Bioassay for Thrombopoietin Utilizing Mice in Rebound Thrombocytosis[1] (37730)

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It is now clear that platelet production is controlled in part by a thrombopoietic stimulating factor (TSF or thrombopoietin). Characterization of TSF, as well as determination of its site of production, chemical nature, and mechanism of action, has, however, been hampered by the lack of a satisfactory assay technique (1–3).

Early assay techniques (4–7) measured TSF in normal recipients by platelet counting, but this procedure lacks sensitivity and isotope uptake may more directly reflect platelet production rates. Therefore, ^{75}Se-selenomethionine or sodium ^{35}S-sulfate has been used in animals to label platelets for determination of their production by megakaryocytes (8–20). Additional studies have utilized assay animals after anti-platelet serum treatment (13–15, 20) or platelet transfusion (8–12, 19) before administration of TSF test substances and isotopes. These animals have suppressed endogenous TSF levels and while they are thrombocythemic, they produce a more identifiable response to exogenous TSF sources than untreated animals (12, 15). Although transfusion of platelets into assay recipients has unquestionably increased the animal's ability to respond to exogenous TSF, the procedure is very costly and time-consuming; this limits the number of assay animals that can be used. Anti-platelet serum pretreatment of assay recipients has the obvious advantage of decreasing the work required for animal preparation; however, the time schedules used for anti-platelet serum-treated mice in TSF assays are quite critical and require further investigation.

Previous work (20) has dealt with the development of a hemagglutination-inhibition assay for TSF. This immunoassay technique may provide a useful tool in future investigations of TSF. However, suitable bioassay procedures are needed to correlate biological TSF activity with the immunological measurement of TSF. It is the purpose of this report, therefore, to describe the further development of a bioassay procedure for TSF that utilizes immunothrombocythemic mice (20).

Materials and Methods. Sixteen sheep of random breeding were used as sources of TSF-rich sera after a single injection of rabbit anti-sheep platelet serum (ASPS). The ASPS was produced and absorbed as previously described (20). Twenty milliliters of the ASPS injected intravenously into each sheep caused a severe thrombocytopenia (0–14,000 platelets/mm^3) within a few minutes. The platelet-depleted sheep were bled from the jugular vein 2–3 hr after ASPS injection into empty evacuated plasma containers (Abbott Laboratories). The serum that contained TSF was removed by centrifugation and used in the experiments presented herein.

Male C_3H mice weighing 22–25 g were used as assay recipients in these experiments. Some of the mice were injected intraperitoneally with rabbit anti-mouse platelet serum (AMPS) which was prepared and absorbed with mouse RBC as previously described (20, 21) (0.1 ml AMPS was diluted to 0.5 ml in saline prior to injection). Anti-mouse

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platelet serum-injected mice with initial platelet counts (determined 4 hr after AMPS injection by direct phase-contrast microscopy from a single drop of blood obtained by retroorbital puncture) above 50,000/mm^3 were excluded from the assay. By use of this procedure, only 275 of 3,258 mice (8%) used in recent bioassays have been discarded. This strain of mice has a normal platelet count of about 9×10^5/mm^3. In the experiments presented in Figs. 3 and 4, 0.5 ml of test substance (saline or sheep serum diluted in saline, 1.5 ml serum + 0.5 ml saline) was injected into mice subcutaneously four times (two times on days 5 and 6) with the first injection given five days after AMPS for a total dose of 2.0 ml. In all experiments, 25 μCi of $Na_2{}^{35}SO_4$ diluted in 0.5 ml of saline were injected intravenously, and the 24-hr radiosulfate incorporation into platelets was determined. In AMPS-treated mice, the ^{35}S injection was given on day 7. At the time of assay, mice were injected intraperitoneally with 0.5 ml of a heparin–Nembutal–saline solution (1.0 ml heparin, 1,000 U.S.P. units; 1.0 ml Nembutal, 50 mg; 10 ml of saline), and platelet counts and WBC counts were made from a drop of blood obtained by retroorbital puncture. About 10 min later, mice were bled from the heart into plastic syringes containing 1.0 ml of 1.0% disodium ethylenediaminetetraacetic acid (Na_2EDTA) in 0.7% saline. Penington (13) has previously shown that blood obtained from the saphenous vein of mice did not differ in platelet counts from cardiac blood samples. The blood of each mouse was expressed into a 12 × 75 mm plastic tube and mixed with an additional 1.0 ml of Na_2EDTA. Platelets were separated from

