Partial Purification and Characterization of a Growth Factor for Macrophage Progenitor Cells With High Proliferative Potential in Mouse Bone Marrow

By A. B. Kriegler, T. R. Bradley, E. Januszewicz, G. S. Hodgson and E. F. Elms

A population of macrophage progenitor cells, with high proliferative potential, has recently been demonstrated in postfluorouracil-treated and normal mouse bone marrow (BM) in vitro, when a newly discovered growth factor (synergistic activity, SA) is combined with a macrophage colony-stimulating factor (CSF) as a proliferative stimulus. SA, shown to be present in human spleen and placental conditioned media (HSCM and HPCM, respectively), have been studied and found to be unstable to trypsin digestion and to heating at 50°C or above; stable between pH 4 and 9; nonadherent to Con-A-Sepharose; and to have an isoelectric point between pH 5 and 5.8 and a molecular weight of between 14,000 and 21,000 as indicated by gel filtration chromatography. SAs from both HSCM and HPCM have been purified 89- and 122-fold, respectively, by precipitation of extraneous proteins at pH 5 followed by chromatographing twice on Sephacryl S200. Neither of these partially purified SAs contain any CSF for mouse BM. These results indicate that the SAs from HSCM and HPCM may be closely related and that they are structurally different from CSFs derived from various murine sources that have been shown to be stable to proteolytic enzymes and heat.

**Colony-Stimulating Factor (CSF)**

Colony-stimulating factor (CSF), present in extracts of pregnant mouse uterus and embryo (PMUE), stimulates macrophage colony formation by single cells from normal mouse bone marrow when incubated at low cell densities in a semisolid agar culture system. PMUE has been shown to contain only CSF-I, a subclass of CSF that specifically stimulates the formation of macrophage colonies. Recently, a class of colony-forming cells (CFC), with the capacity to form large colonies have been demonstrated in normal and 5-fluorouracil (FU) treated mouse bone marrow, which are responsive only to a combined proliferative stimulus. The combined stimulus demonstrated for successful growth of large colonies from such CFCs consists of PMUE together with an activity (designated as synergistic activity, SA) shown to be present in human, rat, and mouse spleen and human placental conditioned medium. In the present article some physicochemical properties and the partial purification of SA from human placental conditioned medium (HPCM) and human spleen conditioned medium (HSCM) are described.

**Materials and Methods**

**Human Spleen Conditioned Medium**

HSCM was prepared from spleens from patients with Hodgkin's disease essentially as described by Bradley et al. Portions of spleen were minced and sieved and suspended in balanced salt solution (BSS) containing 2% fetal calf serum (FCS) and 50 μg/ml of chlorotetracyclin. The supernatant and the majority of erythrocytes were removed after centrifugation at 1000 g. The white cell preparations were then used to condition alpha medium containing 5% FCS at 16 × 10⁶ cells/ml medium. After 6 days, the conditioned medium was centrifuged, diafiltered against distilled deionized water (DDW), and finally concentrated tenfold using an Amicon XM50 membrane. This preparation was sterile filtered and stored at −20°C.

**Human Placental Conditioned Medium**

After removal of membranes, human placenta was cut into approximately 1 cm pieces under sterile conditions and residual blood washed out in BSS containing 2% FCS and chlorotetracyclin. The tissue was then minced using an MSE homogenizer at full speed for 6 sec. Tissue culture flasks (Costar, 150 sq cm) were each charged with 60 ml of alpha medium containing 5% FCS and approximately 6 ml of minced tissue, gassed with a 5% O₂/10% CO₂ gas mixture, and incubated at 37°C. After 6 days the conditioned medium was centrifuged at 28,000 g, concentrated tenfold by ultrafiltration (Amicon PM 10 membrane), and stored at −20°C.

