Inhibitory Activity on Murine Granulocytic Colony Formation of Bone Marrow Cell-conditioned Medium Obtained from Colony-stimulating Factor-producing Tumor-bearing Nude Mice¹

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ABSTRACT

The effects of bone marrow-conditioned medium obtained from colony-stimulating factor-producing tumor-bearing nude mice (G-BM-CM) on mouse and human granulocyte-macrophage colony formation and mouse erythroid colony and burst formation were studied. Addition of G-BM-CM into the mouse granulocyte-macrophage colony-forming system containing colony-stimulating activity more strongly inhibited granulocyte colony formation than did mixed granulocyte-macrophage and macrophage colony formation, while it did not change the number of granulocyte colonies formed by human bone marrow cells stimulated by human granulocyte colony-stimulating activity. Addition of G-BM-CM slightly increased mouse erythroid colony and burst numbers when it was added into an erythroid colony-forming system stimulated by erythropoietin (1 unit/ml), and into the erythroid burst-forming system stimulated by erythropoietin (1 unit/ml) and 7% spleen cell-conditioned medium. These results might indicate that G-BM-CM mainly blocked commitment of mouse granulocyte-macrophage colony-forming cells to granulocytic progeny.

INTRODUCTION

Marked granulocytosis without evidence of significant infections has been observed in some patients with nonhematological cancers (1, 8, 18-20). In our laboratory, tumor pieces taken from a patient with a lung cancer associated with marked granulocytosis have been successfully transplanted into nude mice and maintained for four years (13, 14, 21). The WBC of these nude mice (G mice³) reached as high as 6 x 10¹¹/liter and consisted of 95% mature neutrophils. Metcalf and Burgess (12) reported that, when intact developing Day 2 and Day 3 colonies were cross-transferred to GM-CSF or M-CSF recipient culture, most M-CSF-initiated clones exhibited irreversible commitment to macrophage formation in GM-CSF cultures, and a high proportion of GM-CSF-initiated clones continued to produce granulocyte progeny after transfer to M-CSF. Suda et al. (21) in our laboratory previously reported that bone marrow cells of G mice could make more granulocyte colonies than those of control nude mice that were not bearing tumor pieces (C mice) when SPCM was used as a source of CSA, whereas a large part of GM progenitors were not definitely committed to differentiate to granulocytes and macrophages, because no significant differences of cellular composition of colonies between G and C mice were observed by the stimulation of L-cell-conditioned medium which mainly contained M-CSF. We reported that a part of GM-CFCs in G mouse bone marrow cells could not produce colonies in a monolayer agar culture containing excess amounts of highly purified CSF-HU (CSF-HU-5) which mainly contained M-CSF as a source of CSA (18). This part of GM-CFC in G mouse bone marrow cells could make granulocytic and GM colonies when G-BM-CM or conditioned medium of G mouse bone marrow cells after irradiation was added, which probably contained G-CSF. It indicates that a part of GM progenitors in G mouse bone marrow cells were not committed to granulocytic series even though GM progenitors were exposed to G-CSF secreted from CSF-producing tumor for a long period. In a previous experiment, we found that addition of G-BM-CM weakly inhibited colony formation by C mouse bone marrow cells when stimulated by CSF-HU-5 which mainly stimulated mouse macrophage colony formation (16).

In this paper, we describe the effect of G-BM-CM on the colony formation stimulated by CSF-HU-5 as well as partially purified CSF-HU (CSF-HU-2) which mainly stimulates GM colony formation and G-T-CM which mainly stimulates granulocyte colony formation, to make clear which types of colonies are inhibited by the addition of G-BM-CM.

MATERIAls AND METHODS

Preparation of Conditioned Medium. To prepare C and G mouse bone marrow cell-conditioned medium, bone marrow cells obtained from C mice and from G mice were suspended into 10 ml McCoy’s Medium 5A without fetal calf serum to give a final concentration of 10⁶ cells/ml and were incubated at 37°C in a fully humidified atmosphere containing 5% CO₂ in air (6, 16). After 3 days of incubation, the supernatant was separated by centrifugation and kept at −20°C until use. To prepare G-T-CM, 50 mg of tumor tissue were cut into small pieces (~1 cm³) and incubated in a culture flask containing 20 ml of McCoy’s Medium 5A for 3 days (20). The medium was collected, and the supernatant of the centrifugation was stored at −20°C until use. HPCM was prepared as described by Burgess et al. (7).

