Immunological and Functional Differences between Human Type I and II Colony-stimulating Factors

MING-CHI WU, ALAN M. MILLER, and ADEL A. YUNIS, *Departments of Medicine, Biochemistry and Oncology, University of Miami School of Medicine, and Howard Hughes Medical Institute, Miami, Florida 33101*

A B S T R A C T Two distinct types of colony-stimulating factor (CSF) have recently been described in human tissues and cultured cell lines. Antisera to purified type I and II CSF were prepared in rabbits. Anti-CSF I antibody inhibits CSF I, but has no effect on CSF II. It cross-inhibits CSF I from several other human sources, but does not inhibit CSF from mouse lung or mouse L cells. Anti-CSF II antibody inhibits the activity of CSF II, but has no effect on CSF I. A radioimmunoassay for CSF I has been established. Competitive binding assay further demonstrated the immunological differences between CSF I and II. When CSF II is used to stimulate human marrow cells fractionated by sedimentation velocity, two populations of CFU-C are separated, one sedimenting at 8 mm/h and forming colonies by day 7, and a second sedimenting at 6.8 mm/h and forming colonies by day 13. In contrast, CSF I does not stimulate colony growth by day 7 but does do so by day 13 in cells sedimenting between 7.2–8.5 mm/h. These results indicate that CSF I and II are distinct in their biochemical, immunological, and functional properties.

INTRODUCTION

We have recently reported that conditioned media prepared from a variety of human tissues and cultured cancer cells exhibit two distinct types of colony-stimulating factor (CSF)[1] which can be separated by isoelectrofocusing and gel filtration chromatography. CSF I shows heterogeneity on IEF with pI in the range of 3.6–4.7 and has a molecular weight of 50,000. CSF II appears as a single peak with pI of 5.7 and has a molecular weight of 27,000. CSF I stimulates mouse bone marrow CFU-C but has little or no activity in human marrow while CSF II is more active in human than in mouse marrow (1).

A number of important questions are raised by our observations particularly concerning the relationship between these two types of CSF and the significance of the so called "mouse" activity of type I CSF. If type I CSF plays any role in granulopoiesis in vivo, it must have biological activity on human marrow CFU-C. In our studies described here we demonstrate that (a) in addition to differences in molecular weight, isoelectric point, and marrow specificity, type I and II CSF are immunologically distinct and (b) the action of type I CSF appears to be directed to a specific subpopulation of human CFU-C.

METHODS

Fetal calf serum and horse serum were purchased from Flow Laboratories, Rockville, Md. Tissue culture medium (Dulbecco's modified Eagle's medium) was from Gibco Laboratories, Grand Island, N. Y. Carrier-free radioactive Na^{125}I was from New England Nuclear, Boston, Mass. All other chemicals were of reagent grade.

CSF assay. The standard method developed by Bradley and Metcalf was used with modification (2). The preparation of mouse and human bone marrow cells and the morphological identification of colonies have been described previously (1). A unit of CSF activity is arbitrarily defined as the amount of CSF which stimulates the formation of one colony under the specified assay conditions.

Production of antibodies against CSF I and II in rabbits. Purified CSF I (0.1 mg, 7 × 10⁶ U) and partially purified CSF II (1.0 mg, 1.2 × 10⁶ U) from cultured human pancreatic carcinoma (MIA PaCa-2) (3) were used to immunize rabbits according to procedures described previously (4). Preimmunized and immunized sera were treated with 33% saturated ammonium sulfate, and the globulin fraction was subjected to DEAE-cellulose chromatography according to the standard procedure. The purified IgG fractions (preimmunized and immunized) were reconstituted to the original serum volume in normal saline solution.

Cross-inhibition by antibodies. The antibodies thus prepared were used to study the cross-inhibition among dif-

[1] *Abbreviations used in this paper:* CFU-C; CSF, colony-stimulating factor; IEF, isoelectrofocusing; MIA PaCa-2, cultured human pancreatic carcinoma.
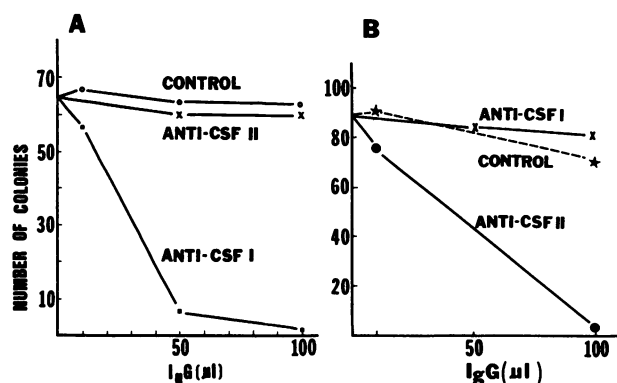
FIGURE 1 Effects of anti-CSF antibodies on CSF activity: CSF I (A) and CSF II (B) were each incubated with either anti-CSF I, anti-CSF II, or control IgG for 30 min at different concentrations and subsequently assayed for the ability to stimulate colony formation on mouse bone marrow (CSF I) or human bone marrow (CSF II). Results are expressed in colonies per 10^5 cells.

