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Neutralizing Antiserum to Thrombopoietin[1] (40245)

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Antisera developed against a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) extracted from sheep plasma have been shown to neutralize the biological activity of other TSF-rich materials (1) and have been used to develop an immunoassay for TSF (2). Other studies (3) showed that antisera raised against TSF-rich kidney cell culture medium and human urinary TSF contained hemagglutinating antibodies and that these antisera could also be used for the immunoassay of TSF found in kidney cell culture medium and human urine fractions. The results of this study (3) indicated similarities in the antigenic determinants of TSF obtained from different sources. These antisera, however, have not previously been tested for their ability to neutralize the biological activity of TSF. Neutralizing anti-TSF sera are needed to continue the study of TSF and its mechanism of action. Therefore, in the work to be presented, antisera raised against TSF from human urine and plasma and kidney cell culture medium were tested for their ability to neutralize the biological activity of TSF in kidney cell culture medium.

Materials and methods. Thrombopoietin for immunization (Table I) was prepared by partial purification of culture medium from human embryonic kidney cells (3, 4), human urine from thrombocytotic patients (5), and plasma from thrombocytopenic patients (5). For absorption of the antisera, control medium, which had not been used for cell culture, was prepared in the same manner as TSF-rich medium. Additional absorbing materials include extracts of normal human urine and normal human plasma.

Immunothrombocythemic mice were used (6) to determine the TSF content of the immunizing proteins (Table I) and to test for TSF in culture medium after incubation with serum from rabbits that had been immunized with TSF-rich materials. For the TSF assay, mice in rebound-thrombocytosis were injected with TSF-test substances, and platelet counts and % ^{35}S incorporation into circulating platelets were determined (6). The data were evaluated by Student's *t* test.

Anti-TSF sera were prepared in rabbits by use of heat-denatured TSF-rich materials coupled to methylated rabbit serum albumin (2). A crude fraction of plasma from a thrombocytopenic patient was used to immunize rabbit No. 006 (Table I). Before immunization, the material was tested for TSF activity by use of the aforementioned TSF immunothrombocythemic mouse assay. The results showed significant increases in % ^{35}S incorporations into platelets of mice injected with Lot 253 Fraction II + III when compared to a similarly prepared fraction of normal human plasma. The other immunizing proteins (shown in Table I) for rabbits No. 042, 054, 072 and 078, were assayed for TSF content and the results have been reported previously (3). The rabbits were injected subcutaneously at multiple sites twice a week for three weeks and were bled seven days after the last dose and after monthly booster doses of the same antigens (2). Normal platelet counts were observed in the immunized rabbits.

In the experiments to be reported, antisera to TSF were tested for their ability to neutralize the biological activity of TSF in kidney cell culture media (4). In all experiments, the anti-TSF sera and normal rabbit serum were heat inactivated by placing them into a 56° water bath for 30 min. After heat inactivation, the antisera were absorbed to remove nonspecific antibodies by incubation with either normal human serum (1:16–1:64, V/V) or control medium (1:16–1:64, V/V) at 37° for 1 hr, followed by additional incubation at 4° for 16 hr. The amount of absorbing material was determined by use of a microabsorption technique that has been described previously (2). After incubation, the incubates were cen-

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TABLE I. IMMUNIZING PROTEIN AND ABSORBING MATERIAL OF ANTI-TSF SERA USED IN THESE STUDIES.

Rabbit No. (antisera)	Immunizing Protein	mg protein/immunization	Absorbing material
006	TSF-rich plasma fraction[a]	20	NHS[e]
042	TSF-rich urine fraction[b]	0.22	NHS
054	TSF-rich fraction from kidney cell culture medium[c]	10	CM[f]
072	TSF-rich fraction from kidney cell culture medium[d]	10	CM
078	TSF-rich fraction from kidney cell culture medium[d]	10	CM
Normal rabbit serum			NHS or CM

[a] Starting material: Lot 253 Fraction II + III; fraction of plasma from a patient with ITP; plt. ct. <10,000/mm^3.
[b] Starting material: Lot 279 A_2; fraction of urine from a thrombocytotic patient; plt. ct. 1×10^6/mm^3 (Ref. 3).
[c] Starting material: Lot 297 Fraction II + III − HP-38; fraction of medium after kidney cell growth (Ref. 3).
[d] Starting material: Lot PM-56 EC; fraction of medium after kidney cell growth (Ref. 3).
[e] NHS—Normal human serum.
[f] CM—Control medium (medium without kidney cell culture).

