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Immunological Studies of Thrombopoietin[1] (38377)

T. P. McDONALD

University of Tennessee Memorial Research Center and Hospital, Knoxville, Tennessee 37920

It is now clear that a thrombopoietic stimulating factor (TSF or thrombopoietin) controls blood platelet production. Recent evidence indicates that TSF can be detected and quantified in whole sera or fractions of plasma by both a bioassay and an immunoassay (1). Also, the presence of TSF has been demonstrated in plasma and sera of thrombocytopenic animals (2, 3) and humans with various hematologic disorders (4). However, at the present time only limited knowledge is available concerning the mechanism of action of TSF, its site of production, its target cell, and its chemical nature.

Previously, antisera to erythropoietin (5) and the renal erythropoietic factor (6) have been described. Antisera to erythropoietin have made it possible to study the action of erythropoietin, to determine its site of formation, and its target cell (7). Similarly, an antiserum to thrombopoietin could be utilized to help delineate the mode of action of TSF. In the present work rabbits were immunized with a TSF-rich fraction and the immune serum was used for further studies. The results of these studies reveal that pretreatment of a potent TSF fraction with the immune serum neutralizes the ability of TSF to stimulate thrombopoiesis in assay mice. The anti-TSF serum does not directly affect platelets, but has the ability to interfere with thrombopoiesis in normal mice—as measured by platelet counts and platelet ^{35}S incorporation.

Materials and Methods. The TSF-rich fraction used for immunization was extracted from the serum of a platelet-depleted sheep (No. 966) by use of diethylaminoethyl (DEAE) cellulose-phosphate cellulose column chromatography, according to a method previously described (8). Twenty mg of this material (Lot 17, Fraction II) caused a significant ($P < 0.025$) increase in ^{35}S uptake values in platelets of assay mice injected with $Na_2{}^{35}SO_4$ when compared to a similar fraction obtained from normal sheep sera. Before immunization, the TSF-rich material was coupled to methylated rabbit serum albumin as previously described (6). The material (20 mg in 2.0 ml) was injected subcutaneously (sc) at multiple sites twice a week for 3 weeks (8). The rabbit (No. 667) was bled 7 days after the last injection. The same antigen was used to give booster doses at 3 week intervals; the rabbit was bled 7 days after booster injections. Normal platelet counts were observed in the immunized rabbit.

Ouchterlony gel diffusion studies (9) were carried out in Agar-gel immunodiffusion plates (Hyland Laboratories, Inc.). The antigens were added 30 min after the antisera had diffused into the Agar. The TSF preparations used in the Ouchterlony experiments were the same as used in later experiments; these fractions were prepared by ethanol (EtOH) precipitation techniques from normal sheep plasma and plasma from platelet-depleted sheep (1).

The effect of anti-TSF on thrombopoiesis in normal mice was investigated. In these experiments, groups of mice were given four daily intraperitoneal (ip) injections of one of the following materials: (a) 0.5 ml of saline; (b) 0.25 or 0.5 ml of normal rabbit serum (normal sheep serum added 1:16 (v/v)); or (c) 0.25 or 0.5 ml of rabbit anti-sheep TSF sera (absorbed 1:16 (v/v) with normal sheep serum). Platelet counts (determined by direct counting under phase microscopy) were performed on some of these mice at

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days 0–4 from a single drop of blood obtained by retroorbital puncture (3). On the third day, the mice were injected intravenously (iv) with 30 μCi of ^{35}S-sodium sulfate and the 24 hr ^{35}S incorporation into platelets was measured (3). At time of assay the mice were injected ip with a heparin-Nembutal solution, blood for platelet counts was obtained, and the mice were bled from the heart into plastic syringes containing EDTA. Platelets were separated by differential centrifugation (3) and resuspended into Isoton (Coulter). Platelet samples were placed in counting vials for determination of radioactivity. Other samples were further diluted in Isoton and platelets counted by use of an Electrozone/Celloscope (Particle Data, Inc.). The percent ^{35}S incorporations of the platelets were calculated as previously described (3).

