Characterization of Colony-stimulating Activity Produced by Human Monocytes and Phytohemagglutinin-stimulated Lymphocytes

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Human colony-stimulating activity (CSA) may support the proliferation of both human and murine granulocyte-macrophage progenitor cells (CFU-C) or, in the case of human urinary CSA, may only stimulate murine bone marrow CFU-C. CSA produced in the culture media of monocytes and macrophages and phytohemagglutinin-stimulated lymphocytes from human peripheral blood was characterized for both human and mouse marrow CFU-C stimulating activities. During the initial phase of a long-term culture of monocytes, both human- and mouse-active CSA (MnCM-HM) were produced. In later phases of culture, however, only mouse-active CSA (MnCM-M) was produced. Fractionation on Sephadex G-150 revealed two functionally distinct species from MnCM-HM and lymphocyte conditioned medium, a high molecular weight factor (MW > 150,000) which stimulated mouse but not human colony formation, and a low molecular weight species (MW 25,000-35,000) which was active against both mouse and human target cells. However, MnCM-M revealed only one high molecular weight species (> 150,000), active only on mouse marrow. The possible biologic significance of such an activity is discussed.

The development of semisolid agar or methylcellulose culture systems which support the proliferation and differentiation of granulocyte-macrophage committed stem cells (CFU-C) has revealed an apparently heterogeneous group of factors with colony-stimulating capacity. Attempts to clone human marrow CFU-C were initially unsuccessful until the development of a culture system employing an underlay of human peripheral blood leukocytes as a source of colony-stimulating activity (CSA). The CSA-producing cells in marrow and blood have been identified as monocytes; however, CSA may also be produced by mitogen stimulated lymphocytes. Human leukocyte conditioned medium contains four apparent molecular species with human colony-stimulating capacity: a low molecular weight, hydrophobic peptide and three activities with apparent molecular weights (MW) of 15,000, 35,000, and 90,000 associated with the cell membrane. Similar heterogeneity has been reported in mitogen-stimulated lymphocyte conditioned media.

CSA purified from human urine has been shown to be a sialic acid containing glycoprotein with an apparent molecular weight of 45,000. This factor, while highly active in stimulating mouse bone marrow colony formation, was almost inactive against human bone marrow. Antiserum prepared against...
human urinary CSA is, however, capable of inhibiting the activity of human serum- or leukocyte-derived CSA.8,20

We report here the production and separation of CSA with both human and mouse marrow-stimulating activity in culture media of monocytes and macrophages (MnCM) and phytohemagglutinin (PHA)-stimulated lymphocytes (LyCM) from human peripheral blood. In addition to confirming the preponderance of human active CSA in a culture medium of mitogen-stimulated human lymphocytes, we report that during the initial phase of culture of monocytes both human and mouse active CSA is produced. In later phases of culture, however, only mouse active CSA is produced. The relationship between human and mouse active CSA is discussed.

MATERIAL AND METHODS

Assay for CSA

Conditioned media were assayed for CSA against 1 x 10^5 fresh human bone marrow or 1 x 10^5 viable nucleated cells from cryopreserved marrow from normal donors cultured in 0.3% agar in supplemented McCoy’s 5A medium containing 15% fetal calf serum.7 To reduce spontaneous colony formation, the human marrow cells were subjected to a preliminary adherence separation as described by Messner et al.21 Conditioned media were also assayed for mouse CSA against 7.5 x 10^5 C57BL/6 marrow cells cultured in McCoy’s agar medium. The cultures were incubated at 37°C in a humidified 8% CO2 atmosphere and were scored for the presence of colonies (greater than 40 cells) and of clusters (3-40 cells) at 7 days for the mouse assay and 10-12 days for the human assay.

Cryopreservation of Human Bone Marrow Cells

Nucleated bone marrow cells were suspended (12 x 10^6 cells/ml) in McCoy’s 5A medium containing 20% fetal calf serum at 5°C-10°C. Equal volumes of cold (5°C-10°C) and serum-free medium containing 20% dimethylsulfoxide were added dropwise. Sterile 2-ml serum vials (NUNC) containing 1 ml of the final cell suspension (6 x 10^6/ml) were stored in liquid nitrogen. When required for CSA assay, the cells were quickly thawed in a water bath at 37°C, diluted ten times by dropwise addition of fresh medium, centrifuged at 413 g for 10 min, washed twice, and re-suspended in the culture medium.