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³ The abbreviations used are: G mouse, nude mouse bearing colony-stimulating factor-producing tumor that developed marked granulocytosis; CSF, colony-stimulating factor; C mouse, control nude mouse not bearing tumor pieces; C-BM-CM, bone marrow cell-conditioned medium obtained from C mouse; G-BM-CM, bone marrow cell-conditioned medium obtained from G mouse; G-T-CM, tumor tissue-conditioned medium obtained from G mouse; CSF-HU, human urinary colony-stimulating factor; HPCM, human placental conditioned medium; GM, mixed granulocyte-macrophage; EP, erythropoietin; M-CSF, macrophage colony-stimulating factor; GM-CSF, mixed granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; SPCM, spleen cell-conditioned medium; CSA, colony-stimulating activity; GM-CFC, granulocyte-macrophage colony-forming cells; G-CFC, granulocyte colony-forming cells; M-CFC, macrophage colony-forming cells; PMN, polymorphonuclear leukocytes.

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GM Colony Formation. GM colony formation was performed in a monolayer agar culture containing 5 x 10^6 C mouse and 1 x 10^6 human phagocyte-depleted bone marrow cells obtained from normal volunteers with informed consent. For mouse colony formation, 3 sources of CSA were used: CSF-HU-5 which had a specific activity of 1 x 10^6 units/mg protein in a mouse standard assay system (15) and mainly stimulated macrophage colony formation; CSF-HU-2 which had a specific activity of 1 x 10^6 units/mg protein and mainly stimulated GM colony formation; and G-T-CM which mainly stimulated granulocyte colony formation. For human colony formation, 2 sources of CSA (G-T-CM and HPCM) were used. Cell aggregates consisting of 50 or more cells were scored as colonies after 7-day cultures. Morphological examination was performed using dual esterase staining of whole-agar-plate specimens according to the methods of Li et al. (11) and Kubota et al. (10). If the most of the cells in a colony were chloroacetate esterase positive or butyrate esterase positive, the colony was considered as a granulocyte or macrophage colony, respectively. If a colony consisted of both chloroacetate esterase-positive cells and butyrate esterase-positive cells, then the colony was considered as a GM colony. C-BM-CM and G-BM-CM were substituted for the culture media when they were added into the colony-forming system. Data were expressed as mean colony number ± S.D. of 3 dishes.

Erythroid Colony and Burst Formation. Erythroid colony formation was performed using the plasma clot culture system reported by Tepperman et al. (22) and modified by Terasawa et al. (23). Phagocyte-depleted bone marrow cells obtained from C mice (1 x 10^6 cells/ml) were embedded in a plasma clot containing EP (1 unit/ml) (Step III, Connaught Laboratories, Toronto, Canada) and cultured for 2 days. At the termination of culture, the plasma clot was fixed with 5% glutaraldehyde, and embedded in a plasma clot containing EP (1 unit/ml) (Step III, Connaught Laboratories, Toronto, Canada) and cultured for 2 days. At the termination of culture, the plasma clot was fixed with 5% glutaraldehyde, and the number of colonies containing 80 or more erythroblasts was counted after hemoglobin and hematoxylin staining. Erythroid burst formation was carried out using the methyl cellulose culture system reported by Ilskov et al. (9). Phagocyte-depleted bone marrow cells obtained from C mice (1 x 10^6 cells/ml) were cultured in 0.8% methyl cellulose containing was carried out using the methyl cellulose culture system reported by Tepperman et al. (22) and modified by Terasawa et al. (23). Phagocyte-depleted bone marrow cells obtained from C mice (1 x 10^6 cells/ml) were embedded in a plasma clot containing EP (1 unit/ml) (Step III, Connaught Laboratories, Toronto, Canada) and cultured for 2 days. At the termination of culture, the plasma clot was fixed with 5% glutaraldehyde, and the number of colonies containing 80 or more erythroblasts was counted after hemoglobin and hematoxylin staining. Erythroid burst formation was carried out using the methyl cellulose culture system reported by Ilskov et al. (9). Phagocyte-depleted bone marrow cells obtained from C mice (1 x 10^6 cells/ml) were cultured in 0.8% methyl cellulose containing 5% C-BM-CM into the mouse GM colony-forming system did not change colony numbers formed by the same number of mouse bone marrow cells, the addition of G-BM-CM into the mouse GM colony-forming system resulted in reduction of colony numbers formed by the same number of mouse bone marrow cells. Addition of 5% G-BM-CM reduced colony numbers formed by C mouse bone marrow cells in the presence of 50 units CSF-HU-5, 50 units CSF-HU-2, and 5% G-T-CM from 45 ± 3 to 33 ± 4 (73.3%), from 38 ± 3 to 29 ± 6 (76.3%), and from 52 ± 2 to 16 ± 2 (30.7%) colonies, respectively. As shown in Table 2, CSF-HU-5 produced 9 GM and 35 macrophage colonies in the absence of G-BM-CM and 4 GM (44.4%) and 29 macrophage (82.6%) colonies in the presence of 5% G-BM-CM. CSF-HU-2 produced 10 granulocyte, 23 GM, and 5 macrophage colonies in the absence of G-BM-CM and 4 granulocyte (40.0%), 20 GM (87.0%), and 5 macrophage (100%) colonies in the presence of 5% G-BM-CM. G-T-CM produced 9 GM and 35 macrophage colonies in the presence of 5% G-BM-CM. These data indicate that addition of G-BM-CM mainly reduced granulocyte colony formation by C mouse bone marrow cells. Large amounts of G-T-CM (10%) could make 92 granulocyte and 24 GM colonies in the absence of G-BM-CM, indicating that G-T-CM contained a large amount of G-CSF and a small amount of M-CSF (Table 3). Addition of 5% G-BM-CM into the C mouse bone marrow colony-forming system in the presence of 10% G-T-CM reduced granulocyte colonies from 92 to 34 (37%) and increased GM colonies from 24 to 48 (200%). Addition of G-BM-CM (up to 10%) into the human bone marrow colony-forming system stimulated by 10% G-T-CM and 10% HPCM did not change colony numbers and morphology (Table 4). Finally, addition of G-BM-CM into a C mouse erythroid colony-forming system stimulated by 1 unit EP and into a C mouse erythroid burst-forming system stimulated by 1 unit EP and 7% SPCM had a tendency to increase erythroid colony and burst numbers (Table 5).

