

Detection of Thrombopoietic Activity in Plasma by Stimulation of Suppressed Thrombopoiesis

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ABSTRACT Rabbits in which thrombocytosis had been produced by five daily transfusions of platelet concentrates had suppressed endogenous thrombopoiesis, as reflected by decreased incorporation of selenomethionine-⁷⁵Se (⁷⁵SeM) into the circulating platelet mass. Rabbits in which endogenous thrombopoiesis had been suppressed by transfusion-induced thrombocytosis were used to detect thrombopoietic activity in rabbit plasma. Thrombopoietic activity was demonstrated in the plasma of both normal and thrombocytopenic donor rabbits. A dose response relationship was observed between the incorporation of ⁷⁵SeM into platelets and the dose of plasma administered. Infusion of 20–150 ml of plasma from thrombocytopenic donors increased the incorporation of ⁷⁵SeM into platelets from 52 to 107% above control values. A dose response effect also was seen after infusion of normal plasma, but normal plasma produced less effect than comparable doses of plasma from thrombocytopenic donors.

Rabbits with transfusion-induced thrombocytosis appear to be more sensitive assay animals for the detection of thrombopoietic activity than animals with normal platelet counts. Changes in the rate of appearance and levels of ⁷⁵SeM may primarily indicate changes in platelet protein or platelet size and are apparently more sensitive indicators of the state of thrombopoiesis than are alterations in the numbers of circulating platelets. The results strongly support the concept of a humoral agent, i.e. thrombopoietin, that acts on megakaryocytes to regulate platelet production.

INTRODUCTION

Evidence for a humoral mechanism for the control of thrombopoiesis has been reviewed recently (2). Evatt

This work was presented in part at the annual meeting of the American Society of Hematology, December, 1969 (1).

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and Levin recently reported that plasma from thrombocytopenic rabbits increased the rate of appearance and levels of selenomethionine-⁷⁵Se (⁷⁵SeM)¹ in the platelets of normal recipients, but normal plasma had no effect (2). Moreover, transfusion-induced thrombocytosis decreased the incorporation of ⁷⁵SeM into platelets, suggesting that endogenous thrombopoiesis had been suppressed.

In the present studies, rabbits in which endogenous thrombopoiesis had been suppressed by transfusion-induced thrombocytosis were used to detect thrombopoiesis activity in rabbit plasma. Stimulation of suppressed thrombopoiesis, as reflected by an increase in the incorporation of ⁷⁵SeM into platelets, was observed after the administration of plasma from thrombocytopenic and normal donors. The results show that rabbits with transfusion-induced thrombocytosis provide a sensitive bioassay for the detection of thrombopoietic activity in plasma. These studies support the concept of a humoral agent that acts on megakaryocytes or their precursors to regulate platelet production.

METHODS

New Zealand white rabbits (1.94 ± 0.20 kg) from a single local supplier were used; their mean platelet count was 628,000/mm³ ± 176,000/mm³ (1 sd). Thrombopoietic activity in plasma was determined by measuring its effect on the per cent incorporation of ⁷⁵SeM into platelets (2). Incorporation of ⁷⁵SeM into the circulating platelet mass was measured serially, after the intravenous injection of 15 μCi of ⁷⁵SeM, as described by Evatt and Levin (2). The technique (2) involves preparation of platelet-rich plasma (PRP) by centrifugation of whole blood. The volume and platelet count of the PRP were measured. Platelets were sedimented by centrifugation of PRP and resuspended in 5 ml of THAM buffer² using a Vortex mixer.³ The resuspended

¹ Selenomethionine-⁷⁵Se, Sethotope, E. R. Squibb & Sons, New Brunswick, N. J. Specific activity, 200–250 mCi/mg.

² 72.8 g of THAM (tris-hydroxymethyl)amino-methane), 18 g of NaCl, 240 ml of 0.2 N HCl, and 40 ml of 4.8% EDTA in 4 liters of distilled H₂O. Fisher Scientific Company, Springfield, N. J.

³ Vortex-Genie, Scientific Industries, Inc., New York.

