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Monoclonal Antibodies to Human Urinary Thrombopoietin[1] (42321)

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Abstract. Monoclonal antibodies (MA) to a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) were obtained from hybridomas derived from the fusion of P3 × 63/Ag 8 cells and spleen cells from TSF-immunized BALB/c mice. The immunizing protein was a partially purified TSF-rich preparation from the urine of a thrombocytopenic patient, and was shown to stimulate platelet production in rebound-thrombocytotic mice; i.e., platelet counts of recipient mice were increased to 133% of control and the values for percentage ^{35}S incorporation into platelets were elevated to 225% of control. Media from several hybrid cultures were tested in a microantibody detection technique that measured the binding of MA to a ^{125}I-purified TSF preparation from human embryonic kidney (HEK) cells. The immune complex was precipitated by the addition of goat anti-mouse IgG serum and centrifugation. One clone gave 25% binding of ^{125}I-TSF after a sevenfold dilution of the medium. This cell line was recloned and four of the subclones produced MA that gave even greater binding capacities. Hybridized cells were injected into "pristane-primed" mice and the antibodies produced in the ascites fluid were also shown to bind the ^{125}I-TSF. Compared to the results of normal mouse serum, ascites fluid containing MA was shown to bind the unlabeled TSF from HEK cells. The TSF activity was significantly reduced in the supernatant fluid after precipitating the TSF–anti-TSF immune complex by a second antibody when tested in an immunothrombocythemic mouse assay. After SDS–PAGE, the precipitate from this TSF–MA conjugate showed that the antiserum bound a single 32,000 mol wt component, indicating the monospecificity of the MA. MA directed toward human TSF will allow studies that were not previously possible.

Heterologous antibodies to a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) have been raised in rabbits against TSF from thrombocytopenic sheep and humans and human embryonic kidney (HEK) cell culture medium (1–4). These antibodies were shown to neutralize the biological activity of TSF (2, 3) and to cause hemagglutination of TSF-sensitized RBC (1, 4). Since other studies have shown that monoclonal antibodies (MA) can be produced against human erythropoietin (5, 6), we attempted to produce MA to human urinary TSF by use of previously described techniques (7). The results show that the MA that were produced will bind both a highly purified ^{125}I-TSF preparation and unlabeled TSF found in crude preparations; the antibodies are of the IgG1 subclass and are monospecific for a 32,000 mol wt component found in crude TSF-rich culture medium.

Materials and Methods. *Immunizing protein.* Mice were immunized with a partially purified TSF-rich preparation (Table I) from urine of a thrombocytopenic patient that was prepared by DEAE-column chromatography (8). As shown, the TSF-rich preparation (TSF Lot 431 A_2) gave increased platelet counts ($P < 0.01$) and elevated the percentage ^{35}S incorporations into platelets ($P < 0.005$) of assay mice when compared to values obtained from other mice that were injected with similar preparations from normal human urine.

Immunization. BALB/c mice were immunized by intraperitoneal (ip) injections of TSF Lot 431 A_2. The antigen was prepared by mixing 0.1 mg of the partially purified TSF with 1% alum solution immediately before injection. After a series of injections (2 times a week for 3 weeks followed by monthly booster injections), plasma from blood obtained by retroorbital puncture was tested for antibodies by immunodiffusion techniques (9). When plasma showed strong precipitating lines against the immunizing protein, the mice were given a final booster of antigen and were killed 3 days later.