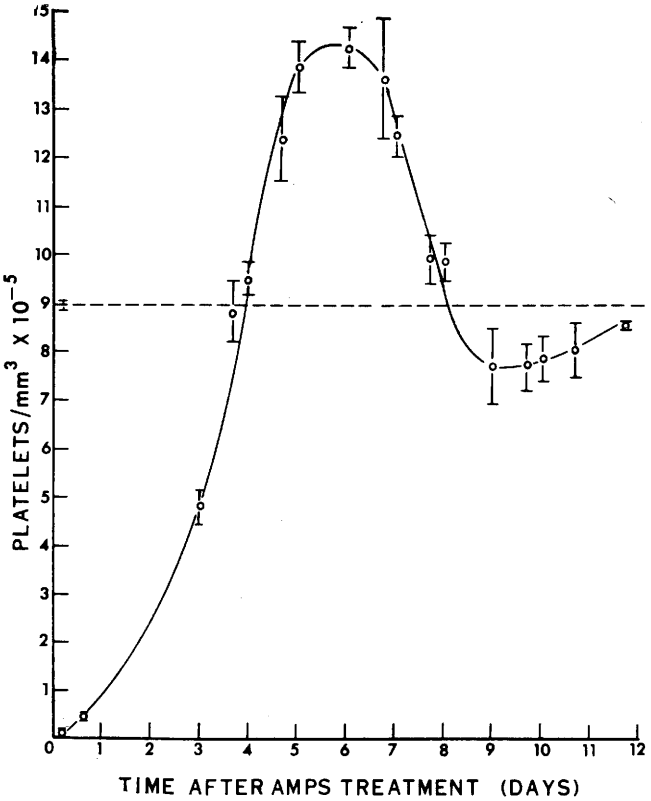

FIG. 1. Peripheral platelet counts of mice after injection of platelet-specific antisera (AMPS). A total of 167 AMPS-treated mice (5–21 mice/point) and 45 normal mice (shown by horizontal dashed line) was used in this experiment. Bars denote standard error of mean (SEM).

the blood by slow centrifugation (30 min at 50*g*) to obtain a platelet-rich plasma layer which was transferred into another tube and then centrifuged at a more rapid rate (15 min at 360*g*) to obtain a platelet button. Platelets from each mouse were washed with 0.5 ml of 1% ammonium oxalate (13, 20), then with a 1.0 ml of saline, and resuspended in 0.35 ml of Isoton (Coulter). Two 100-μl samples of the platelet suspension were placed in plastic counting vials for the determination of radioactivity as previously described (22). In preliminary experiments, radioactivity measurements were also made on samples of the two washes to rule out contamination of platelets with the plasma $Na_2{}^{35}SO_4$. Another sample of the suspension was used for the determination of the number of platelets in the washed platelet suspension. For platelet counting, the suspensions were further diluted (1:2,000) in Isoton and counted (23) by use of an Electrozone/Celloscope (Particle Data, Inc.) with a log converter. In all experiments, the percent yield of platelets in the final platelet suspension of control mice (about 23% of original) did not differ from the platelet yield of mice injected with sera from platelet-depleted sheep. The percent ^{35}S incorporation of the platelets was calculated as follows:

$$\%\ {}^{35}S\ \text{Incorporation} = \left(\frac{\text{cpm of platelet suspension}}{\text{Platelet count of suspension}}\right) (\text{Body weight in grams} \times 7\%) \frac{(\text{Peripheral platelet count/ml})}{\text{cpm injected}} \times 100.$$

Results. Injection of platelet-specific antisera into mice reduced the peripheral platelet count to essentially 0 within 4 hr (Fig. 1). Three days later, the platelet count began to rise sharply, reaching normal counts by day 4, overshooting on days 5–7, and then declining toward normal levels on day 8. On days 9–10, the platelet count again dropped to below the control mouse level ($p < 0.05$).

