet count declined (Fig. 5*B*). At a platelet count of 43% of normal, the apparent megapoietin activity as determined by mean ploidy was half-maximal, close to the platelet count that produced half-maximal removal *in vitro*.

## DISCUSSION

The identification and purification of the physiologically relevant thrombopoietic factor that mediates platelet production by megakaryocytes have been elusive. We have found that thrombocytopenic plasma contains an active substance called megapoietin, which stimulates the number and ploidy of megakaryocytes that grow in bone marrow culture (3). Using this assay, we have determined the time course of megapoietin's appearance in the circulation and shown that its activity has all the characteristics expected of a physiological regulator. We have now created a sheep model of thrombocytopenia, which has provided adequate quantities of thrombocytopenic plasma to permit purification of what we believe is the principle humoral regulator of megakaryocyte endomitosis and platelet production.

Busulfan produced severe thrombocytopenia after 12–15 days with only modest neutropenia and anemia. Plasma from these thrombocytopenic sheep contained megapoietin, since it stimulated an increase in the number and ploidy of megakaryocytes in our bone marrow culture system. As the platelet count declined, the extent of stimulation of the mean ploidy and the number of megakaryocytes in our assay increased proportionally. This reflected an average increase in megapoietin concentration of >100-fold, from 0.25 to 25 pM.

Megapoietin has been purified to homogeneity and is a glycoprotein composed of two species, a major protein of 31,200 Da and a minor protein of 36,700 Da, both with the same specific activity. Like the initially described megapoietin activity (3, 7, 9), the purified megapoietin protein stimulated both the number and endomitotic rate of megakaryocytes *in vitro*. Although sufficient quantities of the final purified product were not available for injection into animals, partially purified protein from thrombocytopenic but not from normal sheep plasma stimulated an increase in the megakaryocyte number, ploidy, and platelet count when injected into rats. Megakaryocyte ploidy increased by day 3 and was maximal by day 5. The number of megakaryocytes increased by day 5 and was accompanied by an increase in platelet count, which became maximal on day 7. The extent of these megakaryocyte changes in the megapoietin-treated rats was indistinguishable from what we have found in rat bone marrow after induction of severe thrombocytopenia.

