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Assay for Thrombopoietin: A New, More Sensitive Method Based on Measurement of the Small Acetylcholinesterase-Positive Cell[1] (41421)

G. D. KALMAZ[2] AND T. P. McDONALD[3]

Department of Medical Biology, University of Tennessee, Center for the Health Sciences/Knoxville Unit, Knoxville, Tennessee 37920

Abstract: A sensitive assay for a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) that utilizes measurement of the small acetylcholinesterase-positive ($SAChE^+$) cell in the marrow of mice is described. The results of this assay were compared with a previously published procedure that measures ^{35}S incorporation into platelets of immunothrombocythemic mice. In the rebound-thrombocytotic mouse assay, TSF from kidney cell culture medium caused a dose-related increase in the amount of ^{35}S incorporation into platelets; the minimum detectable dose of TSF was 15 mg of protein/mouse. For the $SAChE^+$ cell assay, mice received single intraperitoneal injections of TSF or platelet-specific antiserum. Other mice were injected with plasma from thrombocytopenic donor mice. For controls, groups of mice were given saline or normal mouse plasma. Bone marrow from the mice (killed at 10 hr after injection) was taken for smears and stained for acetylcholinesterase. The results show that both TSF and rabbit anti-mouse platelet serum (RAMPS) caused a highly significant dose-dependent increase in the percentage of $SAChE^+$ cells and that plasma from thrombocytopenic mice stimulated an increase ($P < 0.0005$) in the percentage of $SAChE^+$ cells. The minimum detectable dose of TSF in the $SAChE^+$ cell assay was 1.875 mg protein/mouse. Therefore, $SAChE^+$ cells in the marrow of mice can detect smaller doses of TSF than the rebound-thrombocytotic mouse assay.

Previous assays for a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) have utilized platelet counting (1–2), measurement of alterations in platelet labeling with radioisotopes (3–6), changes in platelet sizes (7–9), an immunoassay procedure (10), and increases in alterations of megakaryocytes *in vivo* (11–14) and *in vitro* (15–16). Several techniques are available for the measurement of TSF, but most of the procedures lack sensitivity and reproducibility. Although the measurement of ^{35}S incorporation into platelets of immunothrombocythemic mice gives reproducible results and appears to be more sensitive than are the other currently available techniques (11), the procedure is expensive and time consuming. This expense limits the number of samples that can be assayed; therefore, a more sensitive, reproducible, and inexpensive assay technique for TSF is needed.

Previously, Jackson (12) reported an elevation in the percentage of small acetylcholinesterase-positive ($SAChE^+$) cells in the marrow of rats treated with anti-platelet sera. Long and Henry (13) noted a reduction in the percentage of $SAChE^+$ cells after platelet concentrates had been administered. In a more recent study, Kalmaz and McDonald (14) showed a three-fold increase in the percentage of $SAChE^+$ cells over controls after the mice had been injected with TSF from kidney cell culture medium or platelet-specific antiserum. These findings led to the conclusion that $SAChE^+$ cells are early cells in the megakaryocytic series and appear to be a sensitive indicator of changes in thrombocytopoiesis. Therefore, the measurement of these cells after stimulation with potential thrombocytopoietic agents should be a useful assay for TSF.

Materials and Methods. Eight- to ten-

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[2] Recipient of a National Research Service Award, Fellowship Number HL 06072.

[3] To whom correspondence should be addressed.

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week-old C3H male mice weighing 22–23 g were used. The source of TSF was culture medium from human embryonic kidney cells (17); plasma from thrombocytopenic mice was also used as a thrombocytopoietic agent.

Rebound-thrombocytotic mouse assay. Thrombocytopoietic activity of the TSF was determined by measuring [^{35}S]sulfate incorporation into platelets of mice in rebound-thrombocytosis (6). The procedure was as follows: Mice were given single intraperitoneal (i.p.) injections of rabbit anti-mouse platelet serum (RAMPS) which caused marked thrombocytopenia by 4 hours, followed 5 days later by rebound thrombocytosis. RAMPS was prepared and absorbed as previously described (18). On Days 5 and 6 after receiving the RAMPS injection, the mice were injected subcutaneously with test materials two times each day. Thirty microcuries of $Na_2{}^{35}SO_4$ in 0.5 ml of saline were injected intravenously on Day 7, and the percentage ^{35}S incorporation into circulating platelets in blood samples obtained by cardiac puncture was measured 24 hr later.

