Thrombopoietin Production in Mice Treated with Acetylsalicylic Acid (43303A)

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Abstract. **Recent work revealed that mice in which platelet function was inhibited by acetylsalicylic acid (ASA) treatment showed evidence of increased platelet production. It was proposed that poorly functioning platelets gave rise to elevated thrombocytopoiesis by causing the release and action of thrombopoietin. However, direct evidence is lacking. Therefore, in the work reported here, plasma from mice treated with ASA was injected into normal recipient mice in an attempt to document the existence of the humoral factor. Compared with control mice given normal plasma, the injection of mice with plasma from ASA-treated mice resulted in increased thrombocytopoiesis, as evidenced by significant increases in the percentage of ^{35}S incorporation into platelets, larger platelet size, and elevated megakaryocyte precursor cells (the small acetylcholinesterase-positive cell). For a positive control, additional mice were treated with plasma from animals made thrombocytopenic by an injection of antiplatelet serum. These mice also showed significant increases in thrombocytopoiesis. The results support the hypothesis that platelet production in ASA-treated mice is elevated by release and action of thrombopoietin.** [P.S.E.B.M. 1991, Vol 198]

Platelet production is believed to be regulated by a feedback mechanism that senses a deficiency in the number, mass, or function of circulating platelets to release a humoral factor (thrombopoietin) into the circulation that causes an increase in thrombocytopoiesis (1). Although several examples exist in the literature (1–3) showing that thrombocytopenia leads to thrombopoietin release and action, little data exist indicating that platelet function plays a role in regulating thrombocytopoiesis. In support of the hypothesis that poor platelet function leads to release of thrombopoietin, Jackson and Edwards (4) showed that vincristine, which inhibits platelet function, resulted in increased thrombocytopoiesis. Also, acetylsalicylic acid (ASA or aspirin) has been shown to inhibit platelet function by interfering with thromboxane A_2 production *in vitro* (5), and, in an *in vivo* study, treatment of mice with ASA was shown to increase the percentage of megakaryocyte precursor cells, the small acetylcholinesterase-positive (SAChE+) cells (6). In this work, poor platelet function was linked to increases in blood platelet production, and the results suggested that poorly functioning platelets caused the release and action of thrombopoietin, but direct evidence of production of a humoral factor was not presented.

Therefore, in the present study, donor mice were injected with aspirin and their plasma was collected and injected into recipient mice for the determination of the existence of a humoral platelet-stimulating factor. The percentage of ^{35}S ($\%^{35}S$) incorporation into platelets, platelet size, and percentage of SAChE+ cells were measured in recipient mice. In addition, plasma from mice injected with rabbit antimouse platelet sera (RAMPS) was used as a positive control, and other mice injected with plasma from normal mice served as negative controls. The results showed a significant increase in the platelet production of mice injected with plasma from both ASA-treated mice and RAMPS-treated mice. These data support the theory that thrombopoietin is released in response to poorly functioning platelets and the humoral factor stimulates megakaryocytes to increase platelet production.

Materials and Methods

Animals. Male C3H/HENHSD mice (Harlan Sprague Dawley, Indianapolis, IN) were used through-

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out this study. Mice used as plasma donors weighed about 27 g, whereas mice used for thrombopoietin assays were slightly smaller, averaging about 24 g each.

Donor Mice Treatment. Donor mice were given a single intraperitoneal injection of 5 mg of ASA (Sigma Chemical Co., St. Louis, MO) in a 0.086 *M* NaOH buffer (pH 7.4), as described previously (6). After anesthesia with sodium pentobarbital, the mice were bled by cardiac puncture into heparin-coated syringes at 22 hr after ASA injection. Platelet counts were taken from the retroorbital sinus using 2.08-mm diameter tubing cut at an angle and determined using phase contrast microscopy. Platelet counts for aspirin-treated mice averaged $1.096 \times 10^6 \pm 0.033$ SE/mm^3, compared with $0.944 \times 10^6 \pm 0.027$ SE for untreated normal mice. The blood was kept on ice and the plasma was collected at 4°C and stored at −76°C until injected into recipient mice. Also, samples of blood were collected from several of these mice for aggregation studies with 0.1 mg of collagen in 50 μl (Sigma) to monitor platelet function of donor mice. Mice given injections of aspirin showed a reduced maximum percentage of aggregation (58.7%), as compared with untreated control mice (69.1%). This finding agrees with the data of Sullivan and McDonald (6), which showed a significant reduction in aggregation of platelets at 8 hr after injections of aspirin and a reduced, but not significant, level of aggregation after 24 hr.