**Assay for Synergistic Activity**

Post-FU bone marrow was taken from groups of 5 (BALB/c × C57Bl/6)F₁, 3-mo-old mice 4 days after a single intravenous injection of FU at 150 mg/kg body weight. Agar cultures of these cells (5 × 10⁶ cells/culture) were grown in the presence of a source of CSF essentially as described by Bradley et al. Double-layer cultures (1 ml underlay, 0.5 ml overlayer) in 35-mm Petri dishes were used throughout. PMUE was used as the basic CSF throughout and at a concentration optimal for colony development by normal mouse marrow cells. Three-hundred or 150 μl aliquots of the preparation to be tested for SA, serially diluted (twofold) in normal saline were added to the underlays. Control dishes containing PMUE and 300 or 150 μl of normal saline were included in each assay. The culture medium used was alpha modification of Eagle’s MEM (Flow Laboratories, Detroit, Mich.), supplemented with 20% fetal calf serum. The same batch of FCS (Flow Laboratories batch no. 81224) was used throughout the work and was found to give optimal growth at a concentration of 20%. Cultures were incubated for 14 days at 37°C after gassing with a mixture of 5% O₂ and 10% CO₂. All clones containing more than an estimated 50 cells were counted.
Assay for CSF Activity

The assay for CSF activity for mouse BM was carried out as described above, using 10⁶ BM cells obtained from normal mice (BALB/c x C57BL/6)F₁, per culture. Aliquots (150 μl) of preparation to be tested, serially diluted (twofold) in normal saline, were the only addition to the underlays.

GM-CSF for human BM was assayed in single layer agar (1 ml) cultures using 5 × 10⁶ BM cells/culture. The BM cells were obtained from a pool of normal human BM stored in liquid nitrogen. The medium used was alpha medium supplemented with 20% human serum. Apart from these changes the culture conditions were the same as outlined above.

Trypsin Digestion

Five-milliliter aliquots of concentrated HSCM and HPCM were dialyzed against a 0.02 M Tris/HCl buffer (pH 8.1) containing 0.01 M CaCl₂ using an Amicon PM10 membrane to a final volume of 5 ml. These solutions contain approximately 188 mg protein and were incubated at 37°C with 4 mg Trypsin (TPCK treated, Worthington Biochemicals Corp., Freehold, N.J. 252 U/mg). After 2 hr, an equal amount of Trypsin inhibitor (soybean, Boehringer Mannheim GmbH, Mannheim, West Germany) was added. A control incubation was carried out in exactly the same way using 5 ml of alpha medium containing 5% FCS. Addition of this control incubation mixture to culture dishes containing PMUE together with untreated HPCM or HSCM had no effect on colony growth.

Stability at Different pH Values

Three-milliliter aliquots of HSCM and HPCM were adjusted to a series of pH values between pH 2 and 10 using a 0.1 M NaCl or 0.1 M NaOH solution and stored at room temperature. After 16 hr, the pH of each aliquot was checked and found to be unchanged. Following this the pH of each aliquot was adjusted to 7 using 0.1 M HCl or 0.1 M NaOH, sterile filtered, and assayed.

Con-A-Sepharose Chromatography

Five-milliliter samples of HSCM and HPCM, both of which were not concentrated and contained a total of approximately 20 mg of protein each, were dialyzed against a 0.2 M sodium acetate buffer pH 6 with 1 M NaCl, 10⁻¹ M MnCl₂, MgCl₂, CaCl₂, and 0.1% polyethylene glycol (PEG), and chromatographed on a Con-A-Sepharose column (1.6 × 30 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) as described.⁷⁻⁸

Isoelectric Focusing

Isoelectric focusing was carried out at 4°C by zone convection electrofocusing using a horizontal trough apparatus.⁴ Ten milliliters of HSCM or HPCM (not concentrated, containing approximately 40 mg of protein each) was dialyzed against DDW (PM10 membrane), 0.8 ml of Pharmalyte (pH range 3–10, Pharmacia Fine Chemicals) was added, the volume adjusted to 30 ml with DDW, and the isoelectric focusing carried out at 450 V for 24 h. Fractions were diluted twofold with DDW, sterile filtered, and assayed directly for SA.

