# Demonstration of a Circulating Factor Regulating Blood Platelet Production using $^{35}$S-Sulfate in Rats and Mice[1] (34957)

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Evidence supporting the concept of regulation of blood platelet production through a circulating factor, thrombopoietin has been presented by a number of investigators (1–4). Generally, the injection of massive doses of homologous plasma or serum obtained from animals depleted of platelets by bleeding, exchange transfusions of platelet-free blood, X-irradiation, or injection of anti-platelet serum (APS) produced a significant rise in the platelet count 4 to 5 days after injection into normal recipients. However, a method analogous to the measurement of radioiron incorporation into reticulocytes would have offered a more specific means of assaying platelet production. Platelet counts did not necessarily measure changes in rates of thrombopoiesis since the level of circulating platelets depends on the rate of their destruction as well as production. The release of platelets from sites of sequestration into the circulation could also have occurred.

It has been shown (5) that $^{35}$S-sulfate was permanently incorporated into the developing megakaryocyte in the bone marrow, and the newly formed platelets were thus labeled. Since the platelets already in the circulation did not permanently incorporate the isotope (5), radiosulfate has been used as a specific method for measuring platelet formation (6, 7). In this communication the $^{35}$S-sulfate label was used to demonstrate the presence of thrombopoietin in the plasma of platelet-depleted rats and mice.

*Materials and Methods.* Weanling and adult male Sprague-Dawley rats of a specific pathogen-free strain (CFE) and adult female CF no. 1 mice (Carworth, Inc., New City, N.Y.) were used. Thrombopoietically active plasma was obtained from rats sacrificed 5–18 hr after injection of a rabbit antirat platelet serum (APS), which was prepared as described (1). The donors were bled from the abdominal aorta into siliconized syringes containing 0.15 vol of acid citrate dextrose solution, formula A (ACD) per vol of blood. The blood and plasma were kept at 5° at all times except when boiled or frozen. In some experiments the plasma from normal and from APS-treated rats was acidified to pH 4.5 or 5.5 with 1 *N* HCl and heated in a boiling water bath for 10 to 15 min. The boiled plasma was immediately cooled in an ice bath, and after centrifugation the supernate was adjusted to pH 7.2–7.4. In the other experiments, plasma from APS-treated animals and control rats injected with normal rabbit serum was allowed to stand overnight in the refrigerator with 1/20 vol of goat antirabbit gamma globulin antiserum (GAGGA). Precipitated proteins were removed by sedimentation.

One-third of the boiled or GAGGA-treated plasma was immediately injected ip into assay rats. The remainder was frozen and injected into these animals on the 2 following days, and on the fourth day 1 $\mu$Ci of sodium $^{35}$S-sulfate/g of body wt was injected ip. The animals were exsanguinated and the platelets were separated on the sixth day by a modification of Odell *et al.* (7). Platelet suspensions were washed twice and were counted in a Beckman model LS 250 liquid scintillation counter using 0.5 ml of NCS reagent (Amersham-Searle, Des Plaines, Ill.) and 15 ml of a dioxane scintillator. Quenching was corrected by the external standard

