Acetylsalicylic Acid Stimulates Murine Megakaryocyte Precursor Cells (43081)

PATRICK S. SULLIVAN AND T. P. MCDONALD[1]
Department of Animal Science, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37901–1071

Abstract. **Depression of platelet function with a single intraperitoneal injection of acetylsalicylic acid was found to produce significant increases in several thrombocytopoietic indicators despite no observed change in platelet counts. There was an increase in the number of megakaryocytic precursor cells (small acetylcholinesterase positive or "SAChE+" cells), platelet size, and ^{35}S incorporation into platelets. The results are qualitatively comparable to data from previous experiments showing that treatment of mice with a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) and rabbit anti-mouse platelet serum will elevate thrombocytopoiesis. The results presented herein indicate that interruption of platelet function by aspirin results in the production of new platelets, presumably by the action of a feedback system controlling thrombocytopoiesis.** [P.S.E.B.M. 1990, Vol 194]

Circulating platelet mass and some aspect of platelet function are sensed in the feedback circuit controlling thrombocytopoiesis. An interruption in either of these parameters can signal for the elaboration of a humoral regulatory factor and, thereby, the production of new platelets. Thrombopoietin, or a thrombocytopoiesis-stimulating factor (TSF), has been described as the humoral mediator of this homeostatic mechanism (1). The platelet function mechanism that senses the need for increased platelet production is poorly understood. Moreover, the particular aspect of platelet function which is monitored has not been elucidated. However, some clinical conditions provide insight into this issue. For example, patients with Glanzmann's thrombasthenia have decreased platelet aggregation function that is related to a quantitative or qualitative abnormality in the platelet surface glycoprotein IIb/IIIa complex (2). Patients with congenital deficiencies of platelet cyclooxygenase have prolonged bleeding times (3, 4). Patients with both conditions, however, have normal platelet counts. This lack of increase in platelet counts may indicate that these platelet defects are not sensed as a deficiency of function, or that in a chronic state of decreased function platelet mass assumes a dominant role in regulation of platelet production.

Whether the signal for the production of new platelets comes from a detected insufficiency in platelet mass or function, thrombopoietin acts to increase the numbers of megakaryocytes and, thus, platelets (1). This increased thrombocytopoiesis is detectable both in the peripheral blood and in the marrow. Treatment of animals with exogenous TSF produces significant changes in platelet size (1, 5, 6), radioisotope incorporation into platelets (5, 7), megakaryocyte number, size and ploidy (8, 9), and populations of megakaryocytic precursors in marrow preparations (10). Kalmaz and McDonald (11) have described the use of the latter parameter as an assay procedure for TSF and have described changes in small acetylcholinesterase-positive (SAChE+) cell populations as a very sensitive technique for the detection of thrombopoietin.

Administration of acetylsalicylic acid (ASA) causes the acetylation of a number of enzymes. One of these, cyclooxygenase, is found in platelets and megakaryocytes, and acetylation of the enzyme results in the platelet's inability to produce thromboxane A_2 (12–15). Although there has been some disagreement as to whether aspirin affects bleeding time in rodents (16–18), aspirin has been claimed to decrease the function of circulating platelets by this mechanism. Jackson and Edwards (19) found that inhibition of platelet function with vincristine leads to increased platelet production in the absence of thrombocytopenia, although the func-

[1] To whom all correspondence should be addressed at University of Tennessee, College of Veterinary Medicine, P.O. Box 1071, Knoxville, TN 37901-1071.

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tion of platelets that was monitored for control of platelet production was not identified. Administration of aspirin, therefore, might be expected to influence platelet production indices, i.e., platelet size, SAChE+ cell populations, and ^{35}S incorporation into platelets. Presumably, these changes occur via the release and action of thrombopoietin. In the present study, aspirin administration led to decreased platelet function and increased numbers of megakaryocytic precursor cells in the marrow of treated mice in the absence of thrombocytopenia. Previously described assay techniques for thrombopoietin (platelet sizing and ^{35}S incorporation into platelets) were used to infer that the effects of aspirin on the thrombocytopoietic homeostatic mechanism occurred by release and action of this hormone.

Materials and Methods

Animals and Aspirin. Male C3H mice, aged 6–8 weeks, were used in this work. Depression of platelet function was achieved by a single intraperitoneal injection of acetylsalicylic acid at a dose of 200 mg/kg (16). Crystalline acetylsalicylic acid (Sigma, St. Louis, MO) was dissolved in 0.086 *M* sodium hydroxide, and the pH of the resulting solution was adjusted to 7.40 ± 0.05 using 0.1 *M* NaOH and/or 0.1 *M* HCl. ASA was prepared immediately before each injection, saline (0.9%) was used as a control.

