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Research Article

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Studies of Human Megakaryocytopoiesis Using an Anti-Megakaryocyte Colony-stimulating Factor Antiserum

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Abstract

We produced an antiserum by immunizing rabbits with purified human megakaryocyte colony stimulating factor (Meg-CSF). With the use of an anti-Meg-CSF IgG fraction (A_M-IgG), we detected immunoreactive Meg-CSF both in human aplastic anemia serum (AAS) and normal serum. Based on our immunological and biological analyses, Meg-CSF appeared to be antigenically as well as functionally distinct from human urinary erythropoietin (EPO) and thrombopoietic stimulating factor. The A_M-IgG fraction was able to suppress the ability of both aplastic anemia serum and purified Meg-CSF to promote megakaryocyte colony formation. In addition, the supernatant formed after immune precipitation of the AAS with A_M-IgG no longer possessed Meg-CSF-like activity. The A_M-IgG did not suppress the ability of EPO, phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM), or PHA-LCM + EPO to promote erythroid, granulocyte-macrophage, or mixed colony formation, respectively. The use of this antibody has further defined the dependency of human megakaryocytopoiesis on Meg-CSF.

Introduction

The regulation of human megakaryocytopoiesis and thrombopoiesis is only partially understood. Recently, hematopoietic growth factors able to specifically stimulate megakaryocytopoiesis have been reported to be present in the plasma, serum, and urine of patients with hypomegakaryocytic thrombocytopenia (1–4). It has been suggested that at least two humoral factors, megakaryocyte colony stimulating factor (Meg-CSF)[1] and thrombopoietin (TPO), which exert their effect on different cellular targets, are important in the regulation of in vitro megakaryocytopoiesis (5).

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1. *Abbreviations used in this paper:* AAS, aplastic anemia serum; A_M-IgG, anti-megakaryocyte colony stimulating factor IgG fraction; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; CFU-GEMM, colony forming unit-granulocyte-erythroid-macrophage-megakaryocyte; CFU-GM, colony forming unit-granulocyte-macrophage; CFU-M, colony-forming unit-megakaryocyte; EPO, erythropoietin; IL-3, interleukin 3; Meg-CSF, megakaryocyte colony stimulating factor; PAGE, polyacrylamide gel electrophoresis; PHA-LCM, phytohemagglutinin-stimulated leukocytic conditioned medium; TSF, thrombopoietic stimulating factor.

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Meg-CSF appears to be distinct from TPO, a substance present in thrombocytopenic plasma, which is responsible for megakaryocyte polyploidization. The effect of TPO is quantitated by its ability to stimulate the incorporation of radioisotopes into newly synthesized platelets in recipient animals (6). Meg-CSF influences megakaryocytopoiesis by stimulating the differentiation and proliferation of the colony forming unit-megakaryocyte (CFU-M) (4, 7). Thrombopoietic stimulatory factor (TSF) purified from human embryonic kidney cell conditioned medium, by contrast, does not alter CFU-M cloning efficiency, (7, 8) but accelerates the development of small immature megakaryocytes into single, large, easily recognizable megakaryocytes. Vainchenker et al. (9) have reported that erythropoietin (EPO) promotes the formation of human megakaryocyte colonies, which suggests that EPO may also play a role in the regulation of megakaryocytopoiesis. This observation is of interest since it may provide experimental support for the clinical observation that active megakaryocytopoiesis and thrombopoiesis often accompany active erythropoiesis.

We have recently purified human Meg-CSF into homogeneity. Meg-CSF increases megakaryocyte colony formation in culture but is incapable of supporting erythroid or granulocyte-macrophage colony formation. The available evidence suggests that the active molecule plays an important role in the regulation of human megakaryocytopoiesis. To further define the relationship between purified Meg-CSF, EPO, and TSF, we have succeeded in preparing a polyclonal IgG fraction against the purified human Meg-CSF. We report here an immunological analysis of a number of potential sources of Meg-CSF-like activity. In addition, we show that the anti-Meg-CSF IgG fraction (A_M-IgG) reduces the ability of aplastic anemia serum (AAS) and purified Meg-CSF to enhance CFU-M cloning efficiency, confirming the pivotal role that this factor plays in the regulation of the early events occurring during human megakaryocytopoiesis.

Methods

Human subjects. Bone marrow aspirations were obtained from the posterior iliac crests of hematologically normal individuals. Human AB serum was obtained from normal volunteers while AAS was obtained from patients with severe bone marrow aplasia. Sera were stored at −20°C in aliquots before use. The bone marrow and serum specimens were gathered after informed consent was obtained according to the guidelines of the Indiana University Committee for Protection of Human Subjects.

Source of Meg-CSF. Meg-CSF was purified according to the method of Hoffman and co-workers (7). In addition, the biologically active fraction obtained from affinity chromatography was further loaded onto a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis to recover the 46,000-mol-wt protein and to assure its homogeneity.

Recovery of biologically active factor from SDS-polyacrylamide gel electrophoresis (PAGE) gel. Samples (2 ml) of concentrated fractions obtained after wheat germ lectin sepharose CL-6B chromatography were

run on slab gels (1.5 × 140 × 110 mm) with standard molecular weight markers. At the end of the run, the distance from the tracking dye to the top of the running gel was carefully measured. A strip of the gel was cut vertically for staining as a template and the other part was retained for measurement of biological activity. The unstained part of the gel was sliced horizontally in sections according to the position of bands on the stained gel. The gel slices were shaken overnight at 4°C in 10 ml of 0.02 M sodium phosphate buffer, pH 7.2. The buffer solution was decanted and saved. This extraction step was repeated once and the gel slices were removed and discarded. These supernatants were combined and centrifuged to further remove gel particles and were dialyzed against double distilled water for 24 h with three changes of water, followed by ultrafiltration using an Amicon cell model 52 concentrator (Amicon Corp., Danvers, MA). They were then lyophilized and stored at −20°C until reconstitution in alpha-media for use in the biological assay or for use as an immunogen. When comparing the unstained portion of the gel with the biologically assayed portion, we took gel swelling into account.