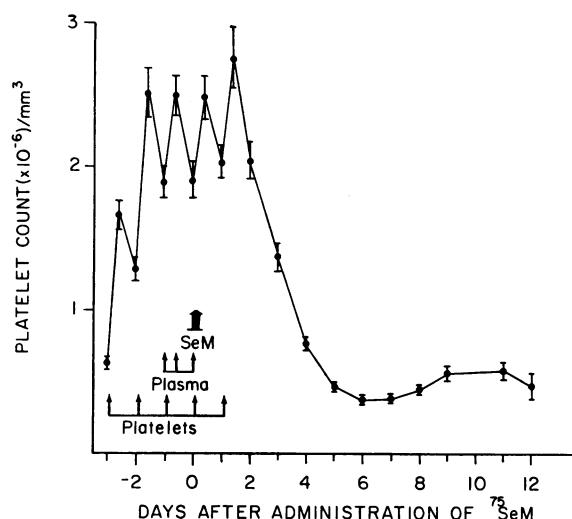


FIGURE 1 Experimental model and effect of hypertransfusion of platelets upon peripheral platelet counts. Platelet counts of 19 rabbits that received five daily transfusions of platelet concentrates at the times indicated are plotted. The mean ± 1 SE is shown. ^{75}SeM was administered 6 hr after the fourth transfusion of platelet concentrates, on day zero. Plasma, when administered, was given in three equally divided doses at the times indicated.

platelets were poured onto a 1.2μ Millipore filter.⁴ The platelets which were trapped on the filter were immediately washed with 30 ml of THAM buffer. Radioactivity of the washed platelets and of 1 ml of the platelet-free plasma was determined. The contamination of the filter by radioactive plasma proteins was calculated for each sample and used to derive the true (corrected) cpm/platelet (2). To allow for differences in circulating platelet mass and to permit comparison between different experimental groups, incorporation of ^{75}SeM was calculated as the per cent administered dose in circulating platelets as follows: (cpm/platelet (corrected) \times total circulating platelets⁵ $\times 100$) / (total cpm ^{75}SeM injected).

Assay animals. Thrombocytosis (platelet counts 2–4 times normal) was produced and maintained by five daily injections of platelet concentrates. All blood components were collected in siliconized glassware.⁶ Blood was obtained by intracardiac puncture from normal, unanesthetized animals using acid-citrate dextrose⁷ as anticoagulant (1 part ACD:9 parts whole blood). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 g for 30 min at room temperature. Additional platelets were harvested from blood cells that had been sedimented during preparation of PRP by adding an equal volume of THAM buffer to these cells, and centrifuging at 160 g for 20 min. The platelet-rich plasma and platelet-rich buffer were pooled, and buf-

fered EDTA⁸ was added (1 part EDTA:20 parts platelet-rich solution). The platelets then were sedimented by centrifugation at 1100 g for 20 min at room temperature, resuspended in 5–12 ml of THAM buffer, and immediately injected into recipient rabbits. Platelets from 10 donor animals were required daily for each assay animal.

Donor animals. Rabbits were rendered thrombocytopenic (platelet counts less than 35,000/mm³) by intravenous injection of 0.5–1.0 ml of guinea pig antirabbit platelet serum (2) and were bled 3–4 hr later. Blood was obtained from unanesthetized animals by intracardiac puncture, using siliconized glassware and acid-citrate dextrose as anticoagulant. Platelet-free plasma (PFP), containing less than 5000 platelets/mm³, was prepared by centrifugation of blood for 40 min at 1100 g at room temperature and was pooled and stored at 4°C for periods of up to 48 hr.

Normal plasma was prepared in the same way from normal rabbits that had not been given platelet antiserum. Plasma from animals with transfusion-induced thrombocytosis was obtained from rabbits that were prepared in the same manner as assay animals. Blood was obtained 24 hr after the last of five daily transfusions of platelet concentrates. PFP from normal, thrombocytopenic, or thrombocytotic animals was administered via the marginal ear vein to rabbits with transfusion-induced thrombocytosis; and its effect on the incorporation into platelets of subsequently administered ^{75}SeM was measured as an index of thrombopoietic activity of the infused plasma (2).

RESULTS

Effects of hypertransfusion of platelets upon peripheral platelet counts. Platelet levels 2–4 times normal were achieved in rabbits which received platelet concentrates for 5 days (Fig. 1). After discontinuation of platelet transfusions, the platelet counts of the recipients gradually decreased with an apparent platelet survival time of 4 days. Platelet counts reached subnormal levels approximately 5–6 days after the last transfusion, and subsequently returned to normal. The administration of 20–150 ml of plasma from normal or thrombocytopenic donors to rabbits with thrombocytosis did not significantly alter the levels of platelets achieved after platelet transfusion, the rate of disappearance of transfused platelets, or the subsequent appearance of relative thrombocytopenia.