Platelet Function Analysis. At intervals after treatment, blood was collected into heparin-saline solution (100 units heparin/ml) via cardiac puncture; aggregation of platelets was determined using a Chronolog aggregometer (Chronolog, Havertown, PA). As the goal of this assay was to demonstrate that thromboxane A_2 was inhibited by aspirin therapy, collagen was chosen to induce aggregation. A 50-μl aliquot of collagen (Sigma, St. Louis, MO) was used to induce aggregation, and three parameters were measured from aggregation plots: maximum slope of aggregation curve, percentage of maximum aggregation achieved, and delay time from addition of collagen to beginning of aggregation. For the purpose of measuring maximum percentage of aggregation, the end point of the curve was designated as the point at which the curve achieved a steady transmission value or at 7 min after the addition of collagen, whichever came first.

SAChE+ Cell Analysis. Bone marrow smears were prepared from femoral marrow using the protocol of Kalmaz and McDonald (10) and were stained using the "direct coloring" method described by Karnovsky and Roots (20). Following a 3-hr incubation in the thiocholine stain, the smears were postfixed in 100% methanol for 10 min and in 50% methanol for 30 sec. Slides were evaluated in their entirety, and between 50 and 250 acetylcholinesterase-positive (AChE+) cells were counted for each mouse. An ocular reticule, calibrated to a standard 0.1-mm calibration slide, was used to measure AChE-positive cells of less than 13 μm in diameter (21). These small AChE-positive (SAChE+) cells were expressed as a percentage of total number of AChE+ cells.

Platelet Sizing and ^{35}S Incorporation. Platelet sizing analysis was performed on an aliquot of platelet-rich plasma using the methods described by McDonald (5, 6). Whole blood obtained by cardiac puncture was centrifuged for 4.5 min at 160*g*, and a sample of platelet-rich plasma was diluted in isotonic buffered saline for analysis on an Electrozone Celloscope (Particle Data, Inc., Elmhurst, IL). Additionally, blood harvested from the retro-orbital sinus was used for determination of peripheral platelet counts.

For assay of ^{35}S incorporation into platelets, single intravenous injections of 30 μCi of ^{35}S were given 24 hr before assay. Samples were collected at 24, 32, or 48 hr after administration of ASA; 24 hr postinjection was chosen as the first sample time because previous experiments (22) indicated that earlier time measurements did not measure blood platelet production. Before 24 hr, ^{35}S labels a relatively labile compound that does not appear to be in the form of a sulfated mucopolysaccharide. Percentage of ^{35}S incorporation was calculated using the method described by McDonald (7).

Statistical Analysis. Because the cardiac puncture collection procedure in mice requires the sacrifice of the animals, multiple samples from the same animal were not possible. Therefore, each data point represents a single animal. For all data presented in this work, Student's *t* test was used to determine statistical significance.

Results

At the time of blood and marrow sample collection, a representative sampling of both control and aspirin-treated mice were examined for the presence of any gross pathologic lesions. Inspection of the peritoneal cavity and viscera, in particular, revealed no gross indications of an inflammation or hemorrhagic reaction associated with the intraperitoneal injection of either aspirin solution or saline.

Maximal depression of aggregation parameters (maximum percentage of aggregation, slope, and delay) was observed 8 hr after administration of ASA. All three platelet aggregation parameters were significantly depressed in platelets from aspirin-treated mice as compared with platelets obtained from saline-treated control mice. Table I shows that 8 hr after treatment maximum percentage of aggregation was decreased to 51.8% of control ($P < 0.005$) and slope to 63.0% of control ($P < 0.025$); delay was increased to an average of 148% of control values ($P < 0.05$). This severe depression of function is a short-lived phenomenon, probably because of the relatively short life-span (about

Table I. Aggregation Parameters of Platelets from Mice after Treatment with Acetylsalicylic Acid[a]

Time after ASA (hr)	Maximum % aggregation[b]	Slope (degrees)[b]	Delay (sec)[b]
0 (control)	63.25% ± 4.04	72.14 ± 7.66	14.71 ± 1.94
8	32.76% ± 4.92***	45.43 ± 7.91**	21.86 ± 3.16*
16	48.13% ± 1.87	36.00 ± 4.00	23.00 ± 1.00*
24	47.02% ± 5.84	58.00 ± 12.00	10.50 ± 3.50

[a] Control and 8-hr values represent eight mice each, and 16- and 24-hr values represent two mice each. Significantly different from control: * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$.
[b] Results are expressed as average ± 1 SE.