Protein estimations. Protein concentration was estimated by the method of Lowry et al. (10) using bovine serum albumin (BSA) as a standard. The absorbance at 280 nm was used to monitor the relative protein concentrations of column effluents.

Immunization schedule and antibody isolation. New Zealand white rabbits were immunized by subcutaneous injection of 400 μg of purified Meg-CSF emulsified in 3 ml of Freund's complete adjuvant (Gibco, Grand Island, NY) 4 wk later and after a subsequent 2-wk period, an additional 150 μg of the protein in incomplete Freund's adjuvant was administered. The rabbits were bled 6–14 d after the last booster injections. The antibody-containing serum was heat-inactivated at 56°C for 30 min. The anti-Meg-CSF immunoglobulin fraction was prepared by precipitation with 45% ammonium sulfate followed by subsequent dialysis of the precipitate against phosphate-buffered saline (PBS) (pH 7.4), and purification on a DEAE affi-gel Blue Column (Bio-Rad Laboratories, Richmond, CA). This IgG fraction was designated as A_M-IgG, and was stored in 0.01 M potassium phosphate-buffered saline (pH 7.4) at −80°C before use. The protein concentration was adjusted to 2.7 mg/ml.

Electrophoresis. 10% polyacrylamide gels were run according to the method of Laemmli (11). Before application onto slab (1.5 × 110 × 140 mm) gels containing 0.1% SDS, the samples were heated to 100°C for 3 min in the sample buffer. Duplicate gels were fixed and stained with Coomassie Brilliant Blue R-250 in acetic acid/methanol/water (1:5:4 by volume) or with the highly sensitive silver stain (Bio-Rad Laboratories). Known molecular weight proteins (Bio-Rad Laboratories) were simultaneously run on the gels to serve as molecular weight reference markers. Markers consisted of 5 μg of myosin-H chain (200,000 mol wt), beta-galactosidase (130,000 mol wt), phosphorylase B (92,000 mol wt), BSA (68,000 mol wt), ovalbumin (45,000 mol wt), carbonic anhydrase (32,000 mol wt), and soybean trypsin inhibitor (20,000 mol wt). Preparative slab gel experiments (3 × 110 × 140 mm) were performed in the same manner as analytical gels except the sample remained in its nonreduced form. Two-dimensional electrophoresis was carried out according to the method of O'Farrell (12).

Immunoelectrophoresis was carried out as described by Grabar and Williams (13). Double-immunodiffusion experiments were performed by applying the technique of Ouchterlony (14) using immunoelectrofilms and double-diffusion plates (Kallestad Laboratories, Inc., Austin, TX).

Iodination of human Meg-CSF. A modification of the procedure described by Stanley (15) and Stagg (16) was used to iodinate Meg-CSF. In brief, purified Meg-CSF (1 μg) was resuspended in 40 μl of 0.1 M sodium phosphate, pH 7.6, containing 10% dimethyl sulfoxide and 100 μg/ml of polythylene glycol 6000. 10 μl of ^{125}I-Na containing 1 mCi were added, followed by 20 μl of a solution of chloramine-T (1 mg/ml). The mixture was incubated at 4°C for 5 min, and 20 μl of both sodium metabisulfite (3 mg/ml) and KI (0.1 M) were added; then 500 μl of BSA (10 mg/ml) was added. Iodinated Meg-CSF was separated from unbound ^{125}I by chromatography on 10-ml disposable columns of sephadex G-50 run in 0.01 M sodium phosphate buffer, pH 7.6, containing 0.1 M NaCl.

Immune precipitation of radiolabeled Meg-CSF and Meg-CSF present in human serum. The purified A_M-IgG fraction or a control normal rabbit IgG fraction was diluted in PBS to which either radiolabeled Meg-CSF, human AB serum, or AAS was added in a final volume 0.2 ml. These mixtures were incubated for 2 h at 37°C and then overnight at 4°C. 10 mg of protein A Sepharose swollen in PBS was added and the immune complexes were precipitated. This mixture was allowed to stand at 23°C for 2 h and was subsequently centrifuged at 8,000 *g* for 5 min in a microfuge (Model 59-A; Fisher Scientific Co., Pittsburgh, PA). The precipitates were washed three times with PBS and the complexes were eluted off with 10% SDS and 10% beta-mercaptoethanol at 100°C for 5 min. The complexes were analyzed for radioactivity by using a gamma counter (Model 5500; Beckman Instruments, Inc., Fullerton, CA) and were analyzed by SDS-PAGE by the method of Laemmli (11). After the completion of electrophoresis, the radiolabeled Meg-CSF SDS-PAGE gel was dried and autoradiographed. The polypeptides obtained from the serum specimens were transferred electrophoretically to nitrocellulose paper for Western blotting. The high-affinity binding of ^{125}I-Meg-CSF to A_M-IgG precipitated by protein A was determined by subtraction of low-affinity binding obtained by the addition of 300-fold excess unlabeled Meg-CSF.

Western blotting. Western blotting was performed according to the method of Towbin et al. (17) using a Trans-Blot cell (Bio-Rad Laboratories). Polypeptide transfer to nitrocellulose paper (15 × 15 cm) was accomplished immediately after the termination of electrophoresis and incubated with A_M-IgG for 2 h, then reacted with ^{125}I-protein A (10^5–10^6 cpm; Amersham Corp., Arlington Heights, IL; specific activity, 30 μCi/mg; concentration, 100 μCi/ml). Bound ^{125}I was detected by autoradiography using X-Omat AR film (Eastman Kodak Co., Rochester, NY) and Dupont Lightening (E. I. Du Pont de Nemours & Co., Inc., Wilmington, DE) plus intensifying screens at −70°C for 24–48 h.