trifuged to remove precipitates and the supernatant fluids were removed. The absorbed antisera and normal rabbit sera were each mixed separately with TSF and placed in a water bath at 37° for 1 hr, followed by an additional incubation at 4° for 54 hr. After incubation of TSF with antisera or normal rabbit sera, the mixtures were further absorbed with sheep anti-rabbit gamma globulin (SARGG) for 1 hr at 37° (1 part SARGG to 8 parts anti-TSF or normal rabbit serum, V/V) to remove excess rabbit gamma globulins and/or antisera-TSF complexes (1). Further incubation was at 4° for 16 hr. For positive controls, TSF and normal rabbit sera (normal human serum or control medium was added 1:16, V/V to the normal rabbit serum) were incubated as outlined above and treated in the same manner as TSF-anti-TSF groups. For negative controls, saline was incubated with normal rabbit serum (with control medium added). After refrigeration at 4° overnight, the incubates were centrifuged to remove precipitates and the supernatant fluids were injected into TSF assay mice (6).

Results. Antiserum to TSF was tested for its ability to neutralize the biological activity of TSF by measuring its capacity to block the stimulation of thrombocytopoiesis in assay mice. In the first experiment (Table II), the injection of TSF that had been incubated with normal rabbit serum gave significant ($P < 0.005$) increases in % ^{35}S incorporation into platelets and platelet counts of assay mice when compared to the saline-normal rabbit serum control values. However, when the same TSF was incubated with sera from a rabbit that had been immunized with TSF there was a marked decrease in % ^{35}S incorporation into platelets and platelet counts of mice, indicating that the immune sera neutralized almost all of the biological activity of TSF.

TABLE II. $Na_2{}^{35}SO_4$ INCORPORATION INTO PLATELETS OF MICE AFTER INJECTION OF TSF AND TSF THAT HAD BEEN INCUBATED WITH SERUM FROM A RABBIT IMMUNIZED WITH A FRACTION OF TSF-RICH KIDNEY CELL CULTURE MEDIUM.[a]

Material	plt. cts. $\times 10^{-5}$ ± SE	% ^{35}S incorporation $\times 10^4$ ± SE
[Saline + NRS] + SARGG	7.50 ± 0.25	31.4 ± 1.7
[TSF + NRS] + SARGG	9.51 ± 0.42*	47.5 ± 3.3*
[TSF + AS 072] + SARGG	8.29 ± 0.11^{NS}	38.4 ± 5.9^{NS}

[a] TSF—PM-75-C300, 30 mg/mouse. NRS—Normal rabbit serum, absorbed 1:16 with control medium. Antiserum (AS) 072 (9/14/76) was absorbed with control medium (1:16, V/V). Each mouse was injected with the product of incubation: 0.5 ml of AS and 30 mg of TSF in 1.5 ml of saline. SARGG—Sheep anti-rabbit gamma globulin serum. Both NRS and AS were heat inactivated, absorbed, and incubated with TSF as described in the Methods Section. Five mice were used in each group. Values were significantly elevated when compared to control, * $P < 0.005$; NS, values were not significantly different from control.

Figure 1 shows the results of four additional experiments in which saline, TSF incubated with normal rabbit serum, and TSF incubated with anti-TSF sera were all ab-