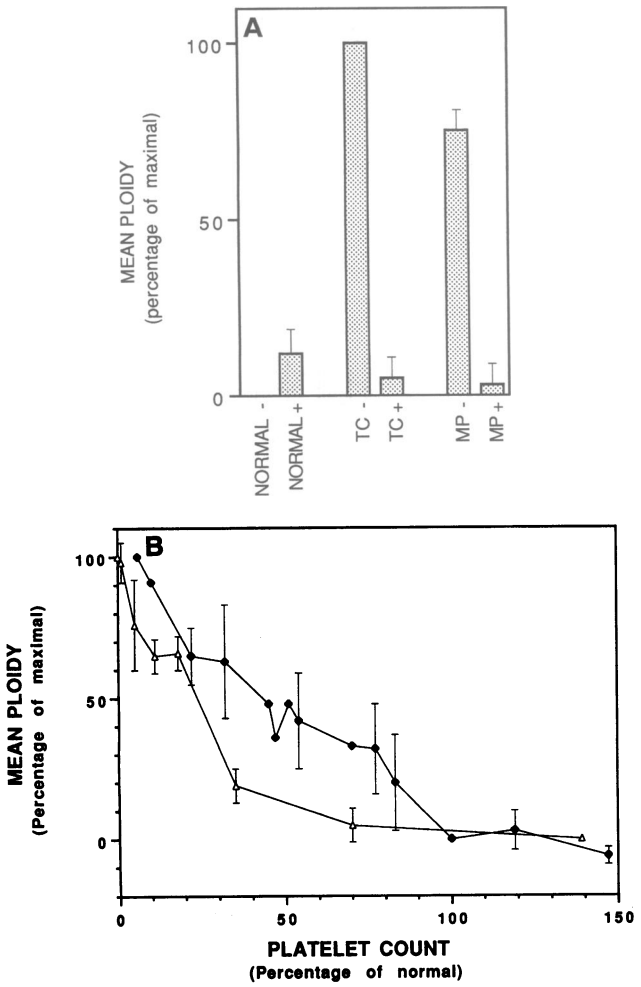


FIG. 5. Sheep platelets removed megapoietin (MP) from plasma. (*A*) Sheep platelets (+) prepared as described (7) or an equivalent amount of buffer (−) were incubated for 1 h at room temperature with normal and thrombocytopenic (TC) sheep plasma as well as with normal sheep plasma into which purified MP had been added (to a level ≈75% that found in the TC plasma). After centrifugation twice at 3000 × *g* for 15 min, the supernatants were assayed for residual MP activity. Relative changes in mean ploidy (±SD) were measured, with 100% being defined as that in TC plasma and with 0% being defined as that in normal plasma. (*B*) MP was again added to normal plasma to approximate the level in TC plasma. Various amounts of platelets were added and the *in vitro* reduction in mean ploidy (±SD) was determined (△) as in *A*. Relative changes in mean ploidy (±SD) for plasma from six TC sheep (◆) were also plotted as a function of the platelet count *in vivo*; 100% was defined as the largest mean ploidy value for any of the six sheep and 0% was defined as the mean ploidy of day 0 samples.

Although we cannot exclude a platelet sensing mechanism analogous to the way the kidney monitors the erythrocyte mass (11), we have suggested (7) that a simpler system may regulate platelet production. A major component of the increase in megapoietin levels during thrombocytopenia may be directly due to the absence of platelets and their associated ability to remove megapoietin from the circulation. To test this hypothesis, we have restored the platelet count to normal in samples of thrombocytopenic plasma and in plasma reconstituted to thrombocytopenic levels with purified megapoietin and found *in vitro* that all megapoietin was eliminated. We then explored the effect of varying the amount of this *in vitro* platelet transfusion and showed that the platelet count required *in vitro* to eliminate half of the megapoietin activity

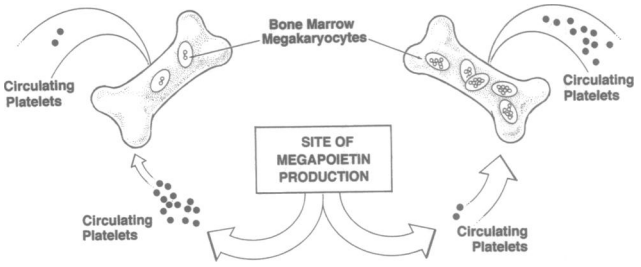


FIG. 6. Proposed mechanism by which megapoietin regulates megakaryocyte growth and platelet production.

was strikingly close to that platelet count required *in vivo* for the half-maximal appearance of megapoietin in the circulation. The small differences between these *in vitro* and *in vivo* platelet counts can be accounted for by taking into consideration some effect of the older age or diminished function of the platelets *in vivo*.

Our data show that megapoietin is the physiologically relevant protein that mediates the feedback loop between the circulating platelets and the bone marrow megakaryocytes. Although the site of its production is not yet known, the effective circulating level of megapoietin is regulated by the ambient platelet mass. With a decline in platelet mass (or possibly a decline in the platelet binding of megapoietin due to increased age or decreased function of platelets), circulating levels of megapoietin increase and stimulate an increase in megakaryocyte number and ploidy, which then increase the production of platelets (see the right side of Fig. 6). With an increase in platelet mass (or possibly an increase in binding of megapoietin due to decreased age or increased function of platelets) circulating megapoietin levels decline and the number and ploidy of bone marrow megakaryocytes decrease with a consequent decrease in platelet production (see the left side of Fig. 6). The availability of purified megapoietin should now allow us to explore further the implications of this model.

This paper was submitted several weeks before the identification of the human c-mpl ligand was reported (12). The amino acid sequences of the three megapoietin peptides described above are >70% identical to residues 122–138, 153–173, and 173–198 in the c-mpl ligand sequence. This sequence homology and the virtually identical range of biological activities demonstrate that megapoietin is the same as the c-mpl ligand.

This work was supported in part by National Institutes of Health Grant HL 39753.

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