SAChE$^+$ cell assay. For the measurement of SAChE$^+$ cells, mice were injected ip with saline, normal mouse plasma, or plasma from thrombocytopenic mice and various doses of TSF-rich culture medium and RAMPS. Ten hours later, blood was collected from the retroorbital sinus for platelet counts. The mice were killed, and the marrow was taken from the femurs and dispersed into a single cell suspension by mixing with a plasma expander (polyvinylpyrrolidone, 3.5% in saline). Marrow smears were made and stained for the presence of AChE activity using the method of Karnovsky and Roots (19). Marrow smears were incubated in the acetylcholine substrate mixture at room temperature for 3 hr. After staining, the smears were postfixed in xylene for 2 min, absolute ethyl alcohol for 30 sec, and then dipped 10 times in water. The preparations were counterstained by use of Gill's hematoxylin No. 2 (Fisher Scientific, Springfield, N.J.) for 3 min and the smears were "blued" in Scott's tap water substitute. All AChE$^+$ cells at the periphery of the smears were counted. SAChE$^+$ cells, expressed as a percentage of the total number of AChE$^+$ cells, were cells that stained positively for AChE and were 13 μm or less in diameter as determined by the use of an ocular reticule at 420× magnification (14).

In a preliminary experiment (Table I) all AChE$^+$ cells were examined on smears, and the percentage of SAChE$^+$ cells on the whole slide was compared to the proportion of SAChE$^+$ cells on the periphery (edges) of the smears. Since about the same proportion of SAChE$^+$ cells were on the edges of the smears as on the entire smear, only the SAChE$^+$ cells at the periphery of the smears were scored in all later experiments.

Comparison of thrombocytopoietic agents from different sources. To determine if the effects of TSF from kidney cell culture medium and the stimulatory effects of plasma from thrombocytopenic mice were similar, assay mice were injected with both preparations. Plasma was obtained from mice made thrombocytopenic with a single ip injection of RAMPS (20). For controls, other mice were injected with normal rabbit serum (NRS). Platelet counts were made on blood taken from the plasma donor mice 4 hr after either RAMPS or NRS injection. Immediately after the blood was taken for platelet counts, the mice were bled from the heart into syringes containing 4% sodium citrate solution; blood was expressed into 50-ml centrifuge tubes immersed in an ice bath. The average platelet counts of donor mice were: $1.09 \pm 0.04 \times 10^6$ after NRS injection and $0.01 \pm 0.00 \times 10^6$ after RAMPS treatment. The plasma was then separated from the blood cells by centrifugation and stored for $\simeq$10 hr at 4° until injected into mice (0.7 ml of plasma/mouse) for the measurement of the percentage SAChE$^+$ cells. Previous experiments (20) had already shown positive thrombocytopoietic effects of plasma from thrombocytopenic mice in the immunothrombocythemic mouse assay.

Statistical analyses were made by use of Student's *t* test and linear regression.

Results. Platelet counts and percentage SAChE$^+$ cells in the marrow of mice 10 hr

TABLE I. A COMPARISON OF THE PROPORTION OF SAChE$^+$ CELLS ON THE WHOLE SMEAR VERSUS THE EDGES OF THE SMEAR

Treatment	Number of specimens	Percentage SAChE$^+$ cells ± SE	
		Periphery	Whole
Saline	6	7.33 ± 0.33	8.00 ± 0.37
RAMPS[a]	6	26.67 ± 3.46	26.17 ± 2.32
TSF[b]	6	26.83 ± 4.33	26.67 ± 3.25

[a] Rabbit anti-mouse platelet serum, injected at 0.1 ml/mouse.
[b] Medium from kidney cell culture, injected at 15 mg/mouse.