In other experiments antiserum to TSF was tested for its ability to neutralize the biological activity of TSF. The TSF-rich fractions were obtained from plasma of platelet-depleted sheep by EtOH extraction (1). The anti-TSF sera (0.25 ml) were absorbed 1:16 v/v with normal sheep sera, mixed with TSF-rich EtOH fractions (2.0 mg of 60–80% EtOH or 7.5 mg 40–60% EtOH fraction/mouse) and incubated for 1 hr at 37° followed by further treatment with sheep anti-rabbit gamma globulin (SARGG; one part SARGG to eight parts anti-TSF, v/v) for 1 hr at 37° and 4° for approximately 16 hr. For positive controls, TSF and normal rabbit serum (normal sheep sera added 1:16, v/v) were incubated as outlined above. For negative controls, a similar fraction of normal sheep plasma and absorbed anti-TSF sera or normal rabbit serum (with normal sheep sera added) were prepared. After refrigeration overnight the incubates were centrifuged to remove the precipitate and the supernatant fluid was injected into assay mice.

Assay mice were prepared as previously described (3). The assay utilizes thrombocythemic mice after a single ip injection of rabbit anti-mouse platelet serum (RAMPS), which produces a characteristic thrombocytopenia within a few hours followed by rebound thrombocytosis. The result is a greater difference between the control and experimental mice because of the lower baseline of ^{35}S incorporation of platelets by these specially prepared mice. One-half ml of the supernatant material of one of the above incubation mixtures was injected sc into assay mice two times on the fifth and sixth days after the start of the assay procedure. Sixteen hours after the last injection, 30 μCi of $Na_2{}^{35}SO_4$ in 0.5 ml of isotonic saline were given iv. The percent ^{35}S incorporation into circulating platelets was measured 24 hr later in blood samples obtained by cardiac puncture as previously described (3).

Results. Immune sera from three bleedings of one rabbit (No. 667) immunized with TSF (Lot 17, F-II) were tested for their ability to form lines against antigens in Ouchterlony gel diffusion tests. The immunizing protein, TSF preparations (obtained by EtOH precipitation techniques) from two different thrombocytopenic sheep, fractions 40–60 and 60–80 EtOH from normal sheep plasma, and normal sheep sera were used as antigens. The results are shown in Table I. Before absorption with normal sheep serum, anti-TSF sera formed precipitin lines with all the antigens used; after absorption of the antisera, however, lines were not detectable against these antigens. The antisera after absorption with normal sheep sera still contained neutralizing antibodies to TSF although precipitin lines were no longer detectable.

Figure 1 shows the results of daily injections of 0.5 ml/day of normal rabbit serum and absorbed anti-TSF sera on platelet counts of normal mice. As shown, platelet counts of control mice varied from 8.25 to 9.00 $\times$ 10^5 platelets/mm^3. The injections of anti-TSF serum into normal mice did not reduce the platelet counts of mice until day 2 (after two daily injections). The platelet counts then decreased at a rapid rate reaching 6.25 $\times$ 10^5 platelets/mm^3 on day 3 and then leveled off at significantly ($P < 0.005$) lower platelet counts on day 4 when compared to the platelet counts of control mice. These data indicated that the action of anti-TSF serum was not on the platelets themselves, but on a precursor of platelet production, probably thrombopoietin.

Figure 2 shows a summary of two experiments in which normal mice were injected ip with 0.5 ml of normal rabbit serum or anti-TSF serum for 4 consecutive days (total dose of 2.0 ml of sera/mouse). Twenty-four hours after the $Na_2{}^{35}SO_4$ injection and the last serum injection the mice were killed and the percent ^{35}S incorporation measured. A significant depression ($P < 0.0005$) in platelet production, as measured by ^{35}S incorporation, was found after injections of anti-TSF serum as compared with con-

TABLE I. Summary of Precipitin Lines Formed by the Immunodiffusion Technique using Anti-TSF Sera Before and After Absorption Against TSF-Rich Fractions, Fractions of Normal Sheep Plasma, and Normal Sheep Sera.