Megakaryocyte colony stimulating factor assay. Meg-CSF was assayed in plasma clot cultures containing 5 × 10^5 bone marrow mononuclear cells (18). The bone marrow aspirate was diluted 1:1 with alpha-medium minus nucleosides (Gibco, Grand Island, NY), containing preservative-free sodium heparin at 20 U/ml and layered over an equal volume of Ficoll-Paque (specific gravity, 1.077 g/cm^3; Pharmacia Fine Chemicals, Piscataway, NJ). Density centrifugation was performed at 500 *g* for 25 min at 4°C in a Beckman model TJ-6R centrifuge (Beckman Instruments, Inc.). The interface mononuclear cell layer was collected and washed with alpha-medium minus nucleosides containing 2% fetal calf serum. Mononuclear cells at 5 × 10^5 cells/ml were cultured in 1-ml volumes in 35-mm petri dishes. The plasma clot technique of McLeod et al. (18) was modified by the substitution of heat-inactivated human AB serum for fetal calf serum, and alpha-medium minus nucleosides for NCTC-109 medium and Eagle's minimal essential medium with Hank's balanced salt solution. Samples to be assayed for Meg-CSF activity were diluted in alpha-medium minus nucleosides at several dilutions. The final 1-ml aliquot of each culture contained the following supplements: alpha-media minus nucleosides, sodium bicarbonate (1.2%), nonessential amino acids (0.01 mmol/ml), L-glutamine (0.4 mmol/ml), and sodium pyruvate (0.2 mmol/ml). Culture dishes were incubated for 10–12 d (unless otherwise stated) at 37°C in a 100% humidified atmosphere of 5% CO_2 in air. Harvesting was performed by in situ fixation with methanol/acetone (1:3) for 20 min, washing with 0.01 M PBS, pH 7.2, then distilled water, and subsequently air dried. Plasma clots were stored frozen at −20°C until immunofluorescent staining was performed.

Immunofluorescent identification of human megakaryocyte colonies. Whole rabbit anti-PGP antiserum, previously established as a megakaryocyte specific marker (8), was diluted in PBS (1:200), layered over the fixed plasma clot culture, and incubated for 60 min at room temperature in 100% humidified air. After washing three times with PBS, the specimens were reincubated for an additional 60 min with fluorescein-conjugated goat anti-rabbit IgG (Meloy Laboratories, Inc., Springfield, VA) diluted in PBS, final concentration, 0.36 mg protein/ml. After being washed with PBS, the specimens were counterstained with 0.125% Evan's Blue for 5 min, washed with distilled water, and mounted in isotonic barbital buffer, pH 8.6, in glycerol (1:3).

Plasma clot cultures were scored in situ in order to enumerate fluorescein positive colonies. The 35-mm petri dishes were inverted and

the base area was completely scanned with a fluorescence microscope at 100 × (Zeiss standard microscope 18 with IV FL vertical fluorescent illuminator; Carl Zeiss, Inc., Thornwood, NY). A megakaryocyte colony was defined as a cluster of three or more intensely fluorescent cells. Each study was performed in duplicate to quadruplicate. Specific activity of Meg-CSF preparations was calculated from the number of megakaryocyte colonies per milligram protein from the linear portion of the dose response curve.

Assay for other hematopoietic colonies. Both BFU-E and CFU-E derived colony assays were performed in plasma clot cultures as previously described (19). Human EPO, specific activity, 30–50 U/mg of protein (Toyobo Co., LTD, Osaka, 530 Japan) was used in these studies. Plasma clots were fixed in situ with 5% glutaraldehyde in 0.01 M phosphate buffer (pH, 7.0–7.2). Colony forming unit-erythroid (CFU-E) and burst forming unit-erythroid (BFU-E) derived colonies were harvested after 7 and 12–14 d of incubation, respectively. Cultures were stained with 1% benzidine and hematoxylin as previously described (19). Maximum growth of CFU-E and BFU-E derived colonies in this modified plasma clot assay system was seen at EPO concentrations of 0.5 and 1.0 IU/ml, respectively.

The colony assay for granulocyte-macrophage colony-forming units (CFU-GM) was carried out according to the method of Fauser and Messner (20). In brief, marrow mononuclear cells were suspended in 35 mm Lux standard tissue culture dishes, containing a 1-ml mixture of Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories), 0.8% methylcellulose, 30% fetal bovine serum, 10% medium conditioned by leukocytes from patients with hemochromatosis in the presence of 1% phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM), and 5×10^{-5} M 2-mercaptoethanol. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Colonies were scored with an inverted microscope after 13–14 d of incubation. Clusters of ≥40 cells, many of which had typical band or segmented polymorphonuclear leukocyte morphology, were defined as CFU-GM derived colonies. The assay for human granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) derived colonies as described by Fauser and Messner (21) was used to assess the effect of growth factors on multipotential colony forming unit cells.