Other donor mice were given a single intraperitoneal injection of 0.05 ml of RAMPS in 0.5 ml of saline which was absorbed with mouse red blood cells, as described previously (7). Four hours after RAMPS injection, platelet counts were taken. The animals were bled as described above, and the blood from 56 animals having a platelet count of below 50,000/mm^3 (average 14,000/mm^3) was pooled. The blood was placed on ice and the plasma was collected at 4°C and stored at −76°C. For control plasma, normal mice were bled using the same procedure.

Recipient Mice Treatment. Mice were given a total of four sc injections of plasma from donor mice (0.5 ml of plasma diluted into 1.5 ml of saline) which were administered in the mornings, and afternoons of Days 0 and 1 and followed by an intravenous injection of 30 μCi of $Na_2{}^{35}SO_4$ on Day 2. Mice were sacrificed at 24 hr after isotope injection for measurements of platelet sizes and $\%^{35}S$ incorporation into platelets.

For determination of $\%^{35}S$ incorporation into platelets (8) and platelet size (9), approximately 0.5 ml of blood was drawn from pentobarbital-anesthetized recipient mice via cardiac puncture into syringes containing 1.0 ml of 3.8% sodium citrate. Platelet-rich plasma was obtained by centrifuging the blood at 160*g* for 4.5 min. After an aliquot of platelet-rich plasma was removed for platelet size determination, the platelet-rich plasma was remixed with the sedimented red blood cells and an additional amount of 1% EDTA was added to improve platelet recovery rates. The platelets were harvested by centrifugation at 450*g* for 4.5 min and washed free of red blood cells and plasma, and platelet radioactivity was counted. Calculation of the incorporation of ^{35}S into platelets was determined by the method of McDonald (8).

Platelet size measurements were determined from an aliquot of platelet-rich plasma diluted in isotonic-buffered saline, as described previously (9), using an Electrozone Celloscope (Particle Data, Inc., Elmhurst, IL) with a log converter. The instrument settings were gain of 4 and current of 6. Platelet concentrations were adjusted to less than 20,000/100 μl to reduce errors due to coincidence. The instrument was calibrated using 2.02-μm diameter latex particles. The data were analyzed using a Particle Data computer program and expressed as the arithmetic mean (9).

For determination of SAChE+ cells, other recipient mice were given a single intraperitoneal injection (0.5 ml) of plasma from ASA-treated, RAMPS-treated, or normal mice. Eight hours after plasma injections, mice were bled and marrow was taken from the femurs. Bone marrow smears were made and processed as described previously (10). The "direct coloring" method of Karnovsky and Roots (11) was used to stain the cells. After rinsing in 0.1 *M* sodium phosphate (pH 6.0) for 1 min, the cells were stained in thiocholine stain for 3 hr and fixed in 100% methanol for 10 min, followed by 50% methanol fixation for 30 sec. Cells staining positive for AChE and measuring less than 13 μm in diameter were counted as SAChE+ cells (12) and expressed as a percentage of the total number of cells staining positive for AChE.

Statistical analysis of data was determined by use of Student's *t* test.

Results

Figure 1 shows the results of two experiments in which the incorporation of $Na_2{}^{35}SO_4$ into newly formed platelets was measured after injection of plasma obtained from normal mice, mice injected with ASA, and mice injected with RAMPS. As shown, plasma from ASA-treated mice gave a highly significant ($P < 0.0005$) increase in $\%^{35}S$ incorporation into platelets, as compared with other mice given normal plasma. In one of the two experiments, mice were given plasma from RAMPS-treated mice as a positive control, which was shown previously (2) to contain thrombopoietin. Mice injected with plasma from RAMPS-treated mice also showed a highly significant ($P < 0.0005$) increase in $\%^{35}S$ incorporation into platelets. Although these mice showed elevated ^{35}S incorporation values, platelet counts of these recipient mice were not increased at the time of sacrifice ($0.975 \times 10^6 \pm 0.033$ SE/mm^3 for control mice, $1.007 \times 10^6 \pm 0.026$ SE/mm^3 for ASA-