after injection of various doses of RAMPS are shown in Table II. The data show an inverse relationship between the amount of RAMPS injected and platelet counts of the mice. There also appeared to be a dose-dependent increase in the percentage of SAChE$^+$ cells in the groups injected with various doses of RAMPS; this is indicated by a significantly higher value ($P < 0.025$) in the group receiving a dose of 0.1 ml/mouse. Therefore, as the dose of RAMPS was increased, the percentage of SAChE$^+$ cells was also increased and, at the highest dose of RAMPS, reached ≈2.7-fold increase over control levels. These data show a direct relationship between the degree of thrombocytopenia and the percentage of SAChE$^+$ cells in the marrow of mice.

The results of assaying TSF found in kidney cell culture medium in mice in rebound thrombocytosis utilizing ^{35}S incorporation into platelets are shown in Fig. 1A. A clear dose–response relationship between the amounts of TSF injected and the levels of ^{35}S incorporation into platelets is apparent. The ^{35}S incorporation into platelets was significantly higher than the isotope incorporation into platelets of saline-injected mice after they had received doses of 15 mg protein/mouse ($P < 0.05$) and 30 mg protein/mouse ($P < 0.0005$) of TSF. Also shown in Fig. 1 are the results from the assay of the same preparation of TSF utilizing the SAChE$^+$ cell determination (Fig. 1B). A dose–response relationship was noted between the proportion of these cells and the dose of TSF injected between 1.875 and 7.5 mg protein/mouse. The percentage of SAChE$^+$ cells was significantly higher than for controls ($P < 0.0005$) after doses of 1.875 mg protein/mouse; peak values (three-fold increase over control) were reached with doses of 15.0 mg protein/mouse. A plateau of response was reached after the injection of 7.5 mg protein/mouse of TSF.

The effects of plasma from antibody-induced thrombocytopenic mice on the percentage SAChE$^+$ cells of recipient mice are shown in Table III. The injection of a single dose of plasma significantly ($P < 0.0005$) increased the percentage of SAChE$^+$ cells in recipient animals when compared to that of other mice given an injection of plasma from NRS-treated mice.

Discussion. The present study showed an inverse relationship between platelet counts and the percentage of SAChE$^+$ cells

TABLE II. PLATELET COUNTS AND PERCENTAGE OF THE SMALL ACETYLCHOLINESTERASE-POSITIVE (SAChE$^+$) CELLS IN MICE 10 HR AFTER INJECTION OF RABBIT ANTI-MOUSE PLATELET SERUM (RAMPS)

Treatment	Number of mice	Platelet count ($\times 10^{-5}$/mm^3 ± SE)	Percentage SAChE$^+$ cells ± SE
Saline	9	10.45 ± 0.26	9.00 ± 0.76
0.01 ml of RAMPS	9	6.74 ± 1.25*	23.67 ± 0.76**
0.1 ml of RAMPS	6	0.26 ± 0.09**	27.17 ± 1.22**

* Significantly different from saline-injected mice, $P < 0.05$.
** Significantly different from saline-injected mice, $P < 0.0005$.