Fig. 1 also shows the design of the experimental model. ^{75}SeM was administered on the 4th day of platelet transfusions (day zero). When plasma was given, it was injected intravenously in three equally divided doses during the 30 hr period before injection of ^{75}SeM .

Effects of hypertransfusion of platelets upon incorporation of ^{75}SeM . Six rabbits received daily transfusions of platelet concentrates for 5 days. No plasma was given. The rate and levels of ^{75}SeM incorporated into platelets were significantly decreased in rabbits with thrombocytosis ($P < 0.0005$ at all times measured) when compared to normal animals (Fig. 2). Maximum levels of

⁸ 0.054 M EDTA (2 g of ethylenediaminetetraacetate/100 ml of distilled H₂O) buffered to pH 7.5 with THAM.

⁴ Millipore filter No. 72AW P02500. Millipore Corporation, Bedford, Mass.

⁵ Whole blood platelet count/ml $\times 0.06 \times$ body weight (kg).

⁶ G. E. silicone S.C.-87 Dri-Film, General Electric Company, Schenectady, N. Y.

⁷ ACD (acid-citrate dextrose) solution, USP, formula A, Fenwal Laboratories, Division of Travenol Laboratories Inc., Morton Grove, Ill.

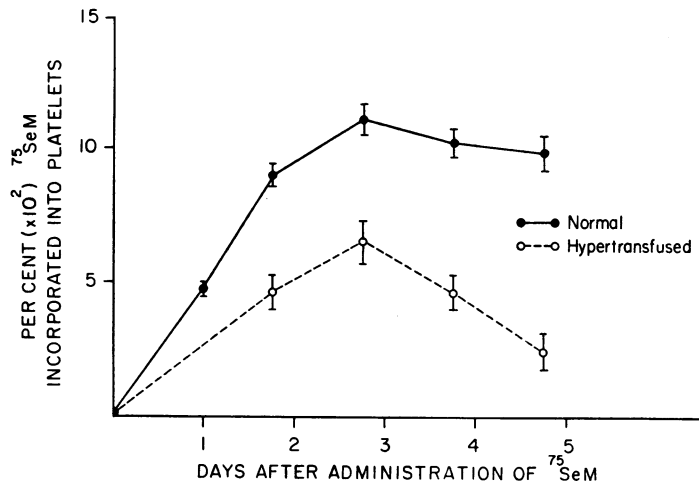


FIGURE 2 Effect of hypertransfusion of platelets upon incorporation of ^{75}SeM into platelets. Thrombocytosis was produced and maintained in six rabbits by five daily transfusions of platelet concentrates. ^{75}SeM was administered after the fourth transfusion of platelets, on day zero (see Fig. 1). 40 normal rabbits did not receive platelets. The mean ± 1 SE is shown. Hypertransfusion of platelets produced a significant decrease in incorporation of ^{75}SeM into platelets ($P < 0.0005$ at all points).

^{75}SeM in platelets occurred 3 days after the administration of the isotope in both groups of rabbits, at which time the amount of ^{75}SeM incorporated in the platelets of rabbits with thrombocytosis was only 59% of that attained in normal rabbits. These results confirmed and extended previous observations in this laboratory (2) and indicated that endogenous thrombopoiesis had been suppressed by platelet hypertransfusion.

Effects of plasma from thrombocytopenic donors, normal donors, or donors with thrombocytosis upon incorporation of ^{75}SeM into the platelets of hypertransfused rabbits. Total volumes of 20, 37.5, 75, or 150 ml of plasma from thrombocytopenic donors were administered intravenously in three equal doses to one, two, two, and four rabbits, respectively, in which thrombocytosis had been produced by transfusions of platelet concentrates (Figs. 1 and 3). ^{75}SeM was administered 6 hr after the last infusion of plasma. The incorporation of ^{75}SeM into the platelets of rabbits that had received plasma from thrombocytopenic donors was significantly greater than in rabbits that had received only transfusions of platelets (Fig. 3 and Table I). A dose response relationship between the volume of infused plasma and the change in maximum uptake of ^{75}SeM into platelets was demonstrated (Table I). Rabbits that received 75 or 150 ml of plasma incorporated significantly more ^{75}SeM into platelets than did rabbits that received 20 or 37.5 ml ($P < 0.01$ at 2 and 4 days, and $P < 0.05$ at 3 days). If expressed as per cent change from hypertransfused controls, the increases after the administra-

tion of 20–150 ml of plasma from thrombocytopenic donors were linear and ranged from 52 to 107%.