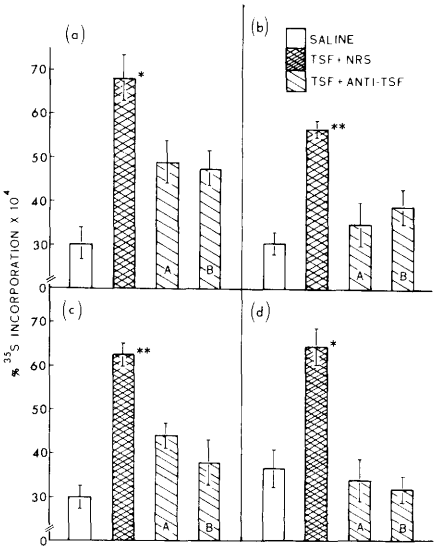

FIG. 1. The % ^{35}S incorporation into platelets of immunothrombocythemic mice after injection of saline, thrombopoietin (TSF) incubated with normal rabbit serum (NRS), and TSF that had been incubated with various anti-TSF sera. Both NRS and anti-TSF sera were heat inactivated, absorbed with control medium (1:16, V/V) or normal human sera (1:16–1:64, V/V), and incubated with TSF (Lot 68-041) as described in the Methods Section. Each bar is the average of five mice and the vertical lines represent the standard errors. The values are significantly different from saline-control; $*P < 0.005$; $**P < 0.0005$. Each panel shows the results of injecting saline, TSF incubated with NRS, and anti-TSF sera incubated with the same TSF on ^{35}S uptake by platelets of assay mice. Panel a, shows the results of Antisera (AS) 072 (5/10/76, Bar A) and AS 078 (4/30/76, Bar B) used at a dose of 0.5 ml/mouse. Panel b, shows the results of AS 006 (11/18/75, Bar A) and AS 042 (6/3/75, Bar B) used at a dose of 0.5 ml/mouse. Panel c, shows the results of AS 078 (4/20/76, Bar A) and AS 054 (12/12/74, Bar B) used at a dose of 0.5 ml/mouse. Panel d, shows the results of AS 042 (3/9/76, 0.125 ml/mouse, Bar A; and 0.25 ml/mouse, Bar B).

sorbed with SARGG, and their supernatant fluids injected into TSF-assay mice. In all cases, the injection of TSF that had been incubated with normal rabbit serum gave highly significant increases in % ^{35}S incorporation into platelets of mice ($P < 0.005$–$P < 0.0005$) when compared to saline-injected control mice. Also, in all experiments, anti-TSF sera caused significant decreases in % ^{35}S incorporation into platelets of mice when compared to mice injected with TSF that had been incubated with normal rabbit serum ($P < 0.025$–$P < 0.0005$). Some of the antisera resulted in greater reduction of ^{35}S values than others, indicating varying antisera titers. However, in all cases the results of injecting mice with TSF that had been incubated with anti-TSF gave ^{35}S values that were not different statistically from values of saline-treated control mice.

Discussion. Results of the present study have shown that antisera developed against TSF from human urine, human plasma, and kidney cell culture medium will neutralize the biological activity of TSF found in kidney cell culture medium. The results of this work agree with previous studies (1) in which it was shown that the thrombocytopoietic 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 fraction from sheep plasma that was rich in TSF.

The data of the present report also extend and agree with previous results (3) which have shown that TSF could be detected in kidney cell culture medium and human urine fractions by both a bioassay and an immunoassay using antisera raised against TSF-rich kidney cell culture medium and human urinary TSF. In these studies, both antisera cross-reacted with the two sources of TSF and gave essentially the same results in the hemagglutination-inhibition assay. These results suggested similarities in the antigenic determinants of these two TSF preparations. Not only did these antisera contain hemagglutinating antibodies, but as shown in the present report, some bleedings of sera from these rabbits also contained anti-TSF neutralizing antibodies.

The data of this study and previous work (3, 4, 7, 8) support the conclusion that TSF in kidney cell culture medium is similar to or identical to TSF from thrmobocytopenic patients and animals, e.g., the medium from kidney cell cultures increases % ^{35}S incorporation into platelets (4) and platelet counts (7). TSF-rich kidney cell culture medium also stimulates megakaryocytopoiesis *in vivo* (Odell et al unpublished observations) and *in vitro* (8) and the material has immunologic similarities (3) to TSF extracted from human urine. It should also be mentioned that the culture media do not contain erythropoietin,

erythrogenin, or *in vivo* white-blood-cell-stimulating factors (4).

The results presented in this work along with other studies (9–12) tend to support the hypothesis that the kidney elaborates TSF. Antisera raised against extracts rich in TSF from (a) plasma of thrombocytopenic patients, (b) human urine, or (c) kidney cell culture media were shown to neutralize the biological activity of potent TSF preparations from kidney cell cultures. The antisera developed in the present study will be valuable for determining the mechanism of action, site of production, target cell, and chemical nature of TSF.

Summary. Potent thrombopoietin (TSF) materials were mixed with normal rabbit serum and serum from rabbits that had been immunized with TSF-rich fractions of human plasma, human urine, or kidney cell culture media; the supernatant fluids from these incubations were then assayed for TSF content by means of the immunothrombocythemic mouse assay. The results indicate that TSF, after incubation with normal rabbit serum, stimulated platelet production in mice, whereas pretreatment of the TSF with anti-TSF serum neutralized the ability of TSF to stimulate thrombocytopoiesis in mice. These data support the hypothesis that TSF found in kidney cell culture medium is immunologically similar to TSF from human urine or plasma.

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