Measurement of ^{35}S uptake by platelets of some of the same mice presented in Fig. 1 is shown in Fig. 2. The 24-hr ^{35}S-sulfate uptake of platelets 3 days (^{35}S injected on

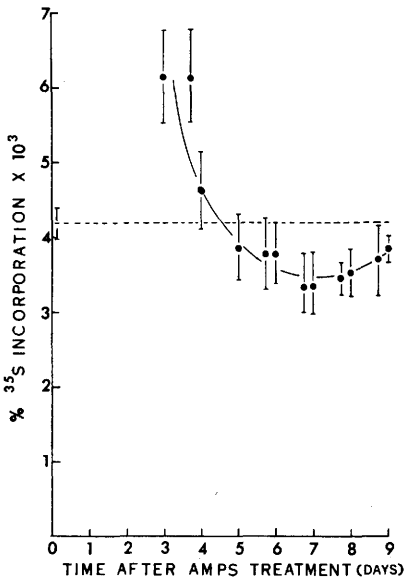

FIG. 2. Twenty-four-hour ^{35}S incorporation of platelets from some of the same mice presented in Fig. 1 (mice shown on day 3 were injected with ^{35}S on day 2, etc.). The horizontal dashed line represents the ^{35}S-sulfate for 27 normal mice and bars denote SEM.

day 2 and ^{35}S uptake measured on day 3) after AMPS treatment was about 1.5 times that of normal controls. The ^{35}S uptakes decreased to almost normal levels on day 4, to below the normal control mouse range ($p < 0.05$) on days 6⅔–7⅔, and then returned toward normal levels again on day 9. These data illustrate that platelet production (as measured by percent ^{35}S incorporation) is suppressed on days 6⅔ and 7⅔ after AMPS injection in mice whose platelet counts were greatly increased on days 5–6.

Figure 3 shows a summary of six experiments in which normal mice and AMPS-treated mice were used for the assay of TSF. When normal mice were used as TSF recipients, there was no statistically significant difference in ^{35}S incorporation by platelets between mice injected with sera from platelet-depleted sheep and those injected with normal sheep sera or saline. However, in the AMPS-treated mice, ^{35}S incorporation was significantly greater ($p < 0.0005$) after injection of sera from platelet-depleted sheep than after either normal sheep sera or saline.

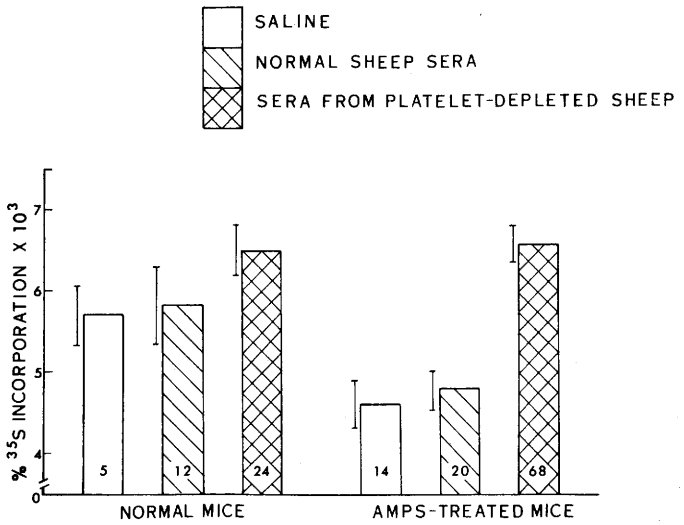

FIG. 3. Sodium ^{35}S-sulfate incorporation by platelets of normal mice and AMPS-treated mice after injections of saline, normal sheep sera, and sera from platelet-depleted sheep. The sheep sera used were from 16 different sheep with platelet counts at the time of bleeding (2–3 hr after ASPS) of 0–14,000/mm^3. The numbers on the bars indicate the number of mice at each treatment and the vertical lines denote SEM.