The presence of carrier ampholytes in these fractions was shown to interfere with 4-day post-FU mouse BM colony growth, by electrofocusing a Pharmalyte solution on its own, and assaying each fraction in the presence of constant maximally stimulating amounts of HPCM and PMUE.

pH 5 Precipitation and Gel Filtration

Ten milliliters of concentrated HSCM or HPCM was cooled in ice and the pH adjusted to 5 with 0.1 M HCl. The resulting precipitate was removed by centrifugation at 9000 g and discarded. The supernatant was adjusted to pH 7, loaded onto a Sephacryl S200 column (2.6 × 86 cm, Pharmacia Fine Chemicals), and eluted with 0.01 M Tris/HCl buffer (pH 7.3) with 2 mM EDTA, 0.15 M NaCl, and 0.1% PEG. Six-milliliter fractions were collected and pooled as shown in Figs. 3A and 4A. Each pool was concentrated threefold by ultrafiltration (PM10 membrane), sterile filtered, and assayed. The pools containing most of the SA were combined, concentrated to 10 ml (ultrafiltration, PM10 membrane), and rechromatographed on the same column as described above. Fractions were pooled as shown in Figs. 3B and 4B and concentrated threefold before assaying.

RESULTS

The ratio of tissue volume to medium volume and the depth of the tissue below the medium was shown to be critical for the production of optimal amounts of GM-CSF for human BM by human placental tissue.¹⁰ The effect of these parameters on the production of SA for mouse BM and GM-CSF for human BM by human

![Graph](https://example.com/graph.png)

Fig. 1. Colony growth of 4-day postfluorouracil-treated mouse BM cells in the presence of a constant maximally stimulating concentration of PMUE and various concentrations of either HPCM (not concentrated, Fig. 1A) or HSCM (concentrated tenfold, Fig. 1B). Numbers of small (diameter <0.25 mm, ■), medium (diameter 0.25–0.5 mm, ▲), and large colonies (diameter >0.5 mm, ○) are shown. Each point is the mean ± SEM of two separate experiments each in triplicate (6 dishes).
placental tissue has been examined in the present study. Maximum amounts of both factors were found to be produced when 1 vol of placental tissue was conditioned in 10 vol of culture medium, and when the medium in the tissue culture flask had a depth of 4 mm. Apart from GM-CSF for human marrow and SA, HPCM also contains CSF for mouse BM, whereas HSCM, prepared as described by Bradley et al., was shown not to contain any CSF for mouse BM or GM-CSF for human BM.

The SA in both HPCM and HSCM was assayed as described by Bradley and coworkers, using 4-day post-FU mouse BM cells, since it has been shown by these authors that the ratio of CFCs responsive to PMUE plus SA to CFCs responsive to PMUE alone is higher in this cell population than in normal BM or in BM at any other time following FU treatment. Colonies were scored after 14 days according to their size. Those with diameter <0.25 mm (containing from 50 to 1000 cells per colony), between 0.25 and 0.5 mm (containing an average of \(5 \times 10^3\) cells per colony), and >0.5 mm (containing an average of \(5 \times 10^4\) cells per colony) were thus scored separately. Bradley et al. have suggested that colonies with diameter >0.5 mm, which only form in response to PMUE plus SA, are derived from high proliferative potential colony-forming cells, while the smaller colonies (diameter <0.5 mm), which form in response to PMUE alone, are derived from low proliferative potential colony-forming cells.

As can be seen in Fig. 1, in the presence of a constant maximally stimulating dose of PMUE, the number of both large (diameter >0.5 mm) and medium-sized colonies (diameter between 0.25 and 0.5 mm) increased with increasing dose of either HPCM (Fig. 1A) or HSCM (Fig. 1B). Some batches of HPCM showed high dose inhibition of large and medium-sized colonies, while all HSCM batches showed this effect. While HSCM had no significant effect on the growth of small colonies (diameter <0.25 mm), the effect of HPCM depended on the particular batch used. The preparation used in Fig. 1A resulted in inhibition of small colony growth at high doses and stimulation at low doses. Some HPCM batches have, however, shown slight inhibition at high doses but no stimulation, while others had no observable effect.

