Effect of Mouse Interferon on Growth and Differentiation of Mouse Bone Marrow Cells Stimulated by Two Different Types of Colony-Stimulating Factor

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The effects of mouse L-cell interferon (IFN) on growth of mouse bone marrow cells and their differentiation into macrophages and granulocytes were investigated in a liquid suspension culture system with two different types of colony-stimulating factor (CSF). Within 7 days, most bone marrow cells differentiated into macrophages in the presence of macrophage colony-stimulating factor (M-CSF) derived from mouse fibroblast L929 cells, but into both granulocytes (40%) and macrophages (23%) in the presence of a granulocyte-macrophage colony-stimulating factor (GM-CSF) from mouse lung tissue. IFN inhibited growth of bone marrow cells with both M-CSF and GM-CSF, but had 20 times more effect on bone marrow cells stimulated with M-CSF than on those stimulated with GM-CSF. A low concentration of IFN (50 IU/ml) stimulated production of macrophages by GM-CSF in liquid culture medium, whereas it selectively inhibited colony formation of macrophages in semisolid agar culture. IFN caused no detectable block of late stages of differentiation; mature macrophages and granulocytes were produced even when cell proliferation was inhibited by IFN. These results indicate that IFN preferentially affects growth and differentiation of the cell lineage of macrophages among mouse bone marrow cells.

Besides having antiviral activity, interferon (IFN) has many effects on cells. It was found to inhibit growth of normal cells and malignant cells and to modulate differentiation of various cells. 

Several investigators have reported that IFN inhibits the formation of macrophage and/or granulocyte colonies in cultures of mouse, rabbit, and human bone marrow cells in semisolid agar. It was also found to inhibit erythroid and megakaryocyte colony formation. 

These effects were thought to be due to inhibition by IFN of growth of precursor cells of these differentiated cells.

However, Verma et al. recently reported that human leukocyte interferon blocks proliferation and differentiation of human bone marrow cells beyond the myelocyte level. On the other hand, we previously found that IFN enhanced induction of differentiation of mouse myeloid leukemic cells (MI) and human promyelocytic leukemic cells (HL-60) into macrophages or granulocytes in vitro by a protein inducer and various other chemical compounds.

In this study, we investigated the effect of IFN on growth and differentiation of mouse normal bone marrow cells in more detail. The IFN used was highly purified by anti-IFN antibody column chromatography. Immature myeloid cells were enriched by elimination of mature macrophages and granulocytes from bone marrow cells. Furthermore, to observe cell differentiation without growth, we mainly used a liquid suspension culture system.

MATERIALS AND METHODS

Preparation of Bone Marrow Cells

Bone marrow cells were obtained from the femurs of 6–10-wk-old ICR mice. Immature myeloid cells were enriched by the method of Lotem and Sachs, with some modifications. Namely, bone marrow cells were washed twice with phosphate-buffered saline (PBS), and

Preparation of Colony-Stimulating Factors (CSFs)

One unit of CSF was defined as the amount stimulating formation of one colony from 10³ bone marrow cells in semisolid agar. The titer of CSF was determined in the concentration range giving a linear dose–response relation.

Macrophage colony-stimulating factor (M-CSF). Mouse fibroblast L929 cells were cultured in serum-free Eagle’s minimal essential medium. CSF was concentrated from the conditioned medium by ammonium sulfate precipitation and fractionated by gel filtration on a Sephadex G-200 column. The final titer of CSF was about 6,600 U/ml.

Granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF was prepared and partially purified from mouse cells (5×10⁵) suspended in 5 ml of PBS were gently layered over 5 ml Lympholyte-M, a separation medium for isolation of mouse lymphocytes, at a density of 1.087–1.088 g/ml (Cedarlane Laboratories, Ltd., Canada). The cells at the interface on centrifugation for 20 min at 400 g were collected, and then mature granulocytes and macrophages, which had Fc surface receptors, were eliminated by Fc rosette formation and density centrifugation. The cells were mixed with erythrocytes coated with antibody against erythrocytes (ratio of erythrocytes to bone marrow cells, 30:1 or more) in 10 ml of PBS, centrifuged for 3 min at 500 g, and incubated at 37°C for 30 min. The cell pellet was dispersed and subjected to a second cycle of Lympholyte-M density centrifugation. The cells from the interface were collected and washed twice with PBS. The yield was about 3%–5% of the initial cell number. The cells obtained in this way consisted of 20% myeloblasts, 46% promyelocytes and myelocytes, 6% monocytes, and 15% mononuclear cells of types that could not be determined from their morphology.
lung tissue by the method of Burgess et al.\textsuperscript{15} The lungs of adult ICR mice were removed, rinsed in PBS, and then transferred to 5 ml of serum-free Eagle’s minimal essential medium. Conditioned medium was heated at 56°C for 30 min and insoluble material was removed by centrifugation at 12,000 g for 15 min. The following procedures were performed in the presence of 0.01% (wt/vol) polyethylene glycol 4,000. The supernatant fluid was dialyzed against deionized distilled water overnight and then centrifuged again to remove the precipitate. The supernatant fluid was applied to a column of concanavalin-A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.2 M sodium acetate buffer, pH 5.0, containing Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, and Mg\textsuperscript{2+} ions (1 mM each). The supernatant fluid applied had been made up to the same final concentration of acetate, using 1 M stock buffer. GM-CSF was eluted from concanavalin-A-Sepharose 4B with the same buffer containing 0.05 M methyl- \alpha-D-glucopyranoside, and dialyzed against PBS. The final titer of GM-CSF was about 7,000 U/ml.