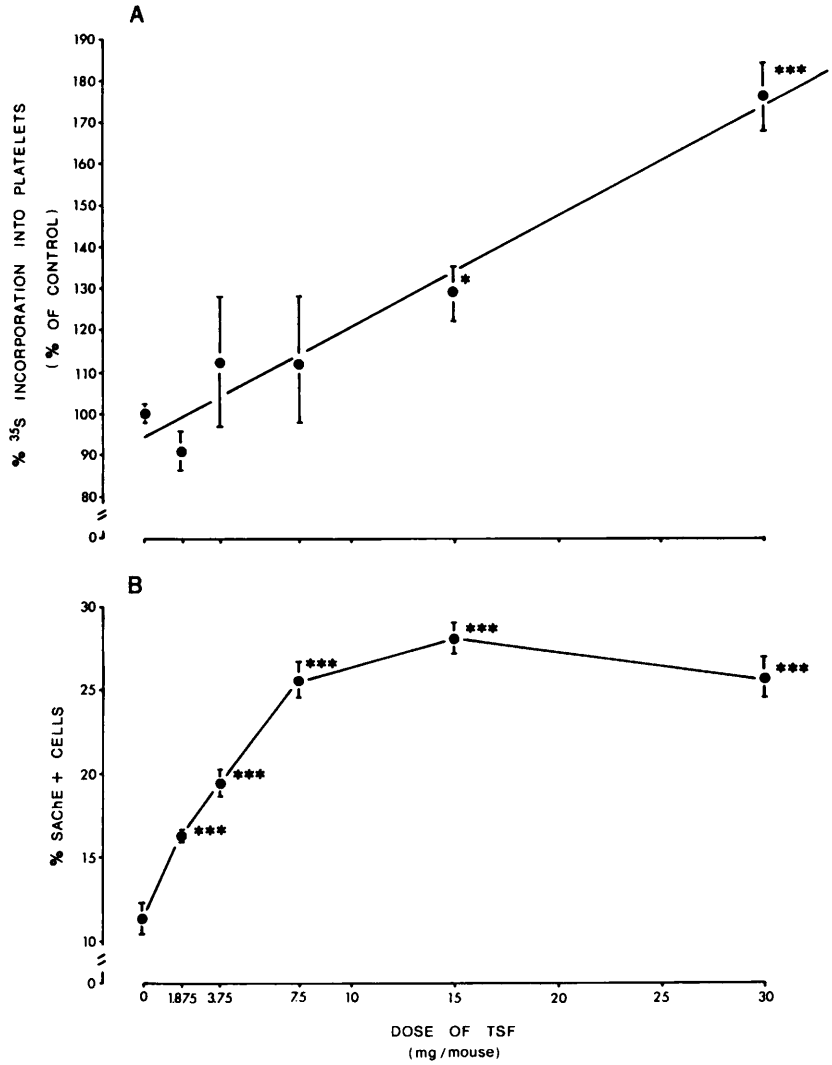

FIG. 1. Changes in percentage ^{35}S incorporation into platelets of rebound-thrombocytotic mice (A) and changes in the proportion of small acetylcholinesterase positive (SAChE+) cells (B) after injection of various doses of TSF-rich kidney cell culture medium. The vertical lines indicate the standard errors. Percentage ^{35}S incorporation into platelets and the proportion of SAChE+ cells were significantly higher than the saline-injected controls: $*P < 0.05$; $***P < 0.0005$. The percentage of ^{35}S incorporation into platelets versus the dose of TSF injected into rebound-thrombocytotic mice was (A): $r = 0.96$; $y = 94.71 + 2.63x$.

in the mice treated with RAMPS. The results also indicated a dose–response relationship between the amounts of TSF-rich culture medium injected and the levels of ^{35}S incorporation into platelets of mice in rebound thrombocytosis; moreover, TSF caused a dose response in the proportion of marrow SAChE+ cells. Plasma from thrombocytopenic mice caused similar increases in percentage SAChE+ cells.

As shown in previous studies (12, 14, 21), the percentage of SAChE+ cells was increased in the marrow of animals that had received single injections of platelet-specific antisera. Jackson (12) reported a 2-fold increase in the number of SAChE+

TABLE III. THE PERCENTAGE OF SAChE$^+$ CELLS IN MARROW SMEARS OF MICE AFTER INJECTION WITH NORMAL MOUSE PLASMA OR TSF-RICH PLASMA

Treatment	Number of mice	Percentage SAChE$^+$ cells ± SE
Normal mouse plasma	11	7.64 ± 0.43
TSF-rich plasma	12	13.83 ± 0.52**

Note. SAChE$^+$ cells = small acetylcholinesterase-positive cells. Donor mice were injected with normal rabbit serum and rabbit anti-mouse platelet serum 4 hr before bleeding from the heart to obtain normal mouse plasma and TSF-rich plasma.