In order to quantitate the SA, only the counts of numbers of large colonies (diameter >0.5 mm) were used. The relative specific activities in Table 1 are expressed as colonies per milligram protein and are calculated from the dose required to stimulate half the maximum number of colonies on the dose–response curve as described for mouse lung CSF. The relative specific activities of the samples, which are compared in Table 1, were all determined in the same assay, i.e., using exactly the same BM.

The SA in both HSCM and HPCM was stable to storage at \(-20^\circ\)C but was lost within 4 mo at 4°C. Trypsin digestion of both preparations resulted in a total loss of SA, indicating that this growth factor contains a peptide moiety and that its instability at 4°C may be due to proteolytic hydrolysis. CSF for mouse BM in HPCM was not affected by Trypsin digestion. The SA in both HSCM and HPCM was unstable to

### Table 1. Partial Purification of Synergistic Activity

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (Colonies)</th>
<th>Relative Specific Activity (Colonies/mg)</th>
<th>Purification (I-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) HPCM (10-fold)</td>
<td>10</td>
<td>400</td>
<td>12,800</td>
<td>32</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(2) pH5 precipitation</td>
<td>10</td>
<td>272</td>
<td>12,240</td>
<td>45</td>
<td>1.4</td>
<td>96</td>
</tr>
<tr>
<td>(3) Sephacryl 5200 (first cycle)</td>
<td>30</td>
<td>16</td>
<td>10,368</td>
<td>648</td>
<td>20</td>
<td>81</td>
</tr>
<tr>
<td>(4) Sephacryl 5200 (second cycle)</td>
<td>10</td>
<td>2.4</td>
<td>9,333</td>
<td>3,888</td>
<td>122</td>
<td>73</td>
</tr>
<tr>
<td>(1) HSCM (10-fold)</td>
<td>10</td>
<td>350</td>
<td>3,500</td>
<td>10</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(2) pH5 precipitation</td>
<td>10</td>
<td>225</td>
<td>3,375</td>
<td>15</td>
<td>1.5</td>
<td>96</td>
</tr>
<tr>
<td>(3) Sephacryl 5200 (first cycle)</td>
<td>22</td>
<td>14.5</td>
<td>2,407</td>
<td>166</td>
<td>17</td>
<td>69</td>
</tr>
<tr>
<td>(4) Sephacryl 5200 (second cycle)</td>
<td>20</td>
<td>2.6</td>
<td>2,301</td>
<td>885</td>
<td>89</td>
<td>66</td>
</tr>
</tbody>
</table>

All samples were assayed in the same assay.

*Pools 6 and 7, Fig. 3A.
†Pool 5, Fig. 3B.
‡Pool 4, Fig. 4A.
§Pools 5 and 6, Fig. 4B.

Protein levels were determined by the technique of Lowry et al.
heating at 50°C or above; stable between pH 4 and 9; and did not bind to Con-A-Sepharose.

Isoelectric focusing indicated that the SA in both preparations had an isoelectric point between pH 5 and 5.8 (Fig. 2). The isoelectric points of CSFs for both human and mouse BM in HPCM overlap with that of the SA (Fig. 2A). No CSF activity for either human or mouse BM can, however, be detected in the fractions from HSCM (Fig. 2B).

The SA in both HPCM and HSCM has been partially purified by precipitating extraneous proteins at pH 5 followed by chromatographing twice on Sephacryl S200 (Table I, Figs. 3 and 4). Precipitation of extraneous proteins by adjusting the pH to 5 results in clarification of the conditioned media with very little loss of activity and facilitates the subsequent gel filtration step. As can be seen in Table 1, the two chromatography steps result in an 89- and 122-fold purification of the SA in HSCM and HPCM, respectively, with overall yields of 66% and 73%, respectively. These partially purified SAs have remained unchanged in activity during storage at 4°C for 4.5 mo.