**Preparation of Mouse IFN**

Newcastle disease virus-induced L-cell IFN was prepared by Dr. Yoshimi Kawade, Institute for Virus Research, Kyoto University, as described previously.\textsuperscript{16} IFN was purified in collaboration with Dr. Y. Iwakura et al.\textsuperscript{17} and affinity chromatography on an anti-L-cell IFN globulin Sepharose column, prepared by Dr. Y. Iwakura et al.\textsuperscript{18} The specific activity of the purified IFN was 10\textsuperscript{8} IU/mg protein. IFN consisted of \(\alpha\)-type (major species) and \(\beta\)-type (minor species).\textsuperscript{19} This preparation of IFN had no CSF activity.

**Liquid Suspension Culture of Bone Marrow Cells**

Bone marrow cells were cultured in RPMI 1640 medium ( Gibco, Grand Island, NY) supplemented with 20% (v/v) heat-inactivated horse serum and various amounts of CSF and IFN. The cells were seeded at a concentration of 2 \times 10\textsuperscript{5} cells/ml in 15-mm tissue culture wells (1 ml/well) (NUNCLO, Denmark). Viable cells and morphological examinations were made on each well after incubation for 7 days unless otherwise noted in the text. The cells adhering to the culture dish were gently removed by pipette. For morphological analysis, more than 200 cells were examined in smear slide preparations stained with May-Grünwald-Giemsa solution.

**Semisolid Agar Culture of Bone Marrow Cells**

Bone marrow cells (10\textsuperscript{6}) were seeded into a 35-mm Petri dish (Falcon Plastics, Oxnard, CA) containing 1 ml of 0.3% agar culture medium supplemented with 20% horse serum and various amounts of CSF and IFN. After incubation for 7 days, the number of colonies containing more than 50 cells was counted under a microscope. The morphology of cells in colonies was determined after staining whole plate preparations with Giemsa solution by the method of Kubota et al.\textsuperscript{20}

**RESULTS**

**Growth and Differentiation of Bone Marrow Cells in Liquid Suspension Culture**

First, we examined the kinetics of growth of mouse bone marrow cells in liquid suspension cultures. As shown in Fig. 1, CSF was essential for survival and growth of bone marrow cells in liquid culture as well as in semisolid agar culture. The viable cell count in control cultures without CSF gradually decreased to 0 on day 5. In cultures containing M-CSF or GM-CSF (800 U/ml), the viable cell number was increased fourfold on day 5 and then began to decrease. The mean viable cell count on day 7 in 6 experiments was 6.8 \pm 2.5 \times 10\textsuperscript{5} cells/ml for M-CSF-stimulated cultures and 4.5 \pm 0.9 \times 10\textsuperscript{5} cells/ml for GM-CSF-stimulated cultures. No significant difference was observed in the growth patterns of bone marrow cells in M-CSF- and GM-CSF-stimulated cultures. The growth of cells depended on the concentration of CSF in the cultures (Fig. 2). Only slight increase in the cell number was observed when the cells were incubated with 200 U CSF/ml. In the following experiments the
culture medium contained 800 U of CSF/ml unless otherwise noted.

Although the peak of cell growth was observed on day 5 on incubation of the cells with CSF, especially in GM-CSF-stimulated cultures, well differentiated mature granulocytes and macrophages were seen on day 7 rather than on day 5. Table 1 shows the cell types of bone marrow cells cultured in the presence of M-CSF or GM-CSF for 7 days. The cells produced in the presence of M-CSF were mainly macrophages, and few mature granulocytes were found in these cultures on day 7. On the other hand, about 40% and 23% of the cells produced in the presence of GM-CSF were granulocytes and macrophages, respectively. In GM-CSF-stimulated cultures, the morphology of the cells depended on the concentration of CSF in the culture medium, as reported by Burgess and Metcalf; at a low concentration of GM-CSF (200 U/ml), more than 60% of the cells were macrophages (data not shown).