ferent types of CSF. CSF I and CSF II from MIA PaCA-2 cells (~100 U) were incubated separately with 100 μl of anti-CSF I antibody, anti-CSF II antibody, or control IgG for 30 min at room temperature and then assayed for activity. Similarly, CSF I from human placenta, lung, urine, leukocyte-conditioned media, and cultured squamous cell carcinoma, as well as mouse lung and L cell CSF, were similarly incubated with anti-CSF I antibody to test for cross-inhibition.

Iodination of CSF I. Purified type I CSF from MIA PaCa-2 cells (5 μg, specific activity, 7×10^7 U/mg) was iodinated with carrier-free Na^{125}I (2 mCi) according to the procedure of Greenwood et al. (5) as modified by Stanley (6). The iodinated ^{125}I-CSF I was then purified on a Sephadex G-25 column (PD-10) followed by an Ultrogel AcA column (10 $\times$ 110 mm, Column volume, 110 ml) equilibrated with phosphate-buffered saline. The ^{125}I-CSF I thus prepared had a specific radioactivity of 10^5 cpm/ng and at least 80% of the initial biological activity. Iodination efficiency was ~10%.

Radioimmunoassay (RIA) of CSF I. CSF samples from different sources were properly diluted with RIA buffer (50 mM phosphate buffer pH 6.5 containing 0.2% bovine serum albumin, 0.1% NaN$_3$, and 0.01% ethylene glycol 6000). 100 μl of sample, 10 μl of ^{125}I-CSF I (18,000 cpm) in RIA buffer, and 25 μl of diluted anti-CSF I antibody were mixed in that order. RIA buffer was added to a final volume of 200 μl. The radioactivity of all samples was counted and the samples were then incubated overnight at 4°C. Undiluted control IgG (100 μl) was added to each tube, followed by 200 μl of saturated ammonium sulfate solution (pH adjusted to 7.0). The precipitates were left in ice for 30 min and then centrifuged at 10,000 rpm (Sorvall RC-5B, SS-34 Rotor). The supernates were removed and the radioactivity of the precipitates measured in a gamma counter (Nuclear Chicago Des Plaines, Ill.). The amount of anti-CSF I antibody used in the assay was equivalent to the concentration which produced a bound/free ratio (B/F) of ~1.0. In our study, the B/F ratio from one batch of ^{125}I CSF I and anti-CSF I could be kept relatively constant for ~1 wk.

Competitive binding of CSF I and CSF II. CSF I from several sources including MIA PaCa-2 cells, human lung, and human placenta were partially purified by isoelectrofocusing and gel filtration chromatography as described

previously (1). Samples containing CSF activities in the range of 0.1 to 1,000 U in 100 μl of RIA buffer were used in the RIA competitive binding studies as described above. Partially purified CSF II from MIA PaCa-2 cells was also used to compare the competitive binding of CSF I and II to the anti-CSF I antibody.

Fractionation of human bone marrow cells by sedimentation at unit gravity. The preparation of human bone marrow cells and fractionation by sedimentation velocity on the Sta-Put apparatus have been described previously (7). A total of 1.4–1.8 $\times$ 10^8 cells were layered over a continuous bovine serum albumin gradient of 0.5 to 2.0% and allowed to sediment for 3.5 h at 4°C. Cell fractions were collected from the bottom of the separation chamber and assayed for CFU-C with either CSF I or CSF II obtained from human lung conditioned medium (8) after isoelectrofocusing and gel filtration chromatography (1).

RESULTS

Inhibition of CSF I and II by antibodies. As shown in Fig. 1, when CSF I was incubated with control IgG, anti-CSF I IgG, and anti-CSF II IgG, the activity as assayed on mouse marrow cells was inhibited only by anti-CSF I; there was no inhibition by anti-CSF II or control IgG. Similarly, the activity of CSF II as assayed on human bone marrow cells was specifically inhibited by anti-CSF II antibody. The results clearly indicate that CSF I and II are immunologically distinct. Anti-CSF I also cross-inhibited CSF I from human lung, placenta, and urine, but showed no effect on CSF from mouse lung and mouse L cells (Table I).