The partially purified SA from HSCM shows a reduced high dose growth inhibitory effect on large and medium-sized colonies (Fig. 5B), indicating that the high dose inhibition, observed with crude HSCM (Fig. 1B), is due to the presence of an inhibitor of cell

During the first chromatographic cycle of HPCM,
the CSF for mouse BM was completely separated from the SA, which has an apparent molecular weight between 14,000 and 21,000. The GM-CSF for human BM, with a reported molecular weight of 30,000 was, however, only partially separated from the SA (Fig. 3). SA from HSCM has the same apparent molecular weight of between 14,000 and 21,000, while no CSF for normal mouse BM can be detected in any of the fractions from the Sephacryl S200 column (Fig. 4).

DISCUSSION

The activity (SA) in HSCM and HPCM, which stimulates the growth of HPP-CFCs in the presence of PMUE, has been partially characterized and purified. The SAs in both preparations have been found to be unstable to trypsin digestion and to heating at 50°C or above; stable between pH 4 and 9; nonadherent to Con-A-Sepharose; to have similar isoelectric points (between pH 5 and 5.8) and molecular weights (14,000–21,000). From these results it can be concluded that both SAs are probably closely related molecules, and that both contain a peptide moiety that can be hydrolyzed by trypsin and which is essential for the activity. The fact that neither of these SA bind to Con-A-Sepharose does not exclude the possibility that they may contain carbohydrate moieties.

Although each of these SAs may possess some heterogeneity in respect to isoelectric focusing, this cannot be concluded from the relatively wide observed isoelectric point ranges, since this may also be due to the type of apparatus used that has a low resolving power. The fact that the SA in crude HPCM and HSCM is retained by an Amicon XM50 membrane, even though their molecular weights are low (14,000–20,000), suggests that they may have an affinity to associate with other proteins in the conditioned media with molecular weights greater than 50,000.

A similar SA obtained from media conditioned by a murine myelomonocytic leukemic cell line (WEHI-3) has recently been described. Like the SAs in HPCM and HSCM, this murine SA has the ability to stimulate cells in normal and FU-treated mouse BM to form very large colonies of macrophages (an average of 50,000 cells/colony) in the presence of preparations containing CSF. SA from WEHI-3 conditioned medium is similar to the HSCM and HPCM activities in that it does not bind to Con-A-Sepharose and is unstable to trypsin. The WEHI-3 activity, however, differs by having a wider isoelectric point range (pH 4–6.5) and molecular weight range (9000–25,000) and is stable over a wider pH range (3–10.6) and temperature range (60°C and below). These differences are not surprising as different species are involved. All three SAs appear to be structurally different from CSFs derived from various murine sources which, in contrast to the SAs, have been shown to be remarkably stable to proteolytic enzymes and heat and to bind to Con-A-Sepharose.

Bol and coworkers have demonstrated the presence of three different subpopulations of granulocyte-macrophage progenitor cells in normal BM, which
they propose from a maturation sequence. The most primitive of these three CFCs, with the highest proliferative potential and forming the largest colonies, require a combination of PMUE and 18 hr postendotoxin mouse serum as a proliferative response. Bertoncello, Bradley, and Hodgson have suggested that the HPP-CFCs, responsive to PMUE plus HSCM or HPCM, is closely related to the most primitive progenitor cell subpopulation described by Bol et al.16,17 Characterization and purification of the factor in postendotoxin serum responsible for this activity, so as to determine its relationship to the SAs, would thus be of great interest.

Further purification of SA is currently in progress and should enable a more detailed study of its function to be carried out.

REFERENCES

Partial purification and characterization of a growth factor for macrophage progenitor cells with high proliferative potential in mouse bone marrow

AB Kriegler, TR Bradley, E Januszewicz, GS Hodgson and EF Elms