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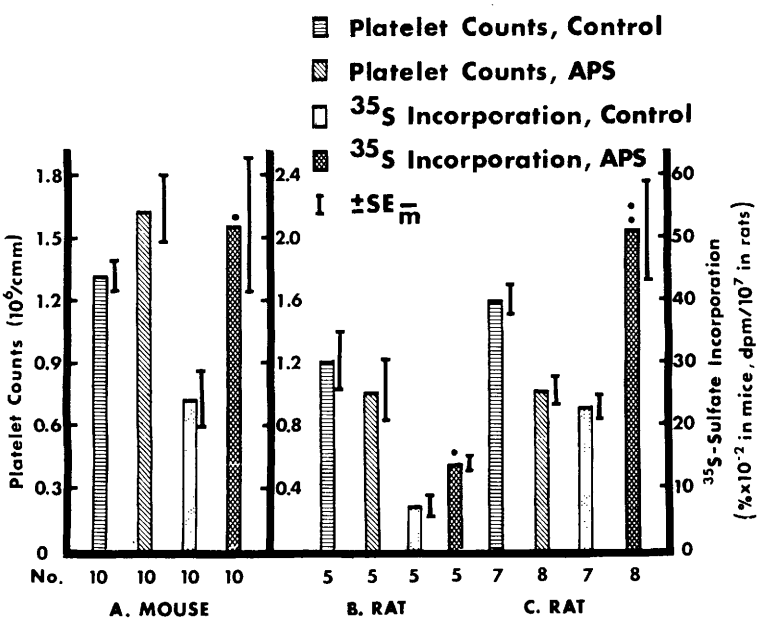


FIG. 1. Platelet counts and $^{35}$S-sulfate incorporation into platelets following antiplatelet serum (APS) injections in adult mice and rats. The radioisotope was administered 2 days (A and B) or 1 day (C) after the APS, and the animals were exsanguinated 48 hr later. • Significantly greater than corresponding control value ($p<.05$); ⁝ ($p<.01$).

channels ratio method.

Adult female mice were injected with 0.015 units of *Clostridium perfringens* neuraminidase (Worthington Biochemical Corp., Freehold, N. J.) and were bled 40 hr later from the heart into syringes containing 0.30 to 0.50 vol of ACD/vol of blood. The plasma was separated by centrifugation in the cold and was immediately injected into assay mice. The animals were given twice daily ip injections of plasma or saline for 2 days, 75 $\mu$Ci of $Na_2{}^{35}SO_4$ on the third day, and were sacrificed on the fifth day. The mice were bled from the freely flowing tail vein for a platelet count, and were then bled from the heart into siliconized syringes containing 1.0 ml of 1.5% disodium ethylenediaminetetraacetate (EDTA). The platelets were separated by a modification of the method previously used with the rats (7). The platelet-rich plasma was transferred to graduated plastic centrifuge tubes, a platelet count was taken (8), and the volume was recorded. The platelets were separated from this plasma using 24-mm diam Millipore filters (pore size: 0.22 $\mu$), and washed 3 times with saline on a 6-place manifold. The filters were placed in a counting vial and counted with 15 ml of a toluene scintillator solution. The Millipore filters allowed complete recovery of the very small volume of platelets obtained in the mouse. Percentage incorporation of $^{35}$S-sulfate into rat and mouse platelets was calculated as described (6). The blood volume was assumed to be 6% of the body weight (6).

*Results.* Adult mice given 0.02 ml of the antirat platelet serum showed a 115% rise in sulfate incorporation into platelets when the $^{35}$S was injected 2 days following the administration of APS (Fig. 1A). Adult rats given 0.2 ml of APS and 1 $\mu$Ci of $^{35}$S-sulfate/g of body weight, 2 (Fig. 1B) or 1 (Fig. 1C) days following the APS, showed a 93 and 130% rise, respectively, in radiosulfate incorporation into rat platelets.

The precipitation and heat denaturation of residual platelet antibody were confirmed by the following experiments. Three ml of APS were added to 9 ml of GAGGA, allowed to stand overnight in the refrigerator, and then centrifuged. The supernate was injected into three normal rats with no reduction of the