Competitive binding of CSF I and II–RIA. Using anti-CSF I antibody and radioiodinated ^{125}I-CSF I, an

TABLE I
The Effect of Anti-CSF I Antibody on CSF from Different Sources

Source of CSF	Control colonies	Colonies AB-treated	Control colonies
			%
MIA PaCa-2 CSF I	97	0	0
MIA PaCa-2 CSF II	71	62	88
HPCM CSF I	95	0	0
HLCM CSF I	110	0	0
Urinary CSF	89	0	0
Mouse lung	75	74	98
Mouse L cells	56	53	96

CSF from different sources was incubated with 100 μl of anti-CSF I antibody or control IgG at room temperature for 30 min in the assay plates and then assayed on mouse marrow. HPCM, human placental conditioned medium; HLCM, human lung conditioned medium; AB, antibody.

RIA has been established. A typical competitive binding curve with CSF I and II is shown in Fig. 2. CSF II from MIA PaCa-2 showed no competition for binding while CSF I from MIA PaCa-2, human lung, and human placenta competed to the same degree. Once again, these results demonstrate the immunological difference between CSF I and II.

Stimulation of different bone marrow CFU-C populations by CSF I and II. The sedimentation pattern of human bone marrow cells as assayed with CSF I and II from human lung is shown in Fig. 3. When CSF II was used to assay the various fractions, two broad CFU-C peaks were observed. One peak, with a sedimentation velocity of 8–8.5 mm/h, formed colonies by day 7 of culture and another peak sedimenting at 6.5 mm/h formed colonies by day 13. In contrast, CSF I stimulated little or no colony formation in any fractions on day 7. Instead, a new, broad CFU-C peak appeared between 7 and 9 mm/h on day 13 of culture.

Colony morphology in fractionated marrow, in response to the two types of CSF, was also different. Colonies formed in response to type II CSF were predominantly granulocytic on day 7 (84%), becoming predominantly macrophagic by day 13. Colonies formed in response to type I CSF on day 13 were 70% granulocytic in the low-sedimenting (6.8 mm/h) and 42% granulocytic in the high-sedimenting (8.5 mm/h) fractions. This was still more than double the percentage seen with type II CSF in the same fractions.

DISCUSSION

A common pattern of two distinct types of CSF in human tissue and cultured cancer cells has been recently reported. CSF I and II can be separated by
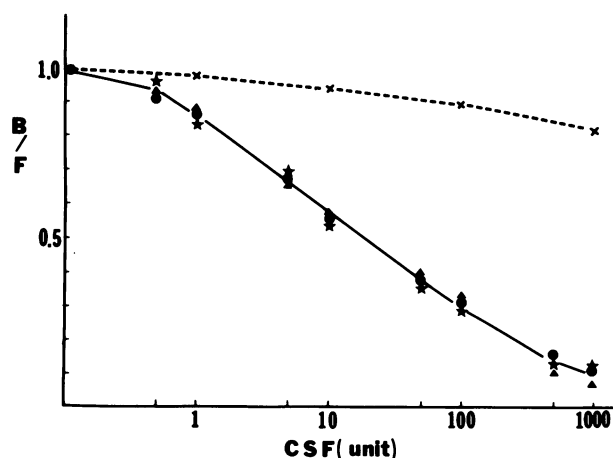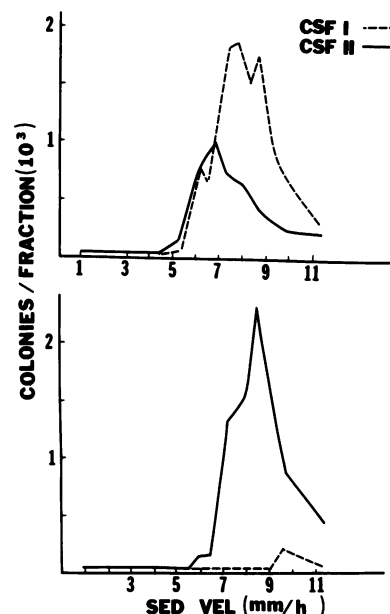
FIGURE 3   Heterogeneity of CFU-C in response to type I and II CSF. Human bone marrow cells were fractionated by sedimentation velocity at unit gravity. Fractions were assayed for colony formation with CSF I and II separately. Plates were read at days 7 (bottom) and 13 (top). The activity of the CSF I and II in unfractionated marrow was, respectively, as follows: day 7, 4.5 and 74.5 colonies/10^5 cells; day 13, 30 and 13 colonies/10^5 cells.