Total volumes of 37.5, 75, or 150 ml of plasma from normal donors were administered to three, seven, and two rabbits, respectively, with transfusion-induced thrombocytosis. 75 or 150 ml of plasma from normal donors

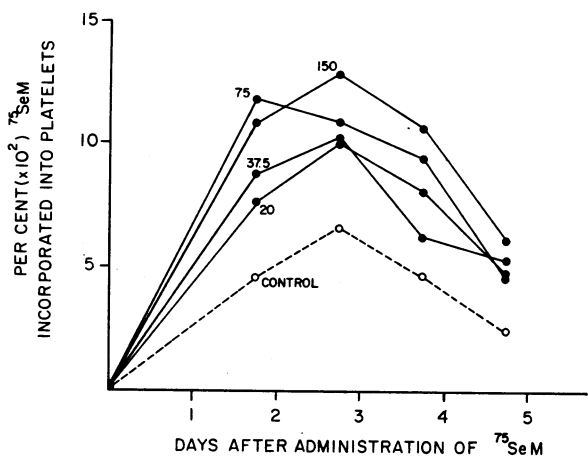


FIGURE 3 Effect of plasma from thrombocytopenic donors upon incorporation of ^{75}SeM into the platelets of hypertransfused rabbits. Total volumes of 20, 37.5, 75, or 150 ml were administered in three equally divided doses to one, two, two, and four rabbits, respectively (\bullet — \bullet). ^{75}SeM was administered 6 hr after the last dose of plasma. Six control rabbits received transfusions of platelets but no plasma (\circ — \circ). The means are shown. There was a significant increase in incorporation of ^{75}SeM into the platelets of rabbits that received plasma (see Table I).

TABLE I
Effect of Plasma from Thrombocytopenic Donors, Normal Donors, or Donors with Thrombocytosis upon Maximum Incorporation of ^{75}SeM into Platelets of Recipients with Suppressed Thrombopoiesis

Dose <i>ml</i>	Thrombocytopenic donors			Normal donors			Thrombocytotic donors		
	Maximum incorporation of $^{75}\text{SeM}^*$	P^\dagger	Per cent increase §	Maximum incorporation of $^{75}\text{SeM}^*$	P^\dagger	Per cent increase §	Maximum incorporation of $^{75}\text{SeM}^*$	P^\dagger	Per cent increase §
150	13.62 \pm 1.01	<0.0005	107	12.72 \pm 0.60	<0.0005	93			
75	11.74 \pm 1.05	<0.005	78	10.01 \pm 0.91	<0.01	52	5.54 \pm 0.08	NS	0
37.5	10.47 \pm 0.05	<0.0025	59	5.83 \pm 1.47	NS	0			
20	9.99		52						
0 (Control)	6.58 \pm 0.76			6.58 \pm 0.76			6.58 \pm 0.76		

* Per cent ($\times 10^3$). The mean ± 1 SE is shown.

† Compares hypertransfused rabbits that received plasma with hypertransfused controls that did not receive plasma.

§ Indicates per cent increase from hypertransfused control rabbits.

also increased the incorporation of ^{75}SeM in recipients with transfusion-induced thrombocytosis (Table I), but 37.5 ml of plasma was without detectable effect. The maximum responses to normal plasma also were dose related and significantly differed from each other at these three doses ($P < 0.01$ – 0.025). Plasma from normal donors had less effect than plasma from thrombocytopenic donors, and the responses to the 37.5 ml dose were significantly different ($P < 0.025$).

75 ml of plasma from donors with transfusion-induced thrombocytosis were administered to two rabbits with transfusion-induced thrombocytosis. Maximum incorporation of ^{75}SeM was $5.61 \times 10^{-2}\%$ and $5.46 \times 10^{-2}\%$, respectively. This was not significantly different from hypertransfused control animals (Table I).

DISCUSSION

Endogenous thrombopoiesis was suppressed in rabbits in which thrombocytosis had been produced by five daily infusions of platelet concentrates, as indicated by the diminished incorporation of ^{75}SeM into the circulating platelet mass. Induction of thrombocytosis for 5 days did not completely suppress platelet production since levels of ^{75}SeM in platelets were low but not absent. Lower levels of ^{75}SeM had been observed when platelets were transfused for 7 days (2). In studies utilizing other isotopes (3–7), platelet production had not been suppressed uniformly with transfusion-induced thrombocytosis.