Hybridization of cells. Mice were killed by cervical dislocation and the spleen was removed aseptically. A single cell suspension was

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TABLE I. ACTIVITY OF A PARTIALLY PURIFIED TSF-RICH HUMAN URINARY PREPARATION USED TO IMMUNIZE MICE AS TESTED IN THE IMMUNOTHROMBOCYTHEMIC MOUSE ASSAY

Material	Dose/mouse (mg)	No. of mice	Platelets ($\times 10^{-6} \pm$ SE)	% ^{35}S incorporation ($\times 10^{3} \pm$ SE)
403 A_2	2.0	5	0.70 ± 0.06	5.6 ± 0.6
431 A_2	2.0	5	0.93 ± 0.03*	12.7 ± 1.2**

Note. 403 A_2 = Fraction of normal urine obtained by DEAE-column chromatography. 431 A_2 = Fraction of urine from a thrombocytopenic patient (91,000/mm^3) obtained by DEAE-column chromatography. Values were significantly increased over controls: *$P < 0.01$, **$P < 0.005$.

obtained and suspended in Dulbecco's modified medium that was supplemented with 20% fetal bovine serum, MEM nonessential amino acids, L-glutamine, and penicillin/streptomycin (all from GIBCO Laboratories, Grand Island, N.Y.). In preparation for fusion, a mouse myeloma cell line P3 × 63/Ag 8 (P3) was passed for 3 consecutive days to produce a culture in log growth phase. Spleen cells (1×10^8) and P3 cells (1×10^7) were washed two times with Dulbecco's modified medium and mixed. The two cell types were fused by adding 1 ml of 50% polyethylene glycol (mol wt 1000) in Dulbecco's modified medium. The reaction was slowed after 1 min by adding Dulbecco's modified medium stepwise to a volume of 50 ml. After washing two times, the hybridized cells were resuspended into Dulbecco's supplemented medium and grown for 24 hr. The cells were then placed in selection medium (HAT medium) which consisted of a 1:100 dilution of Dulbecco's supplemented medium in 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine (Sigma Chemical Co., St. Louis, Mo.).

Cloning. The hybrid cells in HAT medium were diluted into three 96-well microtiter plates and the cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air. The plates were observed daily for cell growth. A sample of the medium from each well that contained a single colony was removed and replaced with Dulbecco's modified medium.

Screening. The media that were removed from the wells of the microtiter plate were tested for the presence of MA by a microantibody detection technique. Cells from wells showing positive titers were plated in a well of a 24-well microtiter plate which contained a feeder layer of spleen cells from a normal mouse. For further cell growth, the cultures were transplanted into either 25- or 80-cm^3 tissue culture flasks.

Iodination of highly purified TSF. A highly purified TSF-rich preparation (10) was iodinated by a previously published technique (11). For iodination, 0.1 ml of methylene chloride and 40 μg of a substituted glycoluril (Iodogen, Pierce Chemical) were placed in a glass tube and rotated in a 37°C water bath until only a thin coating remained on the tube. In another tube, 20 μg of the highly purified TSF, 1.0 μg of potassium iodide, 1.0 mCi of ^{125}I, and 160 μl of a 0.1 M Na borate/0.25 M NaCl solution were mixed. Both tubes were cooled in an ice bath for about 5 min. The reaction was then initiated by transferring the ^{125}I-TSF mixture to the tube containing the Iodogen and allowed to react for 5 min in a gently shaking ice bath. The reaction was terminated by decanting the mixture from the residual Iodogen. The mixture was added to a 10-ml Sephadex G-25 fine column that was equilibrated with a radioimmunoassay diluent (RIAD, consisting of 0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl, 0.1% bovine serum albumin, and 0.1% Na azide) and eluted in 0.5-ml fractions into tubes containing 0.5 ml of collecting buffer (0.01 M phosphate buffer, pH 7.4, 0.15 NaCl, 2.0% bovine serum albumin, and 0.1% Na azide). After collecting the tubes, 10 μl of ^{125}I-TSF from each tube was placed into 0.5 ml of RIAD and counted for radioactivity. The tube that contained the ^{125}I bound TSF with the highest activity was saved, aliquoted into 0.1-ml portions, quick frozen using dry ice and acetone, and stored at −20°C in lead pots.