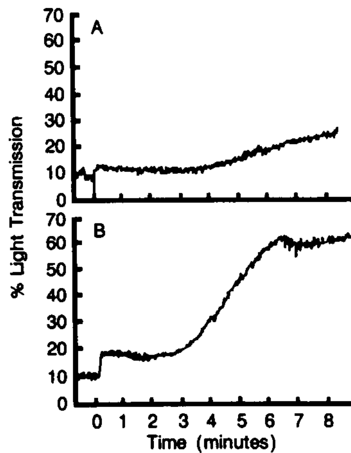

Figure 1. Aggregation tracings from aspirin-treated (A) and saline-treated (B) mice. Both tracings represent measurements taken 8 hr after treatment.

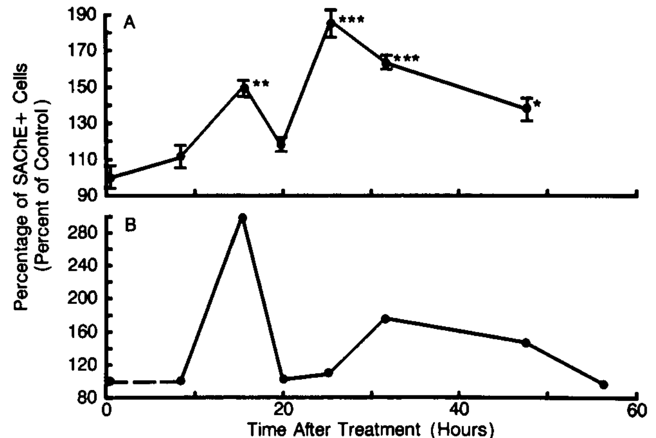

Figure 2. Changes in populations of SAChE+ cells after treatment with acetylsalicylic acid (A) and exogenous TSF (B, reproduced from Kalmaz and McDonald (10)). Total number of cells examined per point ranged from 400 to 1220. Values are expressed as ±1 SE. Significantly different from control: * $P < 0.01$, ** $P < 0.005$, *** $P < 0.0005$.

4 days) of platelets in mice (23). Figure 1 provides examples of aggregation plots from both control and aspirin-treated mice at the 8-hr time period. Platelet counts were not significantly different between control and aspirin-treated mice at any time period measured.

The percentages of SAChE+ cell populations increased significantly following ASA administration, as seen in Figure 2. Maximal effect was seen at the 24-hr time interval (187% of control, $P < 0.0005$), and by 48 hr the levels were approaching control values. Also illustrated in Figure 2 are data from Kalmaz and McDonald (10) illustrating changes in SAChE+ cell populations following administration of exogenous TSF. It should be noted that in both cases a decrease in number of SAChE+ cells was observed at the 20-hr time period.

Platelet sizing data indicated a trend toward larger platelets after treatment with aspirin. Although no significant change in size was observed at 24 hr after administration of ASA, increased platelet sizes were observed at 32 hr (109% of control, $P < 0.005$) and at 48 hr (106% of control, $P < 0.025$) after treatment (Fig. 3).

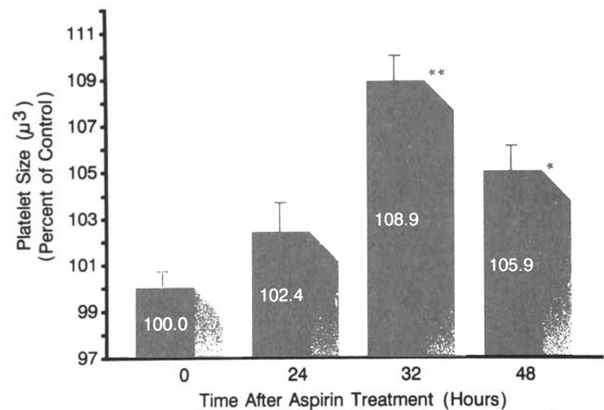

Figure 3. Changes in platelet size following administration of acetylsalicylic acid. Each point represents sizing data from 8 to 23 mice. Values are expressed as ±1 SE. Significantly different from control: * $P < 0.025$, ** $P < 0.005$.