The same cryopreserved marrow was used in the experiments described in Figs. 1 and 4, while fresh marrow was used for other experiments.

Preparation of Conditioned Medium From Human Peripheral Blood Monocytes

Mononuclear cells from normal human heparinized peripheral blood were isolated by the Ficoll-Hypaque method22 and washed twice with McCoy’s 5A medium containing 5% human serum (Flow). Then 15-20 x 10^6 mononuclear cells in 10 ml of medium were inoculated into 25-sq cm culture bottles (Falcon) or 1-2 x 10^6 cells in 1 ml of medium were added to 35-mm plastic Petri dishes (Lux). After 1 hr of incubation at 37°C, the nonadherent cells in media were removed, the adherent cells were washed, and 10 ml of fresh medium was added to the flasks or 1 ml to the Petri dishes. Following incubation at 37°C in 8% CO2 for varying periods of time, the conditioned media were collected, centrifuged at 733 g for 10 min, filtered through 0.22-μm Millipore filters, and stored at -70°C until assayed.

Preparation of Conditioned Media from PHA-stimulated Human Lymphocytes

Ficoll-Hypaque separated peripheral blood mononuclear cells were incubated at a concentration of 15-20 x 10^6 cells/ml in McCoy’s medium in 28 x 120 mm Leighton tubes (Belco) containing 200 mg of sterile carbonyl iron powder (Atomergic Chemical Co.). After thorough mixing of the cells and carbonyl iron, the tubes were incubated for 1 hr and then placed in contact with a magnet while the supernatant was gently decanted. The majority of the monocytes were retained
in the tube and the lymphocyte population obtained was 97%-99% pure. Lymphocytes (1 x 10^6/ml) were cultured in 5 ml of medium with 40 μg/ml of PHA P (Difco) in 17 x 100 mm sterile polystyrene tubes (Falcon) and incubated at 37°C in 8% CO₂ for 5 days. The conditioned medium was collected after centrifugation to remove the cells. The medium was filtered through 0.22-μm Millipore filters and stored at -70°C until assayed.

**Preparation of Monocyte and Leukocyte Feeder Layers**

Monocytes were isolated as adherent cells in 35-mm Petri dishes using a procedure identical to that used to prepare monocyte conditioned medium. For measurement of cumulative CSA production, the adherent cells were overlayed at day 0 with 1 ml of 0.5% agar-McCoy's medium. A target overlay of either 7.5 x 10⁴ mouse marrow cells or 1 x 10⁵ human marrow cells in 1 ml of 0.3% agar-McCoy's medium was then plated over the monocyte feeder layers either at day 0 or following 7- or 14-day preincubation of the feeder layers. Both mouse and human colonies were scored 7 days after plating the marrow cells.

Feeder layers of 1 x 10⁶ unseparated human peripheral blood leukocytes were prepared in 1 ml of 0.5% agar-McCoy's medium as described by Pike and Robinson. Leukocyte feeders were used in parallel with monocyte feeders and were overlayed with mouse or human marrow on days 0, 7, or 14.

Noncumulative monocyte CSA production was measured with adherent cells that had been incubated for 7 or 14 days in 35-mm Petri dishes in 1 ml of liquid medium. Conditioned medium was collected at day 7, and in the 14-day incubation the medium was replaced at day 7 and 7-14 day conditioned medium was subsequently collected. After media removal on day 7 or day 14, the adherent cells were overlayed with 1 ml of 0.5% agar-McCoy's medium and with 1 ml of 0.3% agar-medium containing mouse or human marrow cells. Colony formation was assessed after an additional 7 days of incubation.