Anti-TSF Sera No.	Bleeding date	Lot 17[d] F-II	Lot 275[e] 40–60	Lot 275[f] 60–80	Lot 277[g] 40–60	Lot 305[h] 60–80	NSS[c] 966
667	3/13/72	+[b]	+	+	+	+	+
667	4/4/72	+	+	+	+	+	+
667	11/9/71	+	+	+	+	+	+
667 absorbed with NSS 966 (1:16)	3/13/72	−[a]	−	−	−	−	−
667 absorbed with NSS 966 (1:16)	4/4/72	−	−	−	−	−	−
667 absorbed with NSS 966 (1:16)	11/9/71	−	−	−	−	−	−

[a] −: No lines were formed.

[b] +: One or more lines were present.

[c] NSS: Normal sheep serum.

[d] Lot 17, F-II: Immunizing protein for rabbit No. 667.

[e] Lot 275, 40–60: EtOH fraction from normal sheep plasma.

[f] Lot 275, 60–80: EtOH fraction from normal sheep plasma.

[g] Lot 277, 40–60: EtOH fraction from plasma of sheep No. 733, bled 12 hr after RASPS injection (platelet count 500/mm^3).

[h] Lot 305, 60–80: EtOH fraction from plasma of sheep No. 775, bled 4 hr after RASPS injection (platelet count 3500/mm^3).

trol mice. These data indicated that anti-TSF serum contained a factor which inhibited platelet production.

The experiments outlined above were made in normal mice utilizing a total dose of 2.0 ml of serum/mouse. It seemed possible that lower doses of the antiserum might also reduce the platelet production rates. The experiment presented in Table II shows the results of injecting mice with 0.25 ml of sera/day for 4 days (total dose of 1.0 ml). Platelet counts and ^{35}S incorporations were determined as before. As shown, 4 days after the start of serum injections the platelet counts and percent ^{35}S incorporations of platelets from mice injected with anti-TSF serum were significantly reduced ($P < 0.05$) as compared with control values. The average percent ^{35}S incorporation of mice injected with 1.0 ml of anti-TSF sera was 66% of the control values (Table II), whereas mice injected with 2.0 ml of antiserum had values of 59% of the control (Fig. 2).

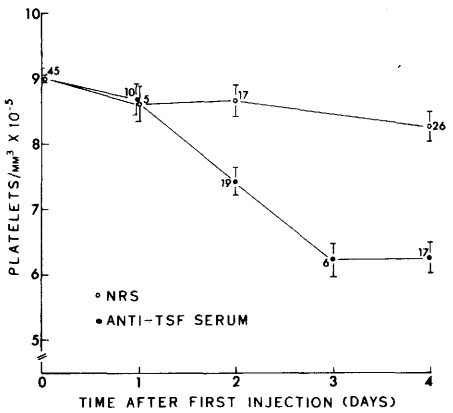

FIG. 1. Peripheral platelet counts of normal mice following injection of normal rabbit serum (NRS) and anti-TSF serum. The numbers next to the points represent the number of mice at each point and the vertical bars indicate the standard error of the mean. Mice were injected ip with 0.5 ml serum/day for 4 days. Antiserum No. 667 (3-13-72) was used after absorption with normal sheep serum (1:16, v/v).

Antiserum to TSF was tested for its ability to neutralize the biological activity of TSF by measuring its capacity to block the stimulation of thrombopoiesis in assay mice. The anti-TSF serum (0.25 ml/mouse) was added to 7.5 mg of 40–60% ethanol fraction of plasma from a thrombocytopenic sheep or 2.0 mg of 60–80% ethanol fraction of plasma from platelet-depleted sheep. For controls normal rabbit serum was added to the same TSF-rich EtOH preparations. The second set of controls included the addition of anti-TSF and normal rabbit serum to similar fractions prepared from normal sheep plasma. Figure 3 shows the results of the two experiments in which the biological activity of thrombopoietin was measured after pretreatment with anti-TSF and precipitation of the antigen-antibody complex with SARGG. As shown, TSF