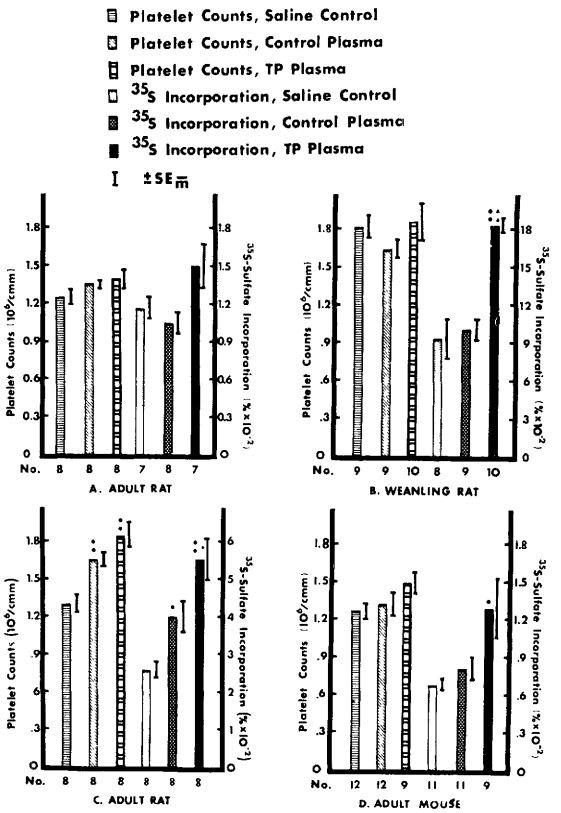


FIG. 2. Thrombopoietin (TP) assays in rats and mice. Rats were injected ip with plasma or saline on three consecutive days, radiosulfate was injected ip on day 4, and the animals were sacrificed on day 6. Mice were given twice daily ip injections of plasma or saline for 2 days, sodium $^{35}$S-sulfate on day 3, and sacrificed on day 5. (A) TP plasma was from antiplatelet serum (APS) treated rats that was adsorbed with goat antirabbit gamma globulin antiserum (B and C) boiled plasma from APS injected rats; (D) plasma prom neuraminidase injected mice. • Significantly greater than corresponding saline control value ($p<.05$); ⁝ ($p<.01$). ▲ Significantly greater than corresponding normal plasma control value ($p<.05$); double triangle ($p<.01$).

platelet levels. Five ml of APS were diluted with 54 ml of normal rat plasma. The pH was adjusted to 4.5 and the mixture was heated in a boiling water bath for 15 min. The cooled plasma was sedimented, the supernate was adjusted to pH 7.2–7.4, and it was injected into five adult rats so that each rat received five times the normal platelet lowering dose. Platelet levels were unchanged.

Twenty-one ml of plasma from APS-treated rats were adsorbed against the GAGGA. The injection of this material into young adult assay rats caused a 49% rise in platelet sulfate incorporation when compared with control GAGGA-treated plasma. There was also a 34% rise in sulfate incorporation when compared with the saline-injected controls (Fig. 2A). The platelet levels were raised slightly over the two control groups.

Ten ml of boiled plasma from APS-treated donors caused 93 and 82% rises in platelet $^{35}$S-sulfate incorporation of weanling rats when compared with saline-injected and boiled plasma-injected recipients, respectively (Fig. 2B). Platelet counts were essentially unchanged. In an experiment carried out on adult rats, (Fig. 2C), 10 ml of boiled normal plasma caused a 56% rise in platelet sulfate uptake when compared to the saline controls and a highly significant 26% rise in platelet counts. Significantly higher activity than that found in the control plasma was detected in

boiled plasma from APS-injected animals. Sulfate incorporation in these recipients was 116% higher than in the saline-injected group, and 39% higher than the control plasma-injected animals. Platelet levels were also higher in the APS group, 42% more than the saline controls.

Sulfate uptake into circulating platelets of mice injected with homologous plasma from neuraminidase-treated donors was significantly increased by 90% over the saline-injected controls (Fig. 2D). There was also a 58% increase in sulfate incorporation in these animals when compared to mice injected with normal plasma.

*Discussion.* These studies have demonstrated that platelet incorporation of $^{35}$S-sulfate was approximately doubled after experimental thrombocytopenia caused by a single injection of APS. These data are in agreement with those of Harker (6) who reported a 3-fold rise in platelet uptake of $^{35}$S-sulfate following 4 days of daily thrombocytopheresis.