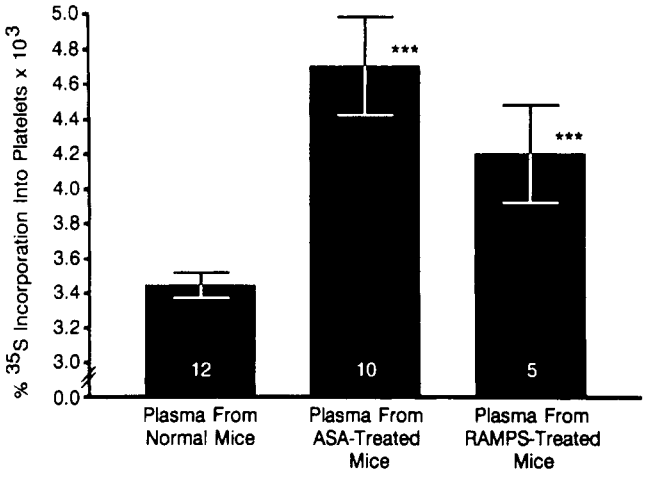

Figure 1. Percentage of ^{35}S incorporation into platelets of mice after injection of plasma from normal mice, plasma from mice injected with 5.0 mg ASA, and plasma from mice injected with RAMPS. Vertical bars represent SE and the numbers on the bars are the number of treated mice per group. Data were significantly higher than values for control mice; *** $P < 0.0005$.

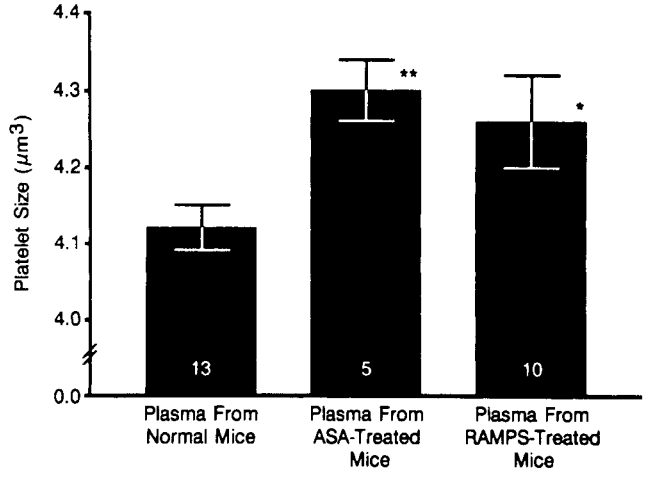

Figure 2. Platelet size determinations of recipient mice after injection of plasma from normal donor mice, plasma from mice injected with 5.0 mg ASA, and plasma from mice injected with RAMPS. Vertical bars represent SE and the numbers on the bars are the number of treated mice per group. Data were significantly higher than values for control mice; * $P < 0.05$, ** $P < 0.005$.

treated mice, and $0.928 \times 10^6 \pm 0.026$ SE/mm^3 for RAMPS-treated mice).

The effects of injecting plasma from normal, ASA-treated, and RAMPS-treated mice on platelet sizes of recipient mice are shown in Figure 2. Platelet sizes of mice injected with plasma from ASA-treated mice were significantly increased ($P < 0.005$), compared with values of other mice injected with normal mouse plasma. Likewise, mice injected with plasma from RAMPS-treated mice (used as a positive control) also showed significant ($P < 0.05$) increases in platelet size, compared with other mice treated with plasma from normal mice.

Figure 3 shows the effect of injecting plasma from

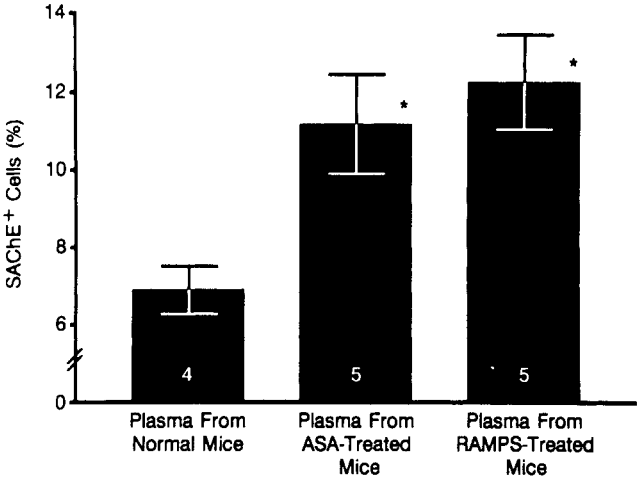

Figure 3. Percentage of SAChE+ cells in the marrow of mice after injection of plasma from normal mice, plasma from mice injected with 5.0 mg of ASA, and plasma from mice injected with RAMPS. The values represent the average of three smears from each mouse. The number of mice used is represented by the number on the bars. Vertical lines represent the SE. Data were significantly higher than values for control mice; * $P < 0.05$.