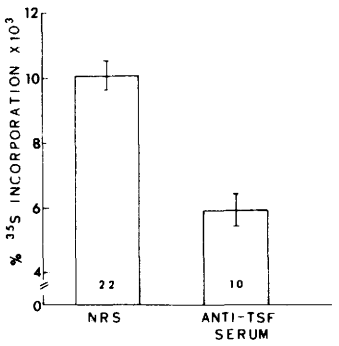

FIG. 2. $Na_2{}^{35}SO_4$ incorporation into mouse platelets following injection of normal rabbit serum (NRS) or anti-TSF serum. The numbers on the bars indicate the number of mice in each treatment group and the vertical lines indicate the standard error of the mean. Each mouse was injected ip with 0.5 ml of serum for 4 consecutive days (total dose 2.0 ml/mouse) and ^{35}S incorporation into platelets measured 24 hr after the last injection. Antiserum No. 667 (3-13-72) was used after absorption with normal sheep serum.

fractions, when incubated with normal rabbit serum, still produced a significant thrombopoiesis in assay mice as compared to control sheep plasma fractions incubated with normal rabbit serum. However, when the same thrombopoietin-rich material was incubated with anti-TSF serum the thrombopoietic activity was abolished.

Discussion. In this report evidence has been presented in support of previous work (8) that antibodies to TSF were produced in rabbits injected with heat denatured TSF conjugated to methylated rabbit serum albumin. Although antisera active against erythropoietin (5) and erythrogenin (6) have been described, neutralizing antibodies to TSF have not been previously reported. Anti-erythropoietin antibodies formed against sheep plasma erythropoietin or human urinary erythropoietin have been shown to neutralize completely the biological activity of erythropoietin *in vitro* (5). Other studies (5–7) have shown these antibodies to be effective in reducing erythropoiesis of intact mice. Moreover, these antibodies have the ability to fix complement and are capable of producing passive cutaneous anaphylaxis. By use of *in vivo* neutralization tests and hemagglutination studies two different types of antibodies to erythropoietin have been described. One type neutralizes the biological activity of erythropoietin and the other causes hemagglutination (5). In the present work antibodies raised against a TSF-rich fraction of sheep serum were shown to be effective in reducing thrombopoiesis of mice (Table II and Fig. 2). In addition, these antisera had no direct effect upon platelets (Fig. 1).

The characteristics of antibodies to the renal erythropoietin factor (REF or erythrogenin) have not been as extensively studied; however, experiments (6) indicated that the erythropoietic activity of REF, as assayed in the polycythemic mouse, was neutralized *in vitro* by addition of serum obtained from a rabbit previously immunized with an REF preparation. These anti-REF sera had no effect on the biological activity of erythropoietin, however, a depression in erythropoiesis was observed after injection of anti-REF into normal mice. These data indicated that the injection of an antibody developed against the REF into normal mice interrupted normal erythropoiesis by reducing REF levels; the reduced REF levels led to decreased amounts of erythropoietin (6).

In agreement with the results obtained for antisera active against erythropoietin and erythrogenin, the results of the present work indicate that serum from the TSF immunized rabbit

TABLE II. Depression of Thrombopoiesis in Normal Mice Following Injections of Sera Obtained from a Rabbit Immunized with a TSF-Rich Fraction.

Material[a]	No. mice	Platelet counts ($\times 10^{-5}$/mm^3 ± SEM) at time of assay	Percent ^{35}S incorporation $\times 10^3$ ± SEM
Saline	5	8.90 ± 1.62	7.742 ± 1.615
Normal rabbit serum + normal sheep serum	5	8.49 ± 0.64	7.032 ± 0.800
Rabbit anti-TSF serum absorbed with normal sheep serum	5	6.11 ± 0.27[b]	4.658 ± 0.635[b]

[a] 1.0 ml/mouse; material was divided into four equal doses and injected ip into mice on 4 consecutive days.
[b] Significantly lower than control ($P < 0.05$). Anti-TSF serum No. 667 (3/13/72) was used.