** Significantly higher than control values ($P < 0.0005$).

cells in rats 6 hr after injection of platelet-specific antisera. A significant increase in the proportion of SAChE$^+$ cells ($P < 0.0005$) was reached at 6 hr with peak values (3-fold increase over control) observed at 8 hr by Kalmaz and McDonald (14). Nakeff and Bryan (21) found a 1.5-fold increase in the number of SAChE$^+$ cells at 6 hr. In agreement with these findings, the results of the present study showed an increase in the proportion of SAChE$^+$ cells in the marrow of mice at 10 hr after RAMPS treatment.

In the present study, platelet counts and the percentage SAChE$^+$ cells had an inverse relationship (Table II), thereby agreeing with the results of previous studies (22, 23) in which the total number of megakaryocytes was determined. The results also indicated that the degree of megakaryocytic response was related to the level of thrombocytopenia. These data, therefore, support the hypothesis that the level of circulating platelets is the major controlling factor in platelet production (22, 24, 25).

A dose–response relationship between the amount of TSF from kidney cell culture medium injected and the level of ^{35}S incorporation into platelets of mice in rebound thrombocytosis is shown in Fig. 1A. This finding confirms earlier studies from this laboratory in which McDonald (26) showed a dose–response relationship between the amount of TSF injected and the level of ^{35}S incorporation into platelets. Similar results between the volume of plasma from thrombocytopenic rabbits, whose platelet numbers were reduced with platelet-specific antibodies, and the incorporation of [^{75}Se]-selenomethionine into platelets of rabbits were observed by Levin *et al.* (27).

In addition to the effects of RAMPS, the injection of TSF-rich culture medium also led to an increase in the percentage of SAChE$^+$ cells in the marrow of mice (Fig. 1B). The percentage of these cells had a dose–response relationship with the amounts of TSF injected; at doses of 1.875 mg protein of TSF, the percentage of SAChE$^+$ cells was significantly higher than for controls ($P < 0.0005$). Peak values (three-fold increase over controls) were observed with doses of 15.0 mg protein/mouse. In agreement with these findings, an increase in the proportion of SAChE$^+$ cells (three-fold over controls) between 6 and 12 hr after TSF injection was noted in a previous study (14).

Plasma from antibody-induced thrombocytopenic mice was tested previously (20) in immunothrombocythemic mice for thrombopoietin; increased platelet counts ($P < 0.025$) and elevated percentage ^{35}S incorporation into platelets ($P < 0.005$) were found when compared to the values of other mice injected with normal mouse plasma. In the present study, both TSF from kidney cell culture medium and plasma from antibody-induced thrombocytopenic mice, which was presumably rich in thrombopoietin (but may have contained residual antibody), increased ($P < 0.0005$) the percentage of SAChE$^+$ cells in recipient mice (Fig. 1 and Table III). These data support the hypothesis that TSF from kidney cell culture medium is similar in its action to the stimulus found in plasma of antibody-induced thrombocytopenic animals.

When the total number of AChE$^+$ cells on the whole slide (Table I) was compared with the number of AChE$^+$ cells on the periphery (edges) of the smears, no significant difference in the percentage of SAChE$^+$ cells was found. It was previously believed that because of their large size, megakaryocytes migrated to the edges during the process of making smears. However, since SAChE$^+$ cells also move to the edges (and apparently in the same propor-

tion), size alone cannot be a factor. The possibility that cell density could be a factor arose when Nakeff and Floeh (28) found that megakaryocytes from a single population, regardless of their maturation state and size, have cell densities that are higher than other nucleated hematopoietic cells. Proof of the hypothesis of higher cell density of the megakaryocytes as a factor in their placement on slides, however, awaits further experimentation.

Previous studies showed that TSF from kidney cell culture medium has biological (29), chemical (29), and immunological (30) properties in common with thrombopoietin produced in thrombocytopenic animals. The present study demonstrates that TSF from different sources will stimulate an increase in the SAChE+ cells of mice and lends support to the hypothesis that kidney cells in culture can produce the TSF.

The results presented herein describe a new assay technique for TSF which is reproducible, inexpensive (when compared to other techniques that require radioisotopes), and (most importantly) is sensitive enough to detect low doses of TSF. This assay procedure should be useful for the study of the site of production and mode of action of TSF.

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