**Fractionation of Conditioned Media**

Conditioned media were spun in an SS34 rotor at 34,858 g for 20 min at 4°C and then concentrated under nitrogen pressure by ultrafiltration in a stirred cell (Amicon) over a UM2 membrane (rated molecular weight cutoff 2000). Concentrates were respun as above and loaded on a Sephadex G-150 column (1.6 x 85 cm, Pharmacia) equilibrated with phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) with a pressure head of 10 cm. Fractions of 2.6 ml were collected. Prior to assay, fractions were sterilized by filtration through 0.22-μm Millipore filters.

Columns were calibrated with the following markers: blue dextran (V₀), bovine serum albumin (MW 67,000), chymotrypsinogen (MW 25,000), cytochrome C (MW 12,500), tyrosine, and 36Cl (V₆). The 36Cl was counted in a Packard tricarb liquid scintillation spectrometer. Pools of the fractions were assayed on mouse and human marrows.

Kd's for the markers were calculated \([ (V_e - V_0)/(V_t - V_0) ]\) and the apparent molecular weights of the active regions determined by comparison to standard curves of \( M^{1/2} \) versus \( Kd^{1/3} \) (Vₚ, elution volume).

**RESULTS**

Conditioned medium from monocyte-adherent cell cultures was collected at weekly intervals for up to 6 wk. After the first week, the monocytes had enlarged and formed a confluent monolayer of macrophages which were actively phagocytic and remained 99%-100% viable throughout the period of study. The MnCM was assayed for CSA against both human and mouse marrow. MnCM collected after the first week stimulated both mouse and human colony formation, with activity against human marrow being more pronounced than that against mouse marrow (Fig. 1). Human and mouse marrow colonies were comprised of granulocytes (25% and 20%, respectively), macrophages (34% and 56%), and mixed granulocytes-macrophages (42% and 36%). At the end of the
second week, production of human-active CSA had declined markedly; it was almost negligible at the end of the third week, and was absent thereafter. In contrast, production of mouse-active CSA increased markedly by the second week of media collection, and during subsequent weeks an increasing and continuous production of this CSA was observed (Fig. 1). Conditioned medium collected after 1 wk (MnCM-HM) was concentrated tenfold by ultrafiltration over a UM2 membrane (Amicon) and titrated for CSA against mouse or human marrow (Fig. 2). Even after tenfold concentration, however, a plateau of colony stimulation was not observed with mouse marrow. The linearity of the titration curve of CSA against mouse marrow indicated that CSA inhibitors were not responsible for the differential responsiveness of mouse and human CFU-C.

Conditioned medium collected between the fourth and sixth weeks (MnCM-M), induced colony growth of mouse marrow only. These colonies were comprised of granulocytes (24%), macrophages (50%), and mixed granulocytes–macrophages (26%). When concentrated sevenfold and titrated against mouse and human marrow (Fig. 3), a sigmoidal dose–response curve was observed with mouse marrow. Human marrow, however, showed no colony for-
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Fig. 3. Dose-response curve of sevenfold-concentrated MnCM-M collected from week 4 to week 6 of culture of monocytes and macrophages against mouse and human marrow.

formation even at the highest concentrations of MnCM-M; only a few clusters were observed and they were composed solely of macrophages.

To test the possibility that human CSA was present in MnCM-M but was masked by a species-specific inhibitor, concentrated MnCM-M was added to human marrow cultures stimulated by human leukocyte feeder layers or by MnCM-HM (Table 1). No evidence of inhibition was observed.

Lipopolysaccharide (LPS) and products of mitogen-activated lymphocytes are known to activate macrophages. Addition of LPS to the monocyte cultures at weekly intervals produced a slight enhancement of production of CSA active against human marrow in the first and second weeks but did not prolong production of this activity (Table 2). Suppression of production of mouse-active CSA was seen with both 10 and 100 μg/ml of LPS and was particularly marked by the third and fourth week of media collection.

Addition of carbonyl iron–purified autologous lymphocytes to monocyte cultures did not significantly alter the magnitude nor the time course of production of human-active CSA, but some augmentation of mouse-active CSA production was observed (Table 2).