In AMPS-treated mice, the percent ^{35}S incorporation of saline-treated mice did not differ from mice injected with normal sheep sera. In agreement with the experiment presented in Fig. 2, the data of Fig. 3 illustrate that at this time period (day 8) AMPS-treated mice injected with either saline or normal sheep sera incorporate less ^{35}S in their platelets ($p < 0.05$) than do normal mice, whereas both kinds of assay mice exhibit a similar uptake after injection of TSF-rich sera. The result is a greater difference in ^{35}S uptake in AMPS-treated mice than in normal mice.

Figure 4 shows a summary of nine experiments to test the influence of cold storage on TSF activity. Thrombopoietic stimulating factor-rich sera were injected into some groups of AMPS-treated mice within 20 hr after separation from the RBC and into other groups after storage at —20° for 1 or 3 weeks. Mice injected with normal sheep sera served as controls. Sera from platelet-depleted sheep when tested before storage caused an average increase in ^{35}S incorporation of platelets to 152% of the control values ($p < 0.005$). However, the sera lost TSF activity upon storage. The value for TSF activity at 1 week of storage (116% of control) was reduced but still significantly higher than control ($p < 0.025$). However, after 3 weeks of storage, the ^{35}S-uptake values for the sera from platelet-depleted sheep were the same as those from normal sheep.

Discussion. In previous studies, TSF was assayed by injecting plasma or serum of platelet-poor donors into normal recipients (1–3) and then performing platelet counts at intervals to determine whether there had been a change in the number of circulating platelets (4–7). A great improvement in thrombopoietin assays was the measurement of thrombopoiesis by the incorporation of ^{75}Se-selenomethionine (8–15) or ^{35}S-labeled sodium sulfate (16–20) as a platelet label. In addition to the use of isotopes, some of these workers (8–12, 19) further increased the sensitivity of TSF bioassays by transfusing the recipient animals with platelets to produce thrombocythemia and thereby suppressing platelet production as measured by isotope incorporation. Moreover, sustained thrombocytosis was apparently a more potent suppressor of thrombopoiesis than acute thrombocytosis, since the duration of thrombocytosis directly influenced the level of sup-

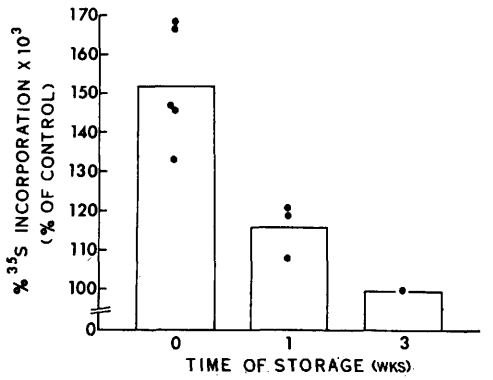

FIG. 4. Sodium ^{35}S-sulfate uptake by platelets of AMPS-injected mice after injections of normal sheep sera or sera from platelet-depleted sheep after various times of storage at —20°. Data are expressed as percent of control (mice injected with normal sheep sera) in each experiment where control values are equal to 100%. Thrombopoietic stimulating factor-rich sera from sheep with platelet counts of 0–10,000/mm^3 were collected 2 hr after ASPS injection. Each point represents the average of 5–8 mice.

pression of thrombopoiesis (12).

Another method of assaying for TSF utilized a hemagglutination-inhibition technique (20); the results indicated that TSF can be detected and quantified in sheep serum. This immunoassay technique may provide an inexpensive method for detection and quantification of TSF, but needs verification by an *in vivo* assay.

The present report has described the use of a specific anti-platelet serum in mice to produce rebound thrombocytosis, and thereby depress thrombopoiesis, as a method of preparation of mice for TSF bioassay. The AMPS consistently produced thrombocytopenia in mice, which was followed by a predictable rebound thrombocytosis (Fig. 1) and a statistically significant ($p < 0.05$) depression of platelet counts about 3 days later (days 9–10). The ^{35}S-sulfate uptake by platelets of these mice showed characteristic changes in production rates: (a) high ^{35}S uptakes when mice were undergoing the most active thrombopoiesis (day 3 after AMPS treatment) and (b) below normal radiosulfate incorporations ($p < 0.05$) on days 6⅔–7⅔ (Fig. 2). Presumably, the thrombocythemia on days 5–6 caused a feedback mechanism (probably the "shutting off" of endogenous TSF production leading to interrupted thrombopoiesis) to depress platelet production for the next 48–72 hr.