Neutralization test. The ability of the anti-Meg-CSF IgG fraction (A_M-IgG, 2.7 mg/ml) to neutralize Meg-CSF activity was assessed using two methods. First, the anti-Meg-CSF IgG fraction (A_M-IgG, 2.7 mg/ml) or the IgG fraction (2.7 mg/ml) of normal rabbit serum was diluted in saline and mixed with an equal volume of either AAS, purified Meg-CSF, PHA-LCM, or a partially purified preparation of human urinary erythropoietin and incubated at 25°C for 1 h before adding to the cultures. Control cultures were established with the same stimulus mixed with an equal volume of physiological saline. The ability of these mixtures to promote megakaryocyte, granulocyte-macrophage, or erythroid-derived colony formation by normal human marrow cells was determined using the various assays described above. The number of each type of hematopoietic colony was determined. The second method for assessing the neutralizing ability of the A_M-IgG was as follows: A_M-IgG and normal rabbit IgG were diluted in saline as above and mixed with an equal volume of AAS and incubated at 25°C for 1 h, followed by an additional incubation at 4°C overnight. The immune complexes that were formed were precipitated by adding 10 mg of protein A-Sepharose swollen in PBS. This mixture was allowed to rotate at 4°C for 2 h and was subsequently centrifuged at 8,000 *g* for 5 min in a Fisher microfuge (Model 59-A). The ability of the supernatants to promote CFU-M derived colony formation by normal marrow cells was determined. In order to further define the specificity of the A_M-IgG neutralizing activity, we assessed the ability of increasing amounts of purified Meg-CSF to overwhelm the antibody effect. The inhibitory activity of the normal rabbit IgG or the A_M-IgG was expressed as the percent of the control number of colonies formed. The titer was expressed as that dilution which caused a 50% reduction in colony numbers.

Statistics. The student *t* test was performed with a statistical package compatible with an Apple II computer (Apple Computer, Cupertino, CA).

Results

The immunogen used to produce the anti-Meg-CSF antibody was purified from the plasma of a patient with hypomegakaryocytic thrombocytopenia according to the methods of Hoffman and co-workers (7). The active Meg-CSF band was cut out and extracted from the preparative SDS-polyacrylamide gel, thus recovering 60–80% of the loaded sample. When 10 μg of the extracted protein was analyzed with two-dimensional gel electrophoresis, it migrated as a single band and showed no evidence of contamination with other proteins (Fig. 1).

Both rabbits immunized with purified Meg-CSF produced antisera to Meg-CSF. IgG fractions were purified from preimmune rabbit serum and antiserum obtained from the immunized rabbits. The preparations revealed a single band in the gamma globulin region when purified IgG fractions were examined in SDS-polyacrylamide gel at pH 8.8. The reactivity of A_M-IgG with Meg-CSF was examined by means of immunodiffusion, immunoelectrophoresis, and Western blotting.

As shown in Fig. 2 *A*, the purified Meg-CSF formed a single precipitin line with the antibody when placed in an immunodiffusion system. As shown in Fig. 2 *B*, the antibody did not form a precipitin line with TSF, purified from human embryonic kidney cell conditioned media (gift of T. P. McDonald, University of Tennessee, Knoxville, TN), or human urinary EPO (specific activity, 500 U/mg of protein, Alpha-Therapeutics Corp., Los Angeles, CA). However, the antibody did form a single precipitin line with Meg-CSF purified by preparative electrophoresis (Fig. 2 *B*) and the fraction obtained after high performance liquid chromatography (Fig. 3), which suggests that the Meg-CSF was immunochemically distinct from both TSF and EPO. The preimmune IgG fraction was run under the same conditions as the immune IgG fraction and at various concen-

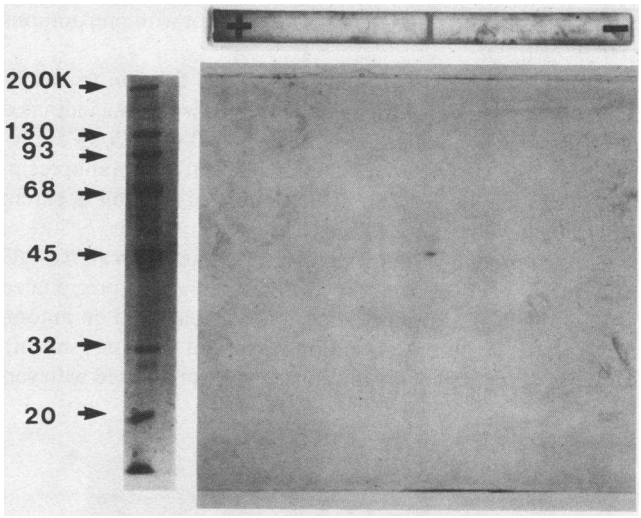

Figure 1. Two-dimensional SDS-PAGE of purified human megakaryocytic stimulating factor. The first dimension was isoelectric focusing in a pH 3.5–10 gradient, 5% acrylamide gel containing 8 M urea and 1% ampholytes. A silver stained gel (5 × 115 mm) is shown horizontally at the top of the electrophoregram. The second dimensional gel of 10% acrylamide, 1.5-mm-thick, was run by loading an isoelectric focusing gel (1 × 115 mm) on the top of the slab SDS-gel. The gel was stained using the Bio-Rad Silver Stain (Bio-Rad Laboratories). At left, known molecular weight proteins are demonstrated.