After iodination, the ^{125}I-TSF was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC). For RP-HPLC, a linear gradient was used; 0.1% trifluoroacetic

acid (TFA) in H_2O was solvent A and 0.1% TFA in acetonitrile (AN) was used as reagent B. All mobile phases were prefiltered (0.22 μm, Millipore) and degassed *in vacuo,* aided by brief sonication. A Waters Associates HPLC system was used that utilized a Model 720 controller, Model 6000 and Model M-45 solvent delivery systems, and a Model U-6K injector capable of injections of 0–2000 μl. A Kratos–Schoeffel Instruments Company Model SF 770 variable wavelength uv absorbance detector was used. A Houston Instruments dual-channel recorder (Fisher Scientific) provided elution profiles. The maximum instrument sensitivity for monitoring proteins was approximately 10 ng. Elutions were made at 1.0 ml/min.

Figure 1 illustrates the elution profile of protein and the emission profile for radioactivity of purified TSF following iodination and RP-HPLC chromatography. ^{125}I-TSF was clearly associated with the protein component which, prior to iodination, was bioactive (10). No secondary γ-emission peaks were produced other than normal residuals expected as peptide and/or oligopeptide fragments due to the iodination procedure. Since a single protein peak was observed, this material was utilized for detection of MA.

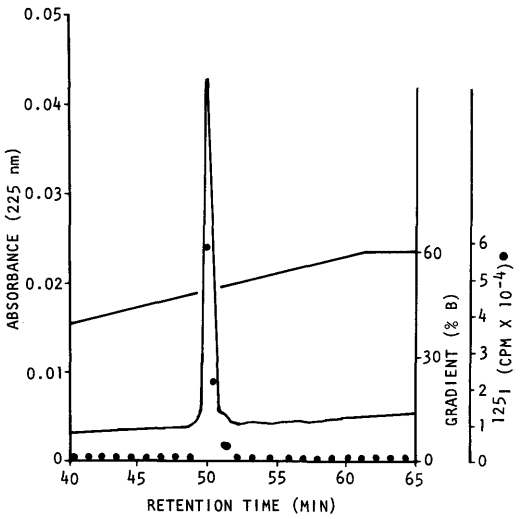

FIG. 1. RP-HPLC profile of ^{125}I-TSF. Injection of 5 μl (140,323 cpm and 1 μg) of ^{125}I-TSF was made on a Brownlee Lab RP-300 column (4.6 × 250 mm). Elution was at 1.0 ml/min. Gradient from 0% A (0.1% TFA/H_2O) to 60% B (0.1% TFA/AN) was at 1%/min. Detection was at A_{225} nm, 0.05 AUFS. ^{125}I counts of TSF were restricted to the single protein peak.

Antibody detection. Fifty microliters of the test materials (primary clone 4IIIA1; reclones R4IIIA1-IE7, -IG2, -IIIF4, and -IIIG2; and ascites fluid HyAb 4-1a) with appropriate controls was added to the first well of rigid U-well microtiter plates. Three additional wells of each row were filled with 25 μl of RIAD. Using a 25-μl dilutor, a serial dilution was then made. Twenty-five microliters of the ^{125}I-TSF, diluted 1:100 in RIAD, was then added to each well. The plate was incubated at 37°C for 1 hr. Following incubation, 0.1 ml of RIAD was added to each well and the plates were stored for 48 hr at 4°C. After storage, 25 μl of either undiluted or a 1:10 dilution of the IgG extract of a second antibody (goat anti-mouse IgG serum, GAMGG) was added to each well. The plate was incubated at 37°C for 1 hr and stored at 4°C overnight. The following day, the microtiter plate was centrifuged at 280*g* for 30 min and 0.1 ml of the supernatant fluid was counted for radioactivity in a γ scintillation counter. Since the supernatant fluid contained the unbound ^{125}I-TSF, the percentage ^{125}I-TSF bound to MA could then be easily calculated.

Ascites fluid production. For ascites fluid production, BALB/c mice were "primed" by an ip injection of pristane (2,6,10,14-tetramethyl pentadecane). Approximately 4 weeks after pristane injection, 1×10^7 hybrid cells were injected ip into these mice and ascites fluid developed in about 2 weeks. Control ascites fluid was obtained from some mice that were injected with pristane but not injected with cells. The ascites fluid from each mouse was collected daily and stored at −76°C until used.