The injection of very large doses of homologous plasma or serum obtained from thrombocytopenic donors into normal recipients has been reported to have caused significant increases in platelet levels 4 to 5 days later (1–4). Evatt and Levin (9) recently studied platelet production in rabbits by measuring the incorporation of $^{75}$Se-selenomethionine. Untreated plasma (75–225 ml) from donors injected with APS 2–4 hr before sacrifice caused a significant 39% increase in $^{75}$Se incorporation into platelets of normal recipients when compared with rabbits given normal plasma.

In the experiments reported here, plasma from donors rendered thrombocytopenic by the injection of platelet antiserum or neuraminidase caused in several instances a rise in platelet levels. However, the measurement of the incorporation of a radioisotope such as $^{35}$S-sulfate into platelets seemed to offer a more sensitive and also a more specific method for assaying thrombopoietic substances. The rise in sulfate incorporation was far greater than was the rise in platelet levels. In the experiment which used weanling rats as assay animals, thrombopoietic plasma caused a significant increase in thrombopoiesis as measured with radiosulfate but no increase in platelet numbers. This may have been due to the rapid growth of the animal. The increased sensitivity of the radioisotope incorporation method may be due to the measurement of rates of thrombopoiesis as distinguished from measurements of the concentration of the circulating platelet pool. Furthermore, the newly formed younger platelets have been reported to be larger than older platelets (10, 11). Therefore, we would expect a greater proportion of larger platelets in the recently stimulated animal, with a greater increase in total platelet mass than increase in number of circulating platelets. This might have been reflected by the proportionately greater increase in the platelet incorporation of sulfate label, than the rise in platelet counts.

Homologous plasma was used for these assays of thrombopoietic activity since foreign proteins have been shown to cause a rise in platelet counts (12). Since plasma from donors rendered thrombocytopenic by the injection of APS was assayed for thrombopoietin by injection into other rats, there was always the possibility that the large doses of plasma needed for these studies might contain residual APS. Platelet antiserum, by initially destroying the recipients' platelets, was shown to actively stimulate thrombopoiesis and sulfate incorporation. Two procedures have been used to remove residual platelet antibody. The first method removed residual antibody by adsorption of test plasma with GAGGA. In order to account for possible platelet altering effects of the small amounts of foreign serum used, control plasma obtained from rats injected with normal rabbit serum was similarly treated with the GAGGA. The second method for inactivating residual antiplatelet serum was to use boiled plasma. The precipitation and also the heat denaturation of platelet antibodies were confirmed by preliminary experiments. The boiled plasma studies confirmed reports that thrombopoietin was heat stable, and implied that thrombopoietin might be a glycoprotein, which often can

withstand boiling.

To avoid the problem of residual platelet antibody, neuraminidase has been used. This enzyme rapidly lowers mouse platelet levels (13) although it will not affect rat platelets except in very large doses. Since the enzyme has been reported to disappear from the mouse circulation in 12–24 hr as determined by assays of enzyme activity (13), the mice were sacrificed approximately 40 hr after injection.

In order to avoid the possibility that the transferred plasma might contain other platelet-lowering agents, control plasma was compared with a saline control.

*Summary.* Platelet counts and $^{35}$S-sulfate incorporation were studied in rats and mice following injection of saline or homologous plasma from normal or platelet-depleted animals. Donor rats were treated with antiplatelet serum 5–18 hr before sarcifice, and donor mice were injected with neuraminidase 40 hr before exsanguination. To prevent the injection of platelet antibody, the simulated and control plasmas were either boiled at acid pH for 10–15 min, or precipitated with goat antirabbit gamma globulin antiserum (GAGGA). The sulfate incorporation into platelets of animals given plasma from depleted donors was significantly increased over control values to levels as high as 116%. These data demonstrated the existence of thrombopoietin control over platelet production. The radiosulfate incorporation method appeared to be more sensitive than assays making use of platelet counts alone.

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