the three groups of mice, i.e., normal mice, mice treated with ASA, or mice treated with RAMPS, on SAChE+ cells in the marrow of mice. Mice given plasma from ASA-treated and RAMPS-treated donors had a significant ($P < 0.05$) increase in the percentage of SAChE+ cells when compared with the values of other mice injected with plasma from normal donors.

Discussion

The present work shows that mice with nonfunctional platelets produce elevated levels of thrombopoietin. Mice that were treated with aspirin (ASA) in doses shown to decrease platelet function were bled, and plasma from these animals injected into normal recipients was shown to contain a factor that stimulated platelet production in recipient mice. We show herein that plasma from ASA-treated mice increased %^{35}S incorporation into newly formed platelets, platelet sizes, and the percentage of SAChE+ cells. These data support the hypothesis that thrombopoietin is released into the circulation in response to nonfunctional platelets and acts on megakaryocytes to increase platelet production.

Platelet counts, which have been used previously as an indicator of thrombopoietin production, were not elevated in mice treated with plasma from ASA-treated mice or RAMPS-treated mice in the present study. Several previous studies have shown that mice treated with thrombopoietin-rich preparations have increased isotopic incorporation without increased platelet counts (1, 3, 13). These studies have shown that platelet counts were not sufficiently sensitive to indicate changes in platelet production of mice given low levels of thrombopoietin. The parameters evaluated in this study (iso-

tope incorporation, platelet size, and SAChE+ populations) have all been described as more sensitive indicators of platelet production (1) than platelet counting. We believe this explains the lack of elevation of platelet counts in the present study.

Sullivan and McDonald (6) presented data linking interruption of platelet function to increased thrombocytopoiesis. In their work, the percentages of SAChE+ cells were elevated after aspirin injection, with the maximal effect seen at 24 hr. Platelet sizes of mice given ASA were elevated at 32 and 48 hr after treatment, and elevation in the $\%^{35}S$ incorporation into platelets was seen at 48 hr after administration of ASA. These observations, along with the present results, indicate that an increase in platelet counts in treated mice is possible, but in both studies the experiments were terminated before the time period (5–6 days) that is required to obtain significant thrombocytosis (8). However, previous studies (6) indicated that over time after ASA injection, thrombopoietin was released endogenously and stimulated platelet production, but these data did not offer evidence of the presence of thrombopoietin. Since ASA has been shown by Demers *et al.* (14) to inactivate cyclooxygenase in the megakaryocytes, Sullivan and McDonald (6) proposed that the early change in SAChE+ cells at 24 hr might be due to either an autoregulation response by the megakaryocyte to the direct effects of ASA, or the release and action of thrombopoietin. An increase in platelet sizes at 32 hr and elevated $\%^{35}S$ incorporation into platelets at 48 hr after ASA injection are consistent with endogenous release and action of thrombopoietin. The present data confirm the conclusion that thrombopoietin was responsible for the endogenous increase in thrombocytopoiesis.

There are several disease states in which platelet counts are elevated and thrombopoietin levels are increased (15). These conditions include hepatoblastoma (16), chronic myeloid leukemia (17), and acute megakaryoblastic leukemia with translocation of chromosome 3q21 (18). Although platelet function studies were not reported in these cases, it is tempting to speculate that the platelets of these patients were poorly functioning and thus were not sensed by the control mechanisms, leading to release of thrombopoietin and, therefore, an increase in thrombocytopoiesis. We show herein that poorly functioning platelets in mice after ASA treatment lead to an increase in thrombopoietin production. We have also observed several cases of chronic myeloid leukemia with elevated platelet counts, abnormal platelet function, and elevated thrombopoietin titers (unpublished observations). Further studies are needed to clarify this hypothesis.