The decline in production of human-active CSA and the increase in production of mouse-active CSA with time (Fig. 1) could be due to the conversion of one factor to the other. To test this possibility, production of CSA by monocytes was determined in semisolid agar without weekly changing of the medium (cumulative feeders), and also with a weekly medium change prior to preparation of feeders (noncumulative feeders). The cumulative feeders, even after 1

Table 1. Effect of Sevenfold Concentrated Mouse-active CSA (MnCM-M) on the Human Marrow Colony Growth Induced by Leukocyte Feeders and Mouse- and Human-active CSA (MnCM-HM)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control</th>
<th>0.1 ml</th>
<th>0.05 ml</th>
<th>0.01 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte feeder</td>
<td>118</td>
<td>124</td>
<td>114</td>
<td>116</td>
</tr>
<tr>
<td>MnCM-HM (0.1 ml of tenfold concentrated)</td>
<td>246</td>
<td>242</td>
<td>240</td>
<td>244</td>
</tr>
</tbody>
</table>

Each value is a mean of colony counts on two plates.
Table 2. Effect of Weekly Addition of LPS or Lymphocytes on the Production of Mouse- and Human-active CSA by Monocytes and Macrophages (0.4 × 10⁶/ml)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>First Week*</th>
<th>Second Week</th>
<th>Third Week</th>
<th>Fourth Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
<td>Mouse</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>Control</td>
<td>62</td>
<td>38</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>Monocytes + LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>65</td>
<td>42</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>72</td>
<td>15</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Monocytes + 1 × 10³ lymphocytes/ml</td>
<td>68</td>
<td>62</td>
<td>3</td>
<td>95</td>
</tr>
<tr>
<td>Monocytes + 1 × 10⁵ lymphocytes/ml</td>
<td>70</td>
<td>75</td>
<td>4</td>
<td>107</td>
</tr>
</tbody>
</table>

Each value is a mean of colony counts/0.1 ml conditioned medium on two plates.

*Time of collection of conditioned medium.

days of preincubation before being overlayed with either human or mouse target cells (CFU-C), exhibited almost the same level of human CSA activity compared to that of the feeders with 0 hr and 7 days of preincubation (Fig. 4II-B). However, the level of mouse-active CSA increased with time in these feeders (Fig. 4II-B). The same was true even with cumulative leukocyte feeders (Fig. 4I). Since the experiments described in Fig. 1 showed that little, if any, human-active CSA was synthesized after day 7, the persistence of a constant level of human activity with an increase in mouse activity in cumulative feeders argued that the mouse-active CSA did not arise from extracellular conversion of human-active CSA to mouse-active CSA.

Fig. 4. CSA activity of cumulative leukocyte or monocyte feeders, noncumulative monocyte feeders, and conditioned media. (I) Human and mouse colony growth induced by 0-hr, 7-day, and 14-day preincubated WBC feeders (cumulative). (II, III) Mouse (II) and human (III) marrow colony growth induced by 0-hr, 7-day, and 14-day preincubated cumulative monocyte feeders (B), and noncumulative monocyte feeders (A) prepared from 0 hr, 7-day and 14-day monocyte cultures maintained in liquid medium (C). Liquid medium (C) collected prior to making noncumulative monocyte feeders at 7 days (days 0–7 of culture) and 14 days (days 7–14 of culture) was also assayed for CSA. Brackets represent ± SEM of triplicate readings of two experiments.
Noncumulative CSA production was studied using monocytes preincubated with liquid medium for 7 and 14 days prior to removal of the medium and overlaying with agar. By definition, the 0 hr noncumulative feeder was identical with the 0 hr cumulative feeder. In these experiments the species specificity of CSA produced by monocytes in a feeder layer situation was studied for 0–7 days, 7–14 days, and 14–21 days of assay. The results (Fig. 411, III) paralleled those obtained in the conditioned media study and showed that production of CSA with mouse specificity increased with time and the production of human activity declined rapidly, as observed earlier in a similar situation (Fig. 1). These results ruled out the possibility that in cumulative feeders human-active CSA was being continuously produced at a rate sufficient both to maintain constant human stimulatory capacity and to permit increasing mouse activity due to continuous conversion of the molecule.