Statistics. In order to compare the data in the presence of G-BM-CM with those in the absence of G-BM-CM (control), Student’s t test was used.

RESULTS

As shown in Table 1, 5 x 10^6 phagocyte-depleted C mouse bone marrow cells could make 45 ± 3, 38 ± 3, and 52 ± 2 GM colonies in the presence of 50 units CSF-HU-5, 50 units CSF-HU-2, and 5% G-T-CM, respectively. Although the addition of 5% C-BM-CM into the mouse GM colony-forming system did not change colony numbers formed by the same number of mouse bone marrow cells, the addition of G-BM-CM into the mouse GM colony-forming system resulted in reduction of colony numbers formed by the same number of mouse bone marrow cells. Addition of 5% G-BM-CM reduced colony numbers formed by C mouse bone marrow cells in the presence of 50 units CSF-HU-5, 50 units CSF-HU-2, and 5% G-T-CM from 45 ± 3 to 33 ± 4 (73.3%), from 38 ± 3 to 29 ± 6 (76.3%), and from 52 ± 2 to 16 ± 2 (30.7%) colonies, respectively. As shown in Table 2, CSF-HU-5 produced 9 GM and 35 macrophage colonies in the absence of G-BM-CM and 4 GM (44.4%) and 29 macrophage (82.6%) colonies in the presence of 5% G-BM-CM. CSF-HU-2 produced 10 granulocyte, 23 GM, and 5 macrophage colonies in the absence of G-BM-CM and 4 granulocyte (40.0%), 20 GM (87.0%), and 5 macrophage (100%) colonies in the presence of 5% G-BM-CM. G-T-CM produced 9 GM and 35 macrophage colonies in the presence of 5% G-BM-CM. These data indicate that addition of G-BM-CM mainly reduced granulocyte colony formation by C mouse bone marrow cells. Large amounts of G-T-CM (10%) could make 92 granulocyte and 24 GM colonies in the absence of G-BM-CM, indicating that G-T-CM contained a large amount of G-CSF and a small amount of M-CSF (Table 3). Addition of 5% G-BM-CM into the C mouse bone marrow colony-forming system in the presence of 10% G-T-CM reduced granulocyte colonies from 92 to 34 (37%) and increased GM colonies from 24 to 48 (200%). Addition of G-BM-CM (up to 10%) into the human bone marrow colony-forming system stimulated by 10% G-T-CM and 10% HPCM did not change colony numbers and morphology (Table 4). Finally, addition of G-BM-CM into a C mouse erythroid colony-forming system stimulated by 1 unit EP and into a C mouse erythroid burst-forming system stimulated by 1 unit EP and 7% SPCM had a tendency to increase erythroid colony and burst numbers (Table 5).
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G-BM-CM Inhibition of Granulocytic Colony Formation in G Mouse