Although unlikely, the possibility exists that ASA in the plasma of donor mice could have caused these changes in platelet production. However, the fact that blood was collected from donor mice at 22 hr after ASA injection seems to rule out this possibility, because the half-life of ASA in the blood is about 12 hr (19). It appears that there was sufficient time for clearance of ASA from the plasma of treated mice before bleeding. In addition, we tested platelets of ASA-treated donor mice for aggregation response to collagen at 22 hr after ASA injection and the platelets were found to have normal function (data not shown). We also found that the platelet aggregation values of recipient mice receiving plasma from ASA-treated mice or RAMPS-treated donors were also not different from those of normal controls. Thus, plasma taken from mice 22 hr after ASA injection did not contain significant amounts of ASA, but a factor we believe to be thrombopoietin found in the plasma of ASA-treated mice stimulated $\%^{35}S$ incorporation into platelets, platelet sizes, and SAChE+ cell production when injected into normal recipient mice. Therefore, the findings of the present report show that poorly functioning platelets lead to the release of thrombopoietin, the natural physiological stimulator of thrombocytopoiesis.

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1. McDonald TP. The regulation of megakaryocyte and platelet production. Int J Cell Cloning **7**:139–155, 1989.
2. McDonald TP, Cottrell M, Clift R. Hematologic changes and thrombopoietin production in mice after x-irradiation and platelet-specific antisera. Exp Hematol **5**:291–298, 1977.
3. Evatt BL, Levin J. Measurement of thrombopoiesis in rabbits using ^{75}selenomethionine. J Clin Invest **48**:1615–1626, 1969.
4. Jackson CW, Edwards CC. Evidence that stimulation of megakaryocytopoiesis by low dose vincristine results from an effect on platelets. Br J Haematol **36**:97–105, 1977.
5. Roth GJ, Stanford N, Majerus PW. Acetylation of prostaglandin synthetase by aspirin. Proc Natl Acad Sci USA **72**:3073–3076, 1975.
6. Sullivan PS, McDonald TP. Acetylsalicylic acid stimulates murine megakaryocyte precursor cells. Proc Soc Exp Biol Med **194**:216–220, 1990.
7. McDonald TP, Clift RE. Mechanism of thrombocytopenia induced in mice by antiplatelet serum. Haemostasis **5**:38–50, 1976.
8. McDonald TP. Bioassay for thrombopoietin utilizing mice in rebound thrombocytosis. Proc Soc Exp Biol Med **144**:1006–1012, 1973.
9. McDonald TP. Effect of thrombopoietin on platelet size of mice. Exp Hematol **8**:527–532, 1980.
10. Kalmaz GD, McDonald TP. Effects of antiplatelet serum and thrombopoietin on the percentage of small acetylcholinesterase-positive cells in bone marrow of mice. Exp Hematol **9**:1002–1010, 1981.
11. Karnovsky MJ, Roots L. A "direct-coloring" thiocholine method for cholinesterases. J Histochem Cytochem **12**:219–221, 1964.
12. Jackson CW. Cholinesterase as a possible marker of early cells of

the megakaryocytic series. Blood **42**:413–421, 1973.
13. Hill RJ, Levin J. Regulators of thrombopoiesis: Their biochemistry and physiology. Blood Cells **15**:141–166, 1989.
14. Demers LM, Budin RE, Shaikh BS. The effects of aspirin on megakaryocyte prostaglandin production. Proc Soc Exp Biol Med **163**:24–29, 1980.
15. McDonald TP. Thrombopoietin: Its biology, clinical aspects, and possibilities. Am J Ped Hematol Oncol (in press).
16. Nickerson HJ, Silberman TL, McDonald TP. Hepatoblastoma, thrombocytosis, and increased thrombopoietin. Cancer **45**:315–317, 1980.
17. Shreiner DP, Weinberg J, Enoch D. Plasma thrombopoietic activity in humans with normal and abnormal platelet counts. Blood **56**:183–188, 1980.
18. Pinto MR, King MA, Goss GD, Bezwoda WR, Fernandes-Costa F, Mendelow B, McDonald TP, Dowdle E, Bernstein R. Acute megakaryoblastic leukaemia with 3q inversion and elevated thrombopoietin (TSF): An autocrine role for TSF? Br J Haematol **61**:687–694, 1985.
19. Flower RJ, Moncada S, Vane JR. Drug therapy of inflammation. In: Gilman AG, Goodman LS, Rall TW, Murad F, Eds. The Pharmacological Basis of Therapeutics. New York: MacMillan Publishing, p686, 1985.