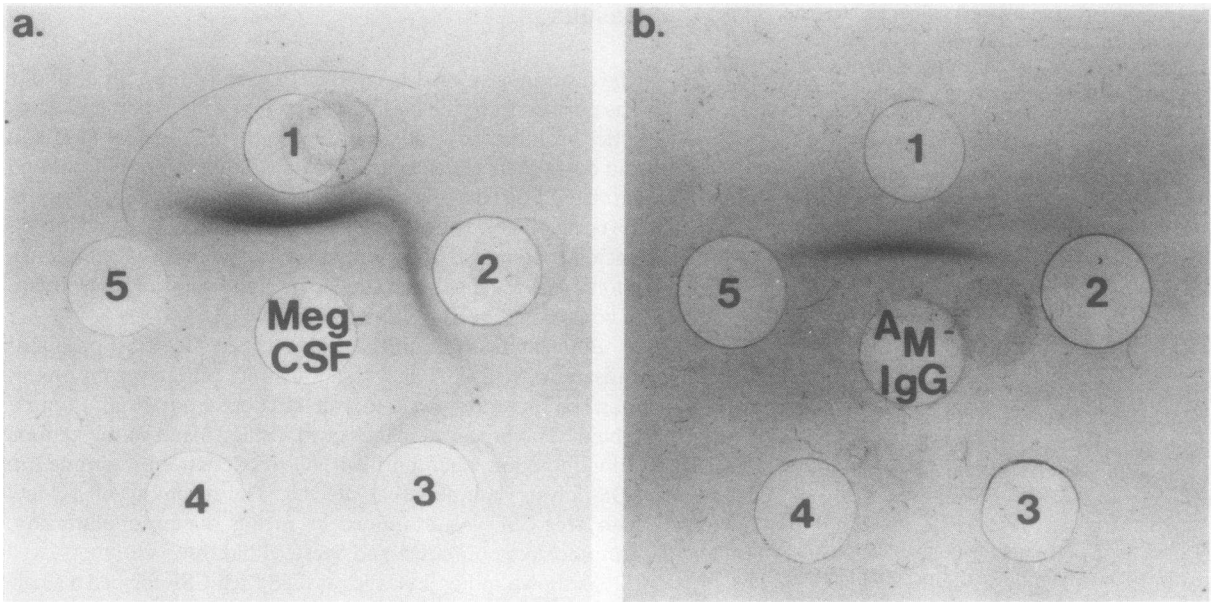

Figure 2. Analysis of various hematopoietic growth factors using immunodiffusion with the anti-Meg-CSF IgG fraction. The experiments were carried out in 1% agarose solution in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.02% NaN_3. (*a*) Center well contains 10 μl of human purified Meg-CSF in a concentration of 0.1 mg/ml and wells 1–5 contain 5 μl of twofold serial dilutions of rabbit A_M-IgG (2.7 mg/ml) starting with undiluted A_M-IgG in well 1. (*b*) Rabbit A_M-IgG (5 μl of 2.7 mg/ml) was placed in center well. Growth factors of comparable concentrations were placed in the outer wells as follows: 1, purified human Meg-CSF (1.0 μg); 2, preimmune rabbit IgG (5.0 μg); 3, TSF (purified from human embryonic kidney cell-conditioned media, 1.0 μg); 4, and 5, human urinary erythropoietin (sp act, 500 U/mg of protein, Lot No. EH009TO, 1.0 and 2.0 μg per well, respectively).

trations of purified Meg-CSF. No precipitin lines formed with the preimmune IgG fraction (data not shown).

Using immunoprecipitation and Western blotting techniques (Fig. 4), several potential sources of hematopoietic growth factors were also examined for reactivity with the anti Meg-CSF IgG fraction. The A_M-IgG formed a precipitin band with AAS, normal human serum, and purified Meg-CSF but not with preparations of TSF or EPO.

In Fig. 5, the ability of purified A_M-IgG fraction to immunoprecipitate iodinated Meg-CSF was examined. The iodinated purified Meg-CSF had a specific activity of 0.45 mCi/μg of protein. The anti-Meg-CSF IgG fraction efficiently immunoprecipitated the iodinated protein which migrated with an apparent molecular weight of 46,000 on SDS-PAGE analysis. ~80% of the labeled protein was maximally precipitated with an A_M-IgG concentration of 0.67 mg/ml. Normal rabbit IgG precipitated <1% of the labeled protein and no detectable band on autoradiography at a concentration up to 2 mg/ml (data not shown). The autoradiograph of the immune complexes formed with various dilutions of A_M-IgG and iodinated Meg-CSF is shown in Fig. 6.

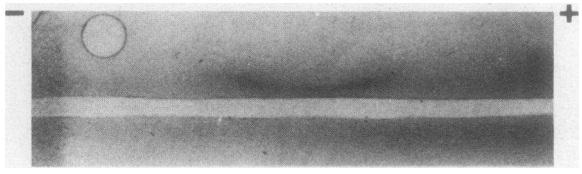

Figure 3. Immunoelectrophoretogram of purified Meg-CSF. Meg-CSF was obtained from the major peak following purification by reverse-phase high pressure liquid chromatography. Trough, A_M-IgG; antigen well, human Meg-CSF. A single precipitin line was revealed.

Neutralization of Meg-CSF activity present in AAS and purified human Meg-CSF by the A_M-IgG fraction is demonstrated in Table I. The A_M-IgG at various concentrations was unable to alter the baseline megakaryocyte colony formation that occurs in the absence of added Meg-CSF (data not shown). The A_M-IgG was able to neutralize the megakaryocyte colony stimulating activity present in AAS or purified Meg-CSF at a statistically significant level ($P < 0.001$). At a dilution of 1:256, the A_M-IgG resulted in a 55% reduction in megakaryocyte colony formation stimulated by AAS. As shown in Table II, this neutralization effect was overcome by adding increasing amounts of purified Meg-CSF. When the supernatants of AAS from which the Meg-CSF had been immune-precipitated were incubated with protein A Sepharose beads to remove free residual A_M-IgG Meg-CSF immune complexes, these fractions still were unable to promote megakaryocyte colony formation. Neutralization of the ability of purified Meg-CSF to promote megakaryocyte colony formation occurred up to a 1:1,024 dilution of A_M-IgG.