Incorporation of ^{35}S into platelets varied significantly from control at two time periods after aspirin administration. Incorporation values were found to be significantly *lower* than control (55.0% of control, $P < 0.005$) at 24 hr after treatment, and the values were significantly *higher* than control (132% of control, $P < 0.05$) 48 hr after injection of ASA. These data are presented graphically in Figure 4.

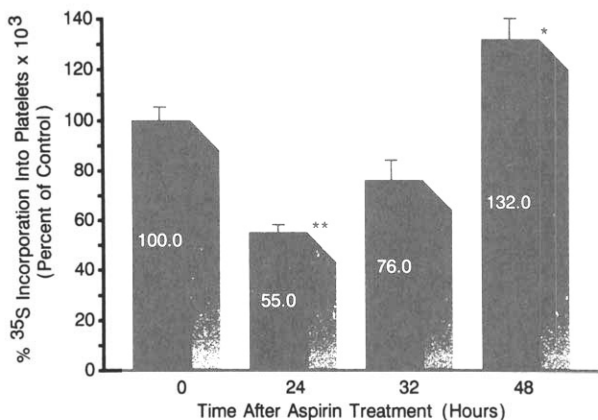

Figure 4. Changes in ^{35}S incorporation into platelets following administration of acetylsalicylic acid. Each point represents incorporation data from 9 to 23 mice. Values are expressed as ±1 SE. Significantly different from control:* $P < 0.05$, ** $P < 0.005$.

Discussion

The parameters considered in this work—changes in SAChE+ cell populations, platelet size, and ^{35}S incorporation into platelets—have all been reported previously as *in vivo* assay methods for thrombopoietin (1). In the current work, injection of acetylsalicylic acid caused significant suppression of platelet function and, subsequently, increases in all three parameters as compared with controls; this suggests that thrombopoietin is released in response to aspirin-related interruption of platelet function.

It could be argued that ASA acts directly at the level of the megakaryocyte and that the changes in SAChE+ cell populations represent a local autoregulation event. However, it seems more likely that the observations presented in this work are the result of release and action of thrombopoietin. A dual mechanism of effect on the thrombocytopoietic system could, however, exist. The decreased ^{35}S incorporation observed at 24 hr after treatment with aspirin may be a result of a direct aspirin-megakaryocyte interaction. Such direct interactions have been proposed previously. Demers *et al.* (24) have suggested that aspirin inactivates cyclooxygenase of megakaryocytes as well as platelets. However, the changes in platelet size and increases in ^{35}S incorporation seen at the later time periods are consistent with changes observed in response to a thrombopoietin-mediated event.

In addition, comparison of current data to previous work supports the assertion that increases in SAChE+ cell populations are, in fact, a result of TSF stimulation. Kalmaz and McDonald (10) noted that changes in SAChE+ cell populations seem to proceed in a cyclical nature after administration of exogenous TSF; these data have been reproduced in Figure 2 along with new data showing changes after ASA treatment for purposes of comparison. An adjustment of 8 hr (dotted line) has been made in the TSF data to account for the time between injection of ASA and maximal depression of function. The changes in SAChE+ cell populations have similar time courses in the two experiments; particularly interesting is the sharp decrease in both plots at 20 hr after the thrombocytopoietic stimuli. It is possible that the first elevation (16 hr) in SAChE+ cells is a *relative* increase secondary to loss of mature megakaryocytes and that the second elevation (24 hr) is an *absolute* increase in SAChE+ numbers. This mechanism would account for the secondary rise in SAChE+ cell numbers seen after administration of TSF, or of other agents by which some mechanism might cause the elaboration of TSF.

Clearly, the interruption of platelet function by acetylsalicylic acid has complex effects on the system controlling platelet production. Because it is known that depressed function can stimulate the production of new platelets (19), it is logical that appropriate doses of aspirin would produce effects similar to those of a platelet inhibitor such as vincristine. This work seems to lend credence to this prediction, as changes in SAChE+ cell populations, platelet size, and ^{35}S incorporation into platelets following ASA administration all showed significant deviations from control values in the same manner as following rabbit anti-mouse platelet serum treatment in previous experiments (5–7, 10). Our observations are consistent with the hypothesis that aspirin causes an interruption of platelet function which is recognized by some sensing mechanism, and that the subsequent release and action of thrombopoietin results in sequential elevations of SAChE+ cell populations, followed by an increase in platelet size and then elevated ^{35}S incorporation into platelets.

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