Gel filtration of conditioned medium from 1-wk monocyte cultures (MnCM-HM) revealed two major regions of activity. The first region (A in Fig. 5), cor-

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**Fig. 5.** Fractionation on Sephadex G-150 of MnCM-HM collected after the first week of culture of monocyte, showing two regions of CSA activities. A, apparent molecular weight > 150,000, active only on mouse marrow; B, apparent molecular weight 25,000, active on both human and mouse marrow.

**Fig. 6.** Dose-response curve of 10x concentrated LyCM against human and mouse marrow.
responding to an apparent molecular weight of greater than 150,000, was found to be active only on mouse marrow CFU-C. The second region of activity (B in Fig. 5), with an apparent molecular weight of 25,000, was active against both mouse and human CFU-C.

LyCM was also active against both mouse and human marrow, and a tenfold concentration of LyCM, when titrated, showed a dose–response relationship (Fig. 6) very similar to that seen with MnCM-HM (Fig. 2). Fractionation
of concentrated LyCM on Sephadex G-150 revealed two regions of activity very similar to those observed with MnCM-HM (Fig. 7). One region (A₁), of apparent molecular weight of 150,000, was active only against mouse marrow, while the second region (B₁), corresponding to an apparent molecular weight of approximately 35,000, was active against both mouse and human marrow.

Gel filtration of MnCM-M containing only a mouse-active CSA gave only one region (A₂ in Fig. 8) of activity against mouse marrow, corresponding to the high molecular weight activity seen in MnCM-HM and LyCM. No human CSA was detected in any fraction even when fractions corresponding to region A of mouse activity or region B of mouse and human activity of MnCM-HM (Fig. 5) were pooled and concentrated tenfold prior to assay.

DISCUSSION

These observations support the concept that monocytes and macrophages are a major source of CSA capable of recruiting additional monocytes and granulocytes by stimulation of the marrow CFU-C compartment. The lower molecular weight species of CSA produced by both monocytes and PHA-stimulated lymphocytes was active against both mouse and human marrow. Its functional properties and apparent size suggest a close identity with CSAs partially purified from human placenta, embryonic kidney, and leukocyte conditioned media. The progressive temporal loss of human CSA in MnCM could not be attributed to production of a CSA inhibitor nor to an enhanced capacity of the macrophages to degrade the activity. The lack of continuous synthesis of this CSA is possibly a function of the age or state of activation of the cultured macrophages, and may suggest a self-limiting mechanism, whereby the positive feedback produced by monocyte CSA production is limited by maturation of the cells to macrophages. In this context, the mechanism of monocyte-derived CSA stimulation of granulopoiesis is also self-limited, since products of mature granulocytes suppress monocyte CSA synthesis and/or secretion.

The second species of CSA elaborated by monocytes and mitogen-stimulated lymphocytes was characterized by its inability to stimulate human colony growth even when concentrated to a level that produced a plateau stimulus in mouse marrow culture. The higher molecular weight and apparent restricted species specificity of this CSA in both MnCM and LyCM distinguished it from the smaller human-active factor. The properties of this CSA were similar to those of CSA purified from human urine. The apparent high molecular weight of urine CSA at an equivalent degree of purification has been attributed to binding of the active 45,000 MW moiety to contaminating proteins.

The production by human monocytes and macrophages of CSA apparently active only in the mouse marrow CFU-C assay raises the question of the biologic significance of such an activity in humans. The CFU-C compartment is heterogeneous by both physical and functional criteria and, in the mouse, CFU-C subpopulations of different buoyant densities exhibit different levels of responsiveness to CSAs of different origins. For example, human urinary CSA stimulated a murine CFU-C subpopulation of high buoyant density which differentiated predominantly into macrophages. It is possible that an equivalent
subpopulation is present in human marrow but that its proliferative potential is such that the clones which develop do not attain sufficient size to be scored as colonies. In this context, while incapable of supporting human colony growth, mouse-active CSA (MnCM-M), like urine CSA, promoted macrophage cluster formation in human marrow culture. Reports that purified CSA can stimulate macrophage proliferation in vitro suggest a biologic role for macrophage-derived or human urinary CSA in stimulating macrophage proliferation independent of recruitment of additional cells from the marrow CFU-C.

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