Tests for antibodies that bind unlabeled TSF. Monoclonal antibodies to TSF were tested for their ability to bind unlabeled TSF by measuring its biological activity in supernatant fluid after precipitating the immune complex (3). The ascites fluid from mice injected with hybrid cells and control BALB/c serum (0.25 ml/mouse) were heat inactivated for 30 min at 56°C. Each specimen was mixed separately with 30 mg of crude TSF and placed in a water bath at 37°C for 1 hr, followed by an additional incubation at 4°C for 54 hr. After incubation of TSF with ascites fluid or

control mouse serum, the mixtures were further treated with GAMGG for 1 hr at 37°C (1 part GAMGG to 8 parts ascites fluid or control serum, v/v) to remove excess mouse γ-globulins and/or MA–TSF complexes (2). After refrigeration overnight at 4°C the incubates were centrifuged to remove precipitates and the supernatant fluids were injected into TSF assay mice (12).

Mouse bioassay for TSF. TSF in supernatant fluids was assayed for bioactivity by the immunothrombocythemic mouse assay (12). A single ip injection of rabbit anti-mouse platelet serum (RAMPS), prepared and absorbed with mouse red blood cells as previously described (13), was administered to groups of 8- to 10-week-old C3H male mice 5 days prior to injection of test materials. Administration of RAMPS induced marked thrombocytopenia within 3–4 hr which was followed by rebound-thrombocytosis. During thrombocytosis, groups of mice were injected subcutaneously (sc) with test materials twice each on Day 5 and Day 6 following RAMPS injection. On Day 7, 30 μCi $Na_2{}^{35}SO_4$ in 0.5 ml saline was injected intravenously (iv), and the percentage ^{35}S incorporation into circulating platelets was measured on Day 8 in blood samples obtained by cardiac puncture; platelet counts were made on blood taken from the retroorbital sinus. The data were evaluated by use of Student's *t* test.

Tests for monospecificity of MA. Monoclonal antibodies to TSF were further tested for their ability to bind specifically the TSF in crude preparations by incubation of the MA with TSF and subjecting the immunocomplex to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Ascites fluid (HyAb 4-la, 20 μl) was mixed with the crude TSF (2.4 μg in 160 μl) and incubated at 37°C for 2 hr. Another tube containing control ascites fluid and TSF was prepared and processed in the same manner as the ascites fluid containing MA. After incubation, the mixtures were further treated with 20 μl of GAMGG diluted 1:1 with RIAD, mixed, and incubated at 37°C for 1 hr. Following this treatment, the incubates were kept at 4°C overnight and the precipitates washed two times with cold water. The immune complexes were solubilized with 2-mercaptoethanol and subjected to SDS–PAGE.

Identification of immunoglobulin subtypes. A sensitive enzyme immunoassay method (HyClone Laboratories, Logan, Utah) that utilizes a double-antibody detection system was used for determination of mouse immunoglobulin subtypes in the culture media. In this test, data were obtained for establishing the specific immunoglobulin subclass, i.e., IgM, IgGI, IgG2a, IgG2b, IgG3, and IgA of the MA. For determination of the antibody type in the ascites fluid, SDS–PAGE was used.

Results. Figure 2 shows the results of testing varying dilutions of culture medium from a single cell clone (4IIIA1) for MA. The medium was incubated with iodinated TSF and the binding capabilities of antibodies to ^{125}I-TSF were measured. When compared to control medium, fluid from this clone gave elevated binding of ^{125}I-TSF after a sevenfold dilution; reduced ^{125}I-TSF binding capacities were observed with greater MA dilutions.