FIGURE 2   Competitive binding curve for CSF from different sources. Ordinate, the ratio of bound ^{125}I-CSF to free ^{125}I-CSF; abscissa, CSF activity in units per assay tube. MIA PaCa-2 CSF II (×); MIA PaCa-2 CSF I (●); human lung CSF I (▲); human placenta CSF I (★).

isoelectrofocusing and gel filtration, and have different pI, molecular weights, and marrow specificity (1). The results presented in this report demonstrate that type I and II CSF are also immunologically distinct. Anti-CSF I antibody inhibits CSF I, but not CSF II activity, whereas anti-CSF II antibody inhibits CSF II but not CSF I. The immunological difference between type I and type II CSF was further demonstrated by the competitive binding experiment using radioiodinated type I CSF.

The non-equivalence of human and mouse marrow cultured in the assay of CSF has been reported previously (9). It is known that human urinary CSF is active in mouse marrow, but that it exhibits little or no activity in human marrow. Our results indicate that human urinary CSF can be totally inhibited by anti-CSF I antibody. Thus, human urinary CSF is biologically and immunologically similar to CSF I. These findings are in agreement with those of Das et al. (10) who recently demonstrated, using a radioreceptor assay method, that human urinary CSF is similar to the mouse active CSF obtained from different human sources.

Because of its low activity in human marrow, CSF I has been referred to as "mouse active," and its role as a stimulator of human CSF-C growth has been neglected. However, it is clear from the present studies

that CSF I is indeed active in human marrow, but that its activity is directed at a subpopulation of CFU-C which is best demonstrated on day 13 of culture and when this subpopulation is concentrated by fractionation.

When human marrow cells are fractionated by sedimentation velocity at unit gravity and cells in the various fractions are cultured in the presence of human lung-conditioned medium as a source of CSF (8), two CFU-C peaks are observed (7), one sedimenting at ~8–8.5 mm/h and forming colonies after 7 d of culture, and a second sedimenting at 6.5 mm/h and not forming colonies until at least the 11th d of culture. A similar CFU-C profile was obtained here using human lung CSF II. In contrast, when lung CSF I was used, essentially one CFU-C peak was obtained, exhibiting colonies on day 13 but having an average sedimentation velocity different from that of the 13-d peak seen with CSF II. Additionally, whereas most of the 13-d colonies with CSF II were macrophagic, those with CSF I were 42–80% granulocytic. The virtual absence of 7-d colonies with CSF I offers a good explanation as to why "human activity" of CSF I has not been evident in the routine 7-d assay in the past.

It is clear from our studies that CSF I and II are biochemically and immunologically distinct and appear to have a different CFU-C specificity. Perhaps more important is that the so called "mouse active" CSF I is also "human active" and should be looked at in a different perspective.

ACKNOWLEDGMENTS

REFERENCES

1. Wu, M-C., and A. A. Yunis. 1980. Common pattern of two distinct types of colony-stimulating factor in human tissues and cultured cells. *J. Clin. Invest.* **65:** 772–775.
2. Ratzan, R. J., M. A. S. Moore, and A. A. Yunis. 1974. Effect of chloramphenicol and thiamphenicol on the in vitro colony forming cell. *Blood.* **43:** 363–369.
3. Wu, M-C., J. K. Cini, and A. A. Yunis. 1979. Purification of a colony-stimulating factor from cultured pancreatic carcinoma cells. *J. Biol. Chem.* **254:** 6226–6228.
4. Fojo, S. S., M-C. Wu, M. A. Gross, Y. Purcell, and A. A. Yunis. 1978. Purification and characterization of a colony-stimulating factor from human lung. *Biochemistry.* **17:** 3109–3116.
5. Greenwood, F. C., W. M. Hunter, and J. A. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89:** 114–123.
6. Stanley, E. R. 1979. Colony stimulating factor (CSF) radioimmunoassay: Detection of a CSF subclass stimulating macrophage production. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 2969–2973.
7. Miller, A. M., M. A. Gross, and A. A. Yunis. 1978. Heterogeneity of human colony forming cells (CFU-C): difference in size, rate of colony formation, and responsiveness to colony stimulating factor. *J. Lab. Clin. Med.* **92:** 38–42.
8. Fojo, S. S., M-C. Wu, M. A. Gross, and A. A. Yunis. 1977. The isolation and characterization of a colony stimulating factor from human lung. *Biochim. Biophys. Acta.* **494:** 92–99.
9. Lind, D. E., M. L. Bradley, F. W. Gunz, and P. C. Vincent. 1974. The nonequivalence of mouse and human marrow culture in the assay of granulopoietic stimulatory factors. *J. Cell. Physiol.* **83:** 35–42.
10. Das, S. K., E. R. Stanley, L. J. Guilbert, and L. W. Forman. 1980. Discrimination of a colony stimulating factor subclass by a specific receptor on a macrophage cell line. *J. Cell. Physiol.* **104:** 359–366.