and Ogawa (17) reported the existence of burst-promoting activity in bone marrow-conditioned medium. The fine mechanism of inhibiting activity of G-BM-CM on mouse granulocyte colony formation was not yet known. Broxmeyer et al. (8) reported that human intact PMN, PMN-conditioned media, and supematant from immediately lysed PMN reduced the number of spontaneous human bone marrow colonies and clusters probably due to nontoxic action on colony-stimulating cells. Because phagocyte-depleted mouse bone marrow cells were used as a source of GM-CFC in our present experiment, inhibition of mouse granulocyte colony formation by G-BM-CM seems not to be due to nontoxic action on colony-stimulating cells. The inhibitory action might be a specific one on mouse GM-CFC because of the following reasons: (a) it decreased the number of granulocyte colonies but increased the number of GM colonies formed by C mouse bone marrow cells when stimulated by 10% G-T-CM; (b) it did not change granulocyte colony number produced by human bone marrow cells when stimulated by G-T-CM and HPCM; and (c) it did not at least reduce erythroid colony and burst numbers formed by mouse bone marrow cells. To answer the question of whether or not the inhibitory activity of G-BM-CM is due to an acidic isoferitinn reported by Broxmeyer et al. (2–5), purification and physicochemical studies of this inhibitory activity are now proceeding.

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| Table 4
| Number of granulocyte colonies formed by 1 x 10^6 phagocyte-depleted human bone marrow cells stimulated by G-T-CM and HPCM in the absence or presence of G-BM-CM (plating efficiency of control cultures, 0.03 to 0.07%)

| Stimulator   | % of G-BM-CM | No. of colonies formed at following % of G-BM-CM | 0%  | 5%  | 10%
|--------------|--------------|-------------------------------------------------|-----|-----|-----
| G-T-CM (10%) |              |                                                 | 25±5 | 30±2 | 28±2
| HPCM (10%)   |              |                                                 | 65±8 | 63±9 | 64±5

* Mean ± S.E.

| Table 5
| Effect of G-BM-CM on erythroid colony and burst formation

<table>
<thead>
<tr>
<th>G-BM-CM (%)</th>
<th>No EP</th>
<th>EP (1 unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of colonies formed/1 x 10^7 cells</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>247 ± 45</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>289 ± 39</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>331 ± 16</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>392 ± 6</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>355 ± 4</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

**DISCUSSION**

In a previous paper (16), we reported that a part of GM-CFC population in G mouse bone marrow cells could not colonize in a monolayer agar culture stimulated by excess amounts of CSF-HU-5 because of a diffusible inhibitor produced by G mouse bone marrow cells. Because CSF-HU-5 mainly contains M-CSF for mouse bone marrow cells, we could not know the effect of G-BM-CM on granulocyte colony formation by C mouse bone marrow cells in monolayer agar cultures. In the present experiment, we used 3 sources of CSA: CSF-HU-5 which mainly stimulated macrophage colony formation (15); CSF-HU-2 which mainly stimulated GM colony formation; and G-T-CM which mainly stimulated granulocyte colony formation. Addition of G-BM-CM weakly reduced GM and macrophage colony numbers and strongly reduced granulocyte colony numbers by C mouse bone marrow cells (Tables 1 and 2). When 5% G-BM-CM was added into a monolayer agar culture containing C mouse bone marrow cells and 10% G-T-CM, G-BM-CM strongly suppressed granulocyte colony formation and increased GM colony formation (Table 3). This may indicate that G-BM-CM strongly blocked commitment of GM-CFC to G-CFC. Although we do not know the reason why the number of GM colonies formed by C mouse bone marrow cells stimulated by 10% G-T-CM increased when G-BM-CM strongly increased granulocyte colony formation, it might be suggested that the part of GM-CFC which should commit to G-CFC in the absence of G-BM-CM formed GM colonies in the presence of G-BM-CM. G-BM-CM weakly inhibited GM-CFC and M-CFC to make colonies in a monolayer agar culture because G-BM-CM slightly reduced GM and macrophage colony numbers in the presence of CSF-HU-5 which had mainly M-CSF activity (Tables 1 and 2). G-BM-CM could not suppress granulocyte colony formation by human bone marrow cells stimulated by G-T-CM and HPCM (Table 4). It indicates that G-BM-CM interacts to murine GM-CFC but not to human GM-CFC. G-BM-CM slightly increased erythroid colony and burst formation by C mouse bone marrow cells probably due to the existence of EP-like activity or burst-promoting activity which might be a nonspecific bone marrow-conditioned media effect unrelated to the presence of granulocytosis-inducing tumor (Table 5). Porter...
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