Recloning of this cell line gave four subclones that also showed binding capabilities; i.e., 22–38% binding of the ^{125}I-TSF by MA was found after a sevenfold dilution; decreased

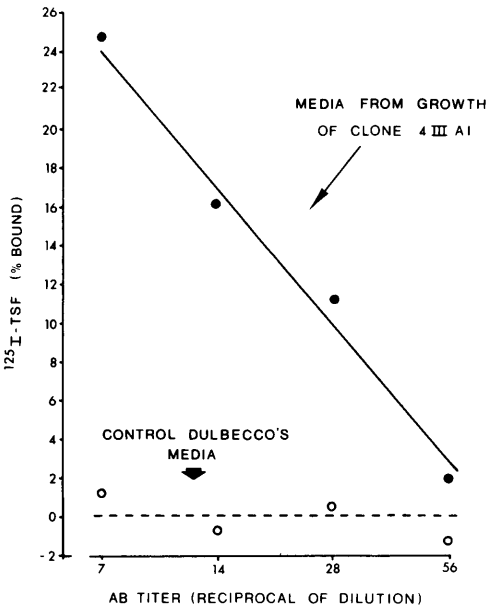

FIG. 2. Medium from a single cell clone (4IIIA1) that was produced by hybridization of spleen cells from a mouse injected with a partially purified TSF with P3 cells in HAT medium. The medium was tested for its ability to bind ^{125}I-TSF, using a microantibody detection technique (see Materials and Methods).

TABLE II. PERCENTAGE ^{125}I-TSF BINDING BY MONOCLONAL ANTIBODIES IN MEDIA OF RECLONED CELL LINES

Reciprocal of dilution	Control Dulbecco's medium	R4IIIA1-IE7	R4IIIA1-IG2	R4IIIA1-IIIF4	R4IIIA1-IIIG2
7	−0.7	37.7	22.1	37.2	29.8
14	−6.9	17.4	13.4	26.2	36.4
28	−0.5	12.5	16.4	2.7	11.0
56	8.2	2.5	14.2	10.5	10.5

binding to 2–14% was found after a 56-fold dilution (Table II).

Table III shows the results of injecting hybrid cells that were producing MA into "pristane-primed" mice and testing the ascites fluid for antibodies. Antibodies in the ascites fluid were shown to bind the ^{125}I-TSF to about 36% regardless of dilution, indicating that the titers of the MA were probably much greater. In each case the titers of the antibodies improved slightly with each more rigid selection procedure; i.e., 25% binding was observed in the original clone, recloning gave 22–38% binding, and antibodies produced in ascites fluid showed 34–37% binding.

Figure 3 shows the results of testing the ascites fluid (HyAb 4-1a) for antibodies that will specifically bind the unlabeled TSF. As shown, TSF alone and TSF incubated with control serum gave significantly ($P < 0.005$) higher percentage ^{35}S incorporations into platelets of assay mice than were found in platelets of mice injected with saline. However, when ascites fluids from mice injected with cells from clone 4IIIA1 were incubated with the TSF, no significant increase in percentage ^{35}S incorporation into platelets was found when compared to the values obtained from mice injected with saline. Moreover, the incubation of HyAb 4-1a with TSF resulted in reduced ($P < 0.05$) incorporation values when compared to results from mice injected with TSF that had been incubated with control serum. These data indicate that MA in ascites fluid will specifically bind the crude-unlabeled TSF, and after addition of the second antibody the TSF can be removed from the supernatant fluid.

The results of subjecting the precipitate of the reaction of the crude TSF-rich material and the ascites fluid (HyAb 4-1a) to SDS–PAGE are shown in Fig. 4. In agreement with the results presented in Fig. 3, these data indicate that a single protein of ~32,000 mol wt was removed from the crude TSF-rich

TABLE III. PERCENTAGE ^{125}I-TSF BINDING OF MONOCLONAL ANTIBODIES FROM ASCITES FLUID PRODUCED BY INJECTING CULTURED CELLS INTO PRISTANE-PRIMED MICE

Reciprocal of dilution	Control ascites	HyAb 4-1a
7	4.2	36.0
14	0.5	34.2
28	−6.1	36.8

Note. Control ascites fluid was produced by the injection of pristane only.