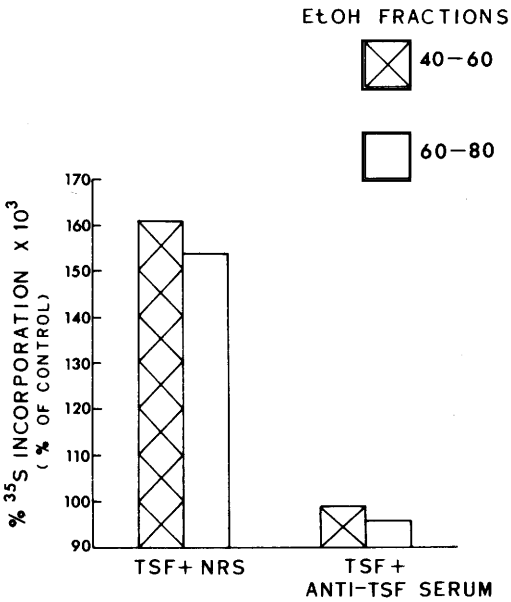

FIG. 3. Two experiments showing sodium ^{35}S-sulfate uptake by platelets of RAMPS-injected mice after injections of EtOH fractions from normal sheep plasma or plasma from platelet-depleted sheep that had been incubated either with normal rabbit serum (NRS) or anti-TSF serum. Data are expressed as percent of control (mice injected with fractions of normal sheep plasma and incubated with either control rabbit serum or anti-TSF serum) in each experiment where control values are equal to 100%. TSF-rich fraction 40–60% (Lot 277) was obtained from a sheep with a platelet count of 500/mm^3 (12 hr after RASPS injection). Anti-TSF serum No. 667 (4/4/72) was used in this experiment. TSF-rich fraction 60–80% (Lot 305) was obtained from a sheep with a platelet count of 3,500/mm^3 (4 hr after RASPS injection). Anti-TSF serum No. 667 (11/9/71) was used in this experiment. Each bar represents the average of five to six mice.

formed precipitin lines against the immunizing material, normal sheep sera, and other control or TSF-rich EtOH fractions of plasma (Table I). However, after absorption with normal sheep sera no lines were formed by the immune sera when tested against these antigens. Although precipitin lines were abolished, the antisera still contained TSF neutralizing antibodies (Fig. 3).

In other studies, a TSF-rich serum fraction was used to immunize rabbits and the immune serum was found to contain hemagglutinating antibodies (8, 10). The hemagglutinating antiserum was absorbed with normal sheep serum to remove antibodies not specific to TSF. The absorbed antiserum containing TSF-specific antibodies was then used to detect TSF in platelet-depleted sheep serum by use of the hemagglutination-inhibition assay for TSF. In the present work, non-precipitating TSF-neutralizing antibodies have been described.

The information in the present report confirms and extends a number of the findings previously reported concerning TSF action. The injection of anti-TSF serum into normal mice resulted in reduced thrombopoiesis. This finding adds to the evidence, along with the demonstration that transfusion (11) or immunothrombocythemia (3) will suppress thrombopoiesis in mice and rabbits, that the TSF participates normally in the day-to-day regulation of thrombopoiesis.

Anti-TSF serum will be valuable in kinetic studies and in determination of the site of TSF production. Although not directly proven, it seems most likely that the neutralization of TSF activity in the experiments reported is the result of an antigen–antibody reaction. The antibody involved may be directed either against the TSF molecule or to the class of proteins to which this factor belongs. The data indicate that the antibody neutralized the biological activity of endogenous TSF; the reduced TSF levels then led to decreased thrombopoiesis.

Summary. Immunological studies of the thrombopoietic stimulating factor (TSF) have been described. The experiments indicated that the thrombopoietic activity of TSF, as assayed in the immunothrombocythemic mouse, was neutralized *in vitro* by addition of serum obtained from a rabbit previously immunized with a TSF-rich preparation. The anti-TSF serum had no effect on platelets; however, a depression in thrombopoiesis was observed after injection of anti-TSF serum into normal mice. It is concluded that the injections into normal mice of an antibody developed against the TSF, interrupts normal thrombopoiesis by reducing endogenous TSF levels.

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