The inability of the anti Meg-CSF IgG fraction to neutralize the effect of EPO in promoting CFU-E and BFU-E derived colony formation is shown in Table III. In addition, treatment with A_M-IgG did not influence CFU-GEMM or BFU-E derived colony formation when stimulated by EPO + PHA-LCM. Similar treatments did not influence the ability of PHA-LCM to promote CFU-GM derived colony formation. This lack of effect on in vitro erythropoiesis, granulopoiesis, and on the formation of mixed colonies suggests that A_M-IgG does not act by a direct toxic action on the colony-forming cells or by reacting with nutrients in the culture medium. Therefore, these experiments

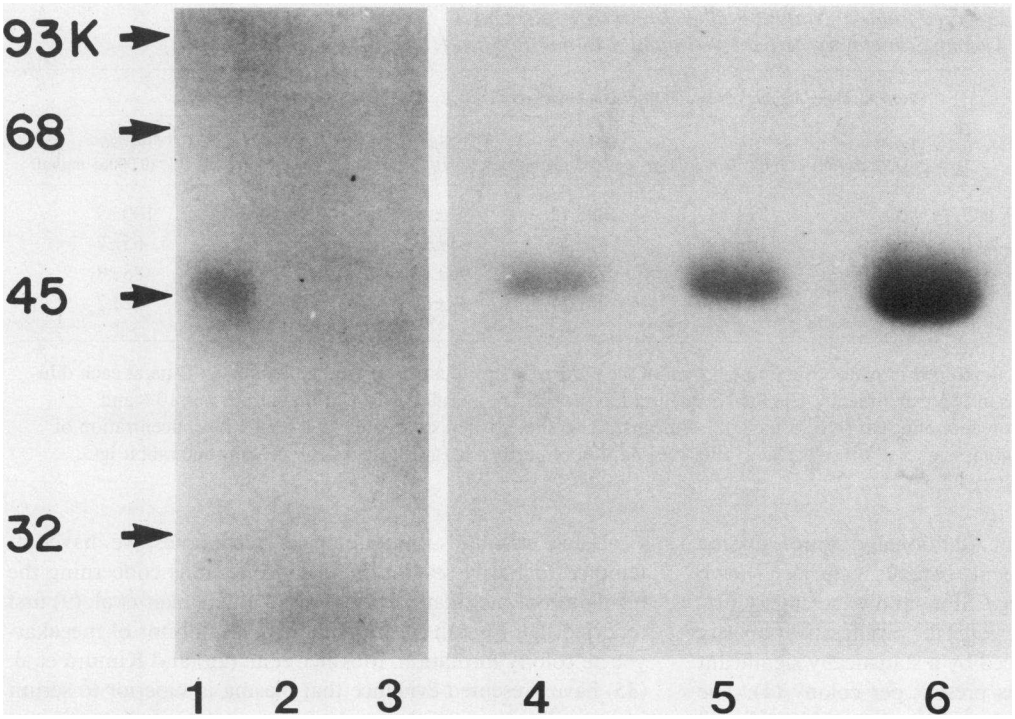

Figure 4. Western blotting of immunoprecipitates of various hematopoietic growth factors and human sera with A_M-IgG. Lanes 1, 2, and 3 represent purified Meg-CSF (2.0 μg), human urinary EPO (7.0 μg), and thrombopoietic stimulating factor (3.0 μg), respectively. Lanes 4, 5, and 6 represent complexes obtained from purified Meg-CSF (5.0 μg), normal AB serum (10%), and AAS (10%) subjected to 10% SDS-PAGE, respectively. Polypeptides were transferred to nitrocellulose paper and the immune-complexes bound to ^{125}I-protein A were detected by autoradiography. Molecular weight markers were indicated by arrows (example: 93 K = 93,000 mol wt).

substantiate the specificity of the A_M-IgG. In this series of experiments and in previously reported studies (4, 8) we have been unable to demonstrate an effect of PHA-LCM or EPO on CFU-M derived colony formation. The effect of A_M-IgG on EPO and PHA-LCM stimulated megakaryocyte colony formation was therefore not assessed.

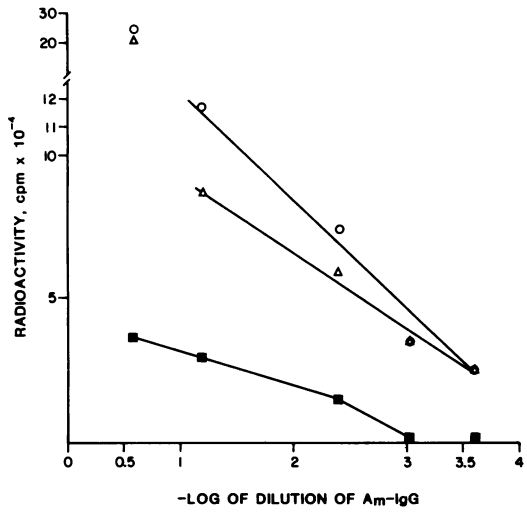

Figure 5. Immune precipitation binding curve of ^{125}I-Meg-CSF to A_M-IgG. ~240,000 cpm of ^{125}I-Meg-CSF (0.4 ng) and 0.1 ml of fourfold serial dilutions of A_M-IgG were used for each point. The total binding radioactivity (○) was determined from the precipitated immune-complexes that were dissociated and eluted off from protein A Sepharose. Low-affinity binding radioactivity was obtained by adding 300-fold excess of unlabeled Meg-CSF to labeled Meg-CSF (■). Specific, high-affinity binding radioactivity (△) was calculated by taking the difference between the total and low-affinity binding.

Discussion

Factors that control human thrombopoiesis and megakaryocytopoiesis have not been well characterized. Recently, clonal culture techniques have been developed that allow the quantitation of human megakaryocyte colony forming units (CFU-M) in bone marrow and peripheral blood (8, 22). A number of investigators have detected factors from a variety of sources that promote megakaryocyte colony formation (23–29). Crude human EPO, sheep EPO (18, 28), homogeneously purified human urinary EPO (27), and TSF (29), have also been shown to enhance

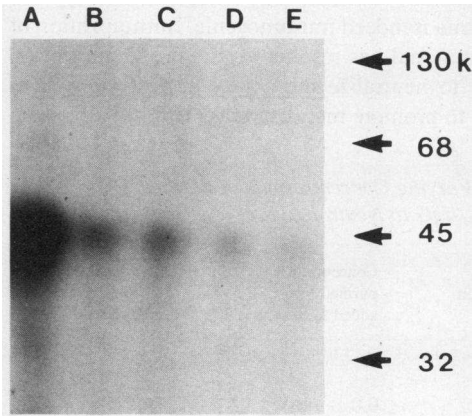

Figure 6. Immune precipitation analysis of ^{125}I-labeled Meg-CSF. Lanes *A* through *E* represent the decreasing concentration of the A_M-IgG (168, 42, 21, 10, and 2.6 μg/ml) used to bind the labeled protein. The iodination, immune precipitation, and subsequent analysis on 10% polyacrylamide gels were done as described in Methods. The gel was dried and exposed to X-Ray film. The position of the indicated standard proteins was determined by Coomassie Blue staining.