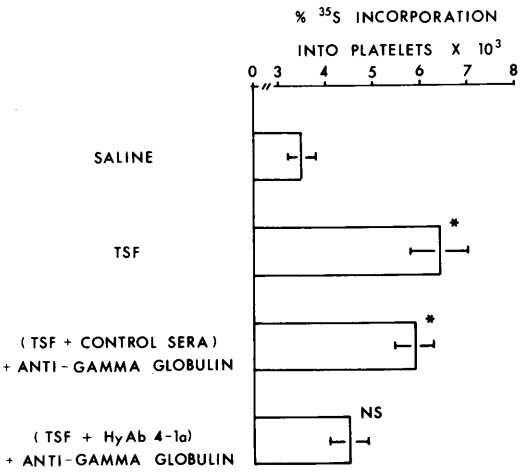

FIG. 3. The effects of ascites fluid (HyAb 4-1a) from a mouse injected with hybrid cells on the biological activity of TSF when tested in the immunothrombocythemic mouse assay. The ascites fluid and control serum (0.25 ml/assay mouse) were heat-inactivated and mixed with TSF (30 mg/mouse) for 1 hr at 37°C, followed by incubation for 54 hr at 4°C. The mixture was further absorbed for 1 hr at 37°C with goat anti-mouse γ-globulin (anti-γ-globulin or GAMGG) to remove antibody–TSF complexes; supernatant fluids were assayed for TSF. Five mice were used in each treatment group and the bars represent the SE; *$P < 0.005$.

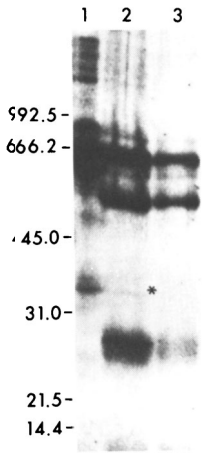

FIG. 4. SDS–PAGE of a crude TSF-rich preparation (lane 1) and precipitates of antibody–antigen reactions of TSF reacted with ascites fluid from a mouse (HyAb 4-1a) that was injected with MA-producing cells (lane 2) and ascites fluid from a control mouse (lane 3). Coomassie blue-stained $NaDodSO_4$/polyacrylamide gel electrophoresis; the molecular weight standards are shown $\times 10^{-3}$ on the left side. A protein band is shown on lane 2 (*) that coincides with a band in the crude TSF-rich preparation (mol wt 32,000) that is not present in the control (lane 3).

preparation by the MA. This protein band was also observed in the crude TSF preparation (lane 1), but not in the control ascites fluid that was incubated with the TSF (lane 3).

The results of determining the type of mouse monoclonal antibody that was present in the culture media of the primary clone and the reclones of hybrid cells, using a commercial subisotyping kit, showed that the media contained a single mouse immunoglobulin of the IgG1 subclass. Also, the control mouse serum contained IgM, IgG1, IgG2a, and IgG2b antibodies. These data support the claim that these cell cultures were developed from a single cell.

Figure 5 shows the results of subjecting control ascites fluid and ascites fluid that developed in mice after injection of MA-producing cells to SDS–PAGE. As shown, two major protein bands, with apparent molecular weights of 52,000 and 23,000, were found in the ascites fluid HyAb 4-1a that were not found in control ascites fluid. These two bands are characteristic of the heavy and light chains of the IgG class of immunoglobulins (5).

Discussion. The present study showed that MA to TSF have been produced. The MA (from the original clone, from recloning the original cell line, and from ascites fluid of "pristane-primed" mice that were injected with cells that were producing MA) were shown to specifically bind an ^{125}I-labeled highly purified TSF preparation. Moreover, the MA found in ascites fluid specifically bound TSF found in a crude preparation.