Anti-mouse platelet serum-treated mice were more responsive, when compared to their controls, than normal mice to exogenous sources of TSF (Fig. 3), just as exhypoxic polycythemic mice have been shown to be more sensitive to erythropoietin than normal mice (24). In this work, a dose of AMPS was used that caused marked thrombocytopenia within 4 hr. In preliminary experiments, lower doses of AMPS resulted in considerable variation in assay data without significant increase in ^{35}S uptake when the mice were injected with platelet-depleted sheep serum. It seems possible that high anti-platelet serum doses which lead to severe thrombocytopenia are required for significant rebound suppression of thrombopoiesis. Ebbe *et al.* (25) have shown that short-lived episodes of thrombocytopenia caused no shift in the megakaryocyte differential count; however, Odell *et al.* (26) found that sustained thrombocytopenia (caused by repeated injections of anti-platelet serum) produced a demonstrable increase in megakaryocytes.

Penington (13–15) also found an increased and accelerated labeling of platelets in anti-platelet serum-treated mice in comparison with normal animals. Moreover, he reported minimum utilization of isotope on day 8 after anti-platelet serum injection. Presumably, this reduced isotope incorporation rate corresponded to minimum platelet production rates and probably lower endogenous TSF levels in the mice at a time when the regenerating platelet count was the highest. He did not, however, make a direct comparison of normal and anti-platelet serum-treated mice as TSF assay animals.

Although the development of bioassays for TSF is only in the earliest stages, it is tempting to speculate that the mechanisms involved in platelet stimulation and suppression are similar to those in erythropoiesis and its control by erythropoietin. Previous studies (24) have shown that the response to erythropoietin is different in mice made polycythemic by

transfusion than in mice made polycythemic by hypoxia. Exhypoxic mice were more sensitive to erythropoietin than hypertransfused mice. These results were interpreted as evidence that erythropoietin action depends upon the proliferative state of the hematopoietic precursor population or length of postmitotic interval (G_1) of the generation cycle of erythropoietin-sensitive cells. Whether similar mechanisms apply to TSF action is unknown, but studies to determine the potency of TSF in platelet hypertransfused, AMPS-pretreated, and normal mice are being made.

Storage at —20° of TSF-rich sera results in loss of TSF activity (Fig. 4). The present work seems to be in disagreement with Van Dyke *et al.* (27) who reported that TSF will withstand storage in a frozen state, but agrees with Schulman *et al.* (28) and Cooney *et al.* (29) who claim that TSF will not withstand storage. Obviously, much more work is necessary to establish how stable TSF is in storage.

The data presented in this report are consistent with the hypothesis of a feedback mechanism by which platelets can affect the rate of thrombopoiesis. Moreover, the observations presented here support the concept of a humoral agent (thrombopoietin) and its control over platelet production.

Summary. A bioassay for the detection of the thrombopoietic stimulating factor (TSF or thrombopoietin) that utilizes thrombocythemic mice has been presented. A single injection of anti-mouse platelet serum (AMPS) to mice produced a characteristic thrombocytopenia that was followed by rebound thrombocytosis. Two to three days later (7–8 days after the AMPS injection), a significantly ($p < 0.05$) depressed thrombopoiesis existed in response to the thrombocytosis. Normal and thrombocythemic mice were injected with normal sheep sera or sera from platelet-depleted sheep and $Na_2{}^{35}SO_4$ incorporation into platelets was measured. A greater difference in ^{35}S uptake existed between AMPS-treated control mice (mice injected with normal sheep serum) and AMPS-treated stimulated mice (mice injected with serum from platelet-depleted sheep) than in normal mice treated with the same sera. Also, TSF in sheep sera was shown to lose activity after storage at —20°.

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