Table I. Effect of the Fraction IgG Prepared from (a) Normal Rabbit Serum (NR-IgG) and (b) Serum of Immunized Rabbit (A_M-IgG) on Colony Stimulatory Activity of Human AAS and Purified Human Meg-CSF

Treatment of hematopoietic growth factor before its addition to culture	Percent colony number of control* (Dilution of IgG preparation)					
	1:4 (0.672 mg/ml)	1:16 (0.168 mg/ml)	1:64 (0.042 mg/ml)	1:256 (0.0105 mg/ml)	1:1,024 (0.0026 mg/ml)	1:4,096 (0.00065 mg/ml)
Human aplastic anemia serum + NR-IgG	93±5	71±13	109±12	92±12	92±9	100±9
Human aplastic anemia serum + A_M-IgG	6±1	14±1	47±12	55±3	55±12	65±7
Purified human Meg-CSF + NR-IgG	92±10	71±16	76±11	96±19	82±5	88±8
Purified human Meg-CSF + A_M-IgG	0	12±2	26±17	37±8	27±2	92±2

* Percentage of colony numbers that developed in plates containing 0.1 ml of the particular stimulus plus 0.1 ml alpha-media. Data at each dilution represent the mean±SD of plates in two experiments. The final concentration of AAS and purified Meg-CSF in culture was 10% and 8×10^{-8} M, respectively. Bone marrow mononuclear cells formed 32±4 means±SD colonies when stimulated by a final 10% concentration of AAS, while 36±2 CFU-M derived colonies per 5×10^5 cells plated after the addition of purified Meg-CSF. NR-IgG, preimmune rabbit IgG.

megakaryocyte colony formation. Additionally, serum, plasma and urine (1, 2) of thrombocytopenic patients have been shown to possess Meg-CSF activity. Meg-CSF not only increases CFU-M cloning efficiency, but also increases the proliferative capacity of individual CFU-M as evidenced by a statistically significant increase in the numbers of cells present per colony (4). The variations in Meg-CSF levels that have been detected in different disease states suggest that alterations in the production of this regulator are physiologically important (2).

Purified regulatory molecules and cell populations must be available before we can unravel the complexities of human megakaryocytopoiesis (29, 30). In the study of erythropoiesis and granulopoiesis, purification of EPO, erythropoietic burst promoting activity, and various subclasses of granulocyte-macrophage colony stimulating factors have provided a better understanding of both granulopoiesis and erythropoiesis (27, 29–34). We have recently adopted this approach and have purified human Meg-CSF using a four-step purification scheme (7). This process has resulted in the isolation of a 46,000-mol-wt glycoprotein that promotes megakaryocyte colony formation. We have now used the purified Meg-CSF to produce an A_M-IgG fraction. The present study shows that Meg-CSF purified from hypomegakaryocytic plasma is indeed immunogenic. Immunization of rabbits with this material has resulted in the production of an IgG fraction able to neutralize the ability of AAS as well as purified Meg-CSF to promote megakaryocyte colony formation.

Table II. Effect of Varying Concentrations of Purified Meg-CSF on the Ability of A_M-IgG to Neutralize Meg-CSF Activity

Treatment before addition to culture	Concentration of purified Meg-CSF added to culture	Mean No. of CFU-M colonies* per 5×10^5 cells plated
	M	
AAS + A_M-IgG	0.0	16.5
AAS + A_M-IgG	1×10^{-8}	33.0
AAS + A_M-IgG	2×10^{-8}	44.0
AAS + A_M-IgG	4×10^{-8}	55.0

* To a mixture of A_M-IgG (1:256 dilution) and 10% AAS, increasing amounts of purified Meg-CSF were added. These mixtures were added to bone marrow cells and then plated in plasma clot cultures. Each value represents the mean of duplicate determinations.

Using standard immunological techniques we have attempted to clarify several important questions concerning the regulation of megakaryocytopoiesis. Vainchenker et al. (9) first reported that human serum contains an inhibitor of megakaryocyte colony formation. Messner et al. (26) and Kimura et al. (35) have presented evidence that plasma is superior to serum in its ability to support megakaryocyte colony formation in a methylcellulose assay system. Kimura et al. (35) also showed that the direct addition of intact or frozen-thawed platelets to methylcellulose assays resulted in a decrease in numbers of megakaryocyte colonies formed. Using a plasma clot culture system, we have not been able to detect the superiority of platelet-poor plasma–derived serum over crude serum or platelet-rich plasma–derived serum in their ability to support megakaryocyte colony formation (Hoffman, R., and E. Bruno, unpublished observations). The work demonstrating differences in the ability of plasma and serum to support colony growth have led Kimura and co-workers (35) to suggest that the enhanced ability of AAS to promote CFU-M cloning efficiency might merely be due to these sera containing less platelet-derived inhibitory activity than serum from patients with elevated platelet counts. This hypothesis would seem to be highly unlikely in light of our observation that the anti-Meg-CSF antibody can neutralize the activity of AAS to promote CFU-M cloning efficiency. In addition, the formation of a precipitin band between the anti Meg-CSF IgG fraction and AAS and normal serum also suggests the presence of an immunologically distinct macromolecule in these specimens which share a common polypeptide segment.