Thrombopoietin, like erythropoietin (14), is a poor antigen (1–4), and development of MA has not been easy. In the present study, a partially purified human urinary TSF preparation (Table I) was used as the antigen. Several other preliminary studies have shown that the use of the more highly purified TSF-rich preparations as antigens did not produce precipitating antibodies on the initial screen. Spleen cells from some of these mice were hybridized with P3 cells and variable results were obtained. In this study, 9 of 15 mice showed positive antibody production after immunization with crude TSF preparations on the initial screen. After nine fusions and screening of about 250 wells/fusion (total of 2500 wells)

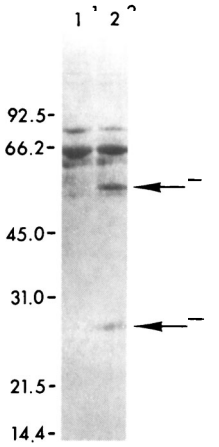

FIG. 5. SDS–PAGE of ascites fluids from a control mouse (lane 1) and from a mouse (HyAb 4-1a) that was injected with MA-producing cells (4IIIA1) (lane 2). Coomassie blue-stained $NaDodSO_4$/polyacrylamide gel electrophoresis showing several protein bands between 92,500 and 45,000 mol wt. Two additional bands are shown in the HyAb 4-1a ascites fluid that are not shown in control ascites fluid, i.e., 52,000 and 23,000 mol wt (arrows). The molecular weight calibration is shown $\times 10^{-3}$ on the left side.

that contained hybridoma cultures, only 54 wells (2%) were positive for the production of MA that were directed against the TSF as measured by the antibody detection technique described herein. Of these positive wells, we have been successful in establishing six cell lines, and the results of one cell line are reported herein.

Partially purified human urinary TSF was used for immunization of the mice and a highly purified TSF from HEK cells was used for the detection of MA, because human urine and serum-free HEK cell culture medium have only a few proteins in common (4), i.e., TSF and possibly CSF, etc. Therefore, this approach has specific immunochemical advantages, and improves the chances of producing a specific MA. As shown herein, MA were produced that bind ^{125}I-TSF, and furthermore, they will selectively bind TSF in a crude preparation.

It was shown in the present report that from 22 to 38% of the ^{125}I-TSF could be bound by the MA. This large variation in binding capacities is probably related to the different titers and dilution of the MA used. For example, further dilutions of the MA will be required (Table III) to reduce the ^{125}I-TSF binding to HyAb 4-1a. It is obvious that reductions in binding capacities are possible, when one considers the control values presented in this table. It is also obvious that the four MA presented in Table II have different antibody titers. Regardless, the goal of demonstrating positive binding, and therefore specificity, of MA to ^{125}I-TSF has been achieved in this study.

Why 100% of the ^{125}I-TSF was not bound is not clear, but the dilution of the culture media by sevenfold before testing, so that enough material would be available for several tests at different dilutions, does not seem to be a reason, since several experiments were performed using very high levels of MA. The results indicate that it was impossible to get 100% binding, regardless of the amount of material used. Moreover, different times of incubation, decreases in the amount of labeled TSF, and different amounts of GAMGG did not result in complete removal of the ^{125}I-TSF. It seems possible, therefore, that TSF may exist in different forms, as has been reported for erythropoietin (15), and the MA may be specific for only one form of the TSF. In agreement with the present results, other studies have noted that only about 45% (5) and 51% (6) of erythropoietin can be bound by MA.

We tested the ability of the MA to react with unlabeled TSF by measuring TSF activity in the supernatant fluids after immunoprecipitation with a second antibody. The results (Fig. 3) showed that when TSF was incubated with ascites fluids from mice injected with cells that were producing MA, the effects of TSF in the mouse bioassay were reduced significantly ($P < 0.05$), but, as expected, not completely. These findings are in agreement with previous results by Weiss *et al.* (5) who showed that incubation of pure erythropoietin with MA directed against erythropoietin resulted in partial removal of the immune complex after precipitation with goat anti-rat immunoglobulin; however, a combination of *Staphylococcus aureus* and rabbit anti-rat immunoglobulin precipitated all of the erythropoietin–antibody complex and the unbound monoclonal antibody, but not all of the ^{125}I bound erythropoietin. Had these workers assayed the supernatant fluids for erythropoietin, there is little doubt that erythropoietin levels would have been reduced. Moreover, the work by Yanagawa *et al.* (6) showed that the erythropoietin–antibody complex was inactive if rabbit anti-mouse IgG absorbed to *S. aureus* was added to specimens containing MA to erythropoietin and unlabeled erythropoietin. Although the two hormones are chemically quite different, it appears that the results of the previous studies are in close agreement with the present work.