Rabbits which had suppressed endogenous thrombopoiesis were used to assay for thrombopoietic activity in plasma. Thrombopoietic activity was detected in the plasma of both normal and thrombocytopenic donors. A dose response relationship was observed between the levels of ^{75}SeM in circulating platelets and the dose of plasma from normal or thrombocytopenic donors that

was administered. However, less thrombopoietic activity was detected in the plasma of normal donors than in the plasma of thrombocytopenic donors. This difference was significant at the lower dose levels tested. At the higher doses tested, this difference decreased in a manner similar to that observed in assays for erythropoietin (8, 9). No thrombopoietic-stimulating activity was detected in the plasma of rabbits with transfusion-induced thrombocytosis at the dose tested.

The volumes of plasma used in these experiments did not significantly affect the numbers of circulating platelets or the rate of disappearance of transfused platelets. Therefore, the difference in incorporation of ^{75}SeM in platelets could not be accounted for by differences in platelet counts. The relative thrombocytopenia which followed the period of thrombocytosis, previously noted by others (5, 10, 11), was not eliminated when thrombopoiesis was stimulated by normal plasma or plasma from thrombocytopenic donors.

Passive transfer of small amounts of residual platelet antiserum in donor plasma might have stimulated thrombopoiesis if it had produced a shortened platelet life span and initial thrombocytopenia in recipients. Mattison has shown that platelet counts do not change after transfusions into normal dogs of large amounts of plasma (250 ml) obtained from thrombocytopenic dogs 15 min after injection of platelet antiserum (12). In the present studies, plasma from thrombocytopenic donors was obtained 3–4 hr after the injection of platelet antiserum. Furthermore, platelet counts were maintained at 2–4 times normal levels, and a shortened platelet life span would not be expected to stimulate thrombopoiesis until platelet counts returned to normal values. Normal counts were not reached until 4 days after administration of the isotope, whereas alterations in levels of ^{75}SeM were present by the 2nd day. Therefore, it is highly unlikely that the stimulatory effect of plasma

from thrombocytopenic donors was due to the passive transfer of small amounts of platelet antiserum.

Rabbits with transfusion-induced thrombocytosis provided a more sensitive assay for thrombopoietic activity than animals with normal platelet counts. When normal rabbits were used as assay animals, thrombopoietic activity was not detected after injection of normal plasma, and a dose response relationship to the administration of plasma from thrombocytopenic donors was not observed (2). In a preliminary communication, Harker reported the detection of thrombopoietic activity in rat plasma using hypertransfused rats as assay animals; however, a dose response effect was not described (13).

The data are compatible with an increased release of platelets and/or an increase in platelet size. Stimulation of thrombopoiesis was not associated with increased platelet counts in normal rabbits (2). Elevation of platelet counts secondary to stimulation of thrombopoiesis might have been undetected in the present studies because of the daily infusion of platelet concentrates with concomitant fluctuations in platelet counts. Young platelets have been reported to be larger and more dense than old platelets and presumably contain more protein (14-16). Stimulation of thrombopoiesis may be reflected by an increase in platelet size (17). Changes in the rate of appearance and levels of ^{54}SeM may primarily indicate changes in platelet protein and are apparently more sensitive indicators of the state of thrombopoiesis than are alterations in the numbers of circulating platelets.

Previous studies have shown that acute thrombocytopenia produces an increase in megakaryocyte size, number, and the rate of maturation (6, 18-20); and Ebbe, Stohlman, Donovan, and Overcash (6) and Odell, Jackson, Friday, and Charsha (20) have suggested that the feedback mechanism that regulates platelet levels is operative at the level of megakaryocytic precursor cells. Our observation that plasma from thrombocytopenic donors produced a greater increase in levels of ^{54}SeM in platelets than equivalent volumes of plasma from normal donors is compatible with a feedback mechanism by which circulating platelets affect the rate of thrombopoiesis. The data strongly support the concept of a humoral agent, i.e. thrombopoietin, that acts on megakaryocytes to regulate platelet production. The site of production, chemical nature, and mode of action of thrombopoietin(s) remain unknown; along with the location and nature of the mechanism that senses platelet levels or total platelet mass.

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