Since a number of investigators have reported that EPO and TSF can support megakaryocyte colony formation (18, 27, 28), it was important to determine the relationship between TSF, EPO, and purified Meg-CSF. We have previously reported that neither TSF nor EPO increases megakaryocyte colony formation (4). The inability of the anti-Meg-CSF antibody to react with either TSF or EPO suggests that these factors are at least immunologically unrelated molecules. The possibility does exist that at high concentrations of TSF or EPO that are artificially created in clonal assay systems, either of these factors might influence in vitro megakaryocyte colony formation. It would appear unlikely, however, that either TSF or EPO are primary physiological regulators of megakaryocytopoiesis at the level of the CFU-M.

Interleukin 3 (IL-3) is a lymphokine first defined by its capacity to induce 20-α-hydroxysteroid dehydrogenase (20-α-SDH)

Table III. Neutralization of CFU-M, CFU-E, BFU-E, CFU-GM, and CFU-GEMM Stimulatory Activity by Normal Rabbit and Anti-Meg-CSF IgG

Prior treatment of hematopoietic growth factor to its addition to culture	CFU-M derived colonies* per 5×10^5 cells plated	CFU-E derived colonies* per 5×10^5 cells plated	BFU-E derived colonies* per 5×10^5 cells plated	CFU-GM derived colonies* per 1×10^5 cells plated	CFU-GEMM per 1×10^5 cells plated
None	3±1	0±0	0±0	6±4	0±0
Human EPO (1 U/ml)	4±2	270±46	119±13	37±10	4±2
Human EPO (1 U/ml) + NR-IgG	NA	288±17, NS	129±4, NS	NA	NA
Human EPO (1 U/ml) + A_M-IgG	NA	294±22, NS	139±29, NS	NA	NA
AAS	36±2	NA	NA	NA	NA
AAS + NR-IgG	33±5, NS	NA	NA	NA	NA
AAS + A_M-IgG	5±0 P = 0.000025	NA	NA	NA	NA
PHA-LCM	1±0	NA	NA	100±24	NA
PHA-LCM + NR-IgG	NA	NA	NA	103±10, NS	NA
PHA-LCM + A_M-IgG	NA	NA	NA	113±15, NS	NA
PHA-LCM + human EPO	NA	NA	66±9	NA	13±4
PHA-LCM + human EPO + NR-IgG	NA	NA	76±10	NA	12±1, NS
PHA-LCM + human EPO + A_M-IgG	NA	NA	87±4	NA	20±5, NS

* Bone marrow cells were incubated with a 1:16 dilution of the A_M-IgG or NR-IgG and either erythropoietin, AAS (10%), or PHA-LCM (5%) and then plated in plasma clot or methylcellulose cultures. Each value represents the mean±SD of either duplicate or quadruplicate cultures. NA, not available. NR-IgG, preimmune rabbit IgG. A_M-IgG, anti-Meg-CSF IgG. NS, not significant.

in spleen cells of neonatal nude mice (37). IL-3 has been purified to homogeneity and its responsible gene cloned (37, 38). IL-3 has been shown to possess a number of hematopoietic growth factor-like activities (39). Quesenberry and co-workers (40) have presented data that suggest that IL-3 is a potent stimulator of in vitro murine megakaryocyte colony formation. IL-3 appears to represent the marrow cell-line inducing activity present in media conditioned by the murine myelomonocyte cell line WEHI-3 (41). WEHI-3 cell conditioned medium has also been used as a source of murine Meg-CSF. Recently, Williams and co-workers (42) have shown that the Meg-CSF in WEHI-3 cell conditioned medium was identical to IL-3. The relationship of human Meg-CSF to IL-3 is unknown. Unfortunately, there is no purified human molecule equivalent to murine IL-3 available with which to define this relationship. However, this issue was explored using the anti-Meg-CSF IgG fraction. Crude PHA-LCM prepared from human mononuclear cells possesses many IL-3-like activities (43). The PHA-LCM had burst promoting activity, granulocyte-macrophage colony stimulating activity, and multipotential colony stimulating activity. The A_M-IgG fraction was able to neutralize the ability of AAS and purified Meg-CSF to stimulate megakaryocyte colony formation but did not alter the ability of PHA-LCM to support CFU-GM, CFU-GEMM, or BFU-E derived colony formation. In addition, using Western blotting, we were unable to detect any Meg-CSF in several different batches of PHA-LCM (data not shown). These data suggest that the purified Meg-CSF is immunologically distinct from the IL-3-like activity present in PHA-LCM.

The detection of Meg-CSF in normal serum using A_M-IgG as an immunological probe was a surprising finding. This data would suggest that detectable levels of this factor are present in most individuals in order to maintain baseline megakaryocytopoiesis and thrombopoiesis. These findings are consistent with previous data published by our laboratory, which suggest that Meg-CSF is required in vitro at higher concentrations than previously recognized for other hematological growth factors (7, 43, 44). The reasons for this discrepancy are unknown to us, but could indicate the unique properties of Meg-CSF.

The data presented in this report clearly demonstrate the presence of Meg-CSF in AAS and normal serum. This factor appears to play a pivotal role in the regulation of megakaryocytopoiesis. Although we have been unable to detect a serum inhibitor of megakaryocyte colony formation, our results do not exclude the presence of such a factor. The possibility arises that the methylcellulose assay system might be more sensitive in the detection of such inhibitors than the plasma clot culture system. Our present studies, however, do clearly define the presence of Meg-CSF-like activity in human serum. Whether the effect of this factor can be blunted by inhibitory molecules originating in platelets is a question that we are now approaching, using a better defined serum-free culture system.

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