In addition to the findings presented in Fig. 3, it should also be mentioned that when the precipitate from a TSF–MA reaction was subjected to SDS–PAGE there was found a single Coomassie blue-positive band with an apparent mol wt of 32,000 that was not found in the control preparation. These data indicate that the MA described herein are monospecific, and they further confirm our previous finding that TSF from HEK cell culture medium has a molecular weight of ~32,000 (10).

The antibodies that are found in the culture media and described in this report are of the IgG1 subclass. In addition, the present study also showed that after subjecting ascites fluid

from mice that were producing MA to SDS–PAGE, two major protein bands with apparent molecular weights of 52,000 and 23,000 were found (Fig. 5). The control ascites fluid did not contain these two protein bands. These data are, therefore, in agreement with previous work by Weiss *et al.* (5) and Yanagawa *et al.* (6). Since the molecular weights of these two protein bands are characteristic of the heavy and light chains of the IgG class of immunoglobulins, and the results of the subtyping experiments showed that the media contained only IgG1 antibodies, it is concluded that the MA described in this report are of the IgG1 subclass.

MA for immunochemical studies of TSF have many advantages over the use of heterologous antibodies. Since TSF in a highly purified state (10) is available only in limited amounts, MA will make possible the large-scale isolation of TSF by immunoaffinity methods. Moreover, MA will aid in further chemical and biological characterization of TSF, make possible the identification of the site of production of the hormone, and provide antibodies for the development of radioimmunoassays for TSF.

1. McDonald TP. The hemagglutination-inhibition assay for thrombopoietin. Blood **41**:219–233, 1973.
2. McDonald TP. Immunological studies of thrombopoietin. Proc Soc Exp Biol Med **147**:513–518, 1974.
3. McDonald TP. Neutralizing antiserum to thrombopoietin. Proc Soc Exp Biol Med **158**:557–560, 1978.
4. McDonald TP, Cottrell M, Nolan C, Walasek O. Immunologic similarities of thrombopoietin from different sources. Scand J Haematol **18**:91–97, 1977.
5. Weiss TL, Kavinsky CJ, Goldwasser E. Characterization of a monoclonal antibody to human erythropoietin. Proc Natl Acad Sci USA **79**:5465–5469, 1982.
6. Yanagawa S-I, Yokoyama S, Hirade K, Sasaki R, Chiba H, Ueda M, Goto M. Hybridomas for the production of monoclonal antibodies to human erythropoietin. Blood **64**:357–364, 1984.
7. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497, 1975.
8. McDonald TP. Assay of thrombopoietin utilizing human sera and urine fractions. Biochem Med **13**:101–110, 1975.
9. Ouchterlony O. Gel-diffusion techniques. In: Ackroyd JF, ed. Immunological Methods. Philadelphia, Davis, p55, 1964.
10. McDonald TP, Cottrell M, Clift R, Khouri JA, Long MD. Studies on the purification of thrombopoietin from kidney cell culture medium. J Lab Clin Med **106**:162–174, 1985.
11. Fraker PJ, Speck CJ, Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Commun **80**:849–857, 1978.
12. McDonald TP. Bioassay for thrombopoietin utilizing mice in rebound thrombocytosis. Proc Soc Exp Biol Med **144**:1006–1012, 1973.
13. McDonald TP, Clift R. Mechanism of thrombocytopenia induced in mice by anti-platelet serum. Haemostasis **5**:38–50, 1976.
14. Lange RD, McDonald TP, Jordan T. Antisera to erythropoietin: Partial characterization of two different antibodies. J Lab Clin Med **73**:78–90, 1969.
15. Miyake T, Kung CK-H, Goldwasser E. Purification of human erythropoietin. J Biol Chem **252**:5558–5564, 1977.

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