**Effect of IFN on Growth of Bone Marrow Cells in Liquid Suspension Culture**

Bone marrow cells were cultured with IFN plus M-CSF or GM-CSF for 7 days, and then the number of viable cells in each culture was counted. As shown in Fig. 3, IFN inhibited the growth of bone marrow cells in both M-CSF- and GM-CSF-stimulated cultures, but it was more inhibitory to cells stimulated by M-CSF than to those stimulated by GM-CSF. The mean titers of IFN required for 50% inhibition of growth in 5 experiments were 43 ± 24 IU/ml for M-CSF-stimulated cultures and 992 ± 79 IU/ml for GM-CSF-stimulated cultures. In GM-CSF-stimulated cultures, the presence of a low concentration of IFN (50–100 IU/ml) slightly enhanced the growth of bone marrow cells.

**Effect of IFN on Differentiation of Bone Marrow Cells in Liquid Suspension Culture**

Table 1 shows the effect of IFN on the differentiation of cells into macrophages in M-CSF-stimulated

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**Table 1. Effect of IFN on Growth and Differentiation of Bone Marrow Cells in Liquid Suspension Culture**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IFN (IU/ml)</th>
<th>Total Viable Cells (x 10⁶/ml)</th>
<th>Cell Type (x 10⁶/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Myeloblasts</td>
</tr>
<tr>
<td>M-CSF</td>
<td>0</td>
<td>7.50 ± 1.13 †‡</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>61.9 ± 18.3</td>
<td>1.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20.4 ± 3.7</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0</td>
<td>54.8 ± 17.1</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>83.7 ± 2.8</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>66.8 ± 13.1</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>26.8 ± 3.4</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>

*Bone marrow cells (2 x 10⁶ cells/ml) were incubated with M-CSF (800 U/ml) or GM-CSF (800 U/ml) in the presence of various concentrations of IFN for 7 days.
†Myeloid cells included promyelocytes, myelocytes, and metamyelocytes.
‡Mean ± SE of duplicate determinations.
§Percent of each cell type of the total cell counts.

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![Fig. 3. Effect of IFN on growth of bone marrow cells stimulated with M-CSF or GM-CSF in liquid suspension culture. Bone marrow cells (2 x 10⁶ cells/ml) were incubated with M-CSF (800 U/ml) or GM-CSF (800 U/ml) in the presence of various concentrations of IFN for 7 days. Then, viable cells were counted by the trypan blue dye exclusion test. Values are mean percentages of the control in duplicate determinations; bars, SE. The cell numbers of the M-CSF-stimulated control culture and the GM-CSF-stimulated control culture were 7.5 x 10⁶ cells/ml and 5.5 x 10⁶ cells/ml, respectively.](image-url)
concentrations of IFN enhanced significantly the production of macrophages. IFN inhibited growth of bone marrow cells in cultures, and the concentration of primitive myeloid cells decreased with increases of granulocytes and more stage of differentiation. Granulocyte differentiation by IFN at their specific the number of the cells were macrophages. 80% of the cells were macrophages was not significantly reduced, and more than the percentage of differentiated macrophage growth, cultures: although addition of IFN (100 IU/ml) to M-CSF-stimulated cultures caused 73% inhibition of cell growth, the percentage of differentiated macrophages was not significantly reduced, and more than 80% of the cells were macrophages.

A low concentration of IFN (50 or 100 IU/ml) enhanced significantly the production of macrophages and monocytes in GM-CSF-stimulated cultures, but the number of macrophages was unchanged at high concentrations of IFN (1,000 IU/ml) (Table 1). On the other hand, the number of granulocytes and more primitive myeloid cells decreased with increases of concentration of IFN. We did not observe block of granulocyte differentiation by IFN at their specific stage of differentiation.

**Effects of IFN on Growth and Differentiation of Bone Marrow Cells in Semisolid Agar Culture**

Bone marrow cells form colonies in semisolid agar culture containing CSF. M-CSF stimulated bone marrow cells to form macrophage colonies and GM-CSF stimulated them to form macrophage, granulocyte, and mixed colonies. We investigated the effect of IFN on formation of these colonies in the presence of M-CSF or GM-CSF. As shown in Fig. 4A, IFN inhibited colony formation with both M-CSF and GM-CSF. In agar cultures, as well as in liquid cultures, IFN inhibited growth of bone marrow cells in M-CSF-stimulated cultures more than that in GM-CSF-stimulated cultures. The morphology of cells in colonies was examined by staining whole plate preparations. In the presence or absence of IFN, M-CSF-stimulated cultures contained only macrophage colonies. On the other hand, the colonies of GM-CSF-stimulated cultures without IFN consisted of 27.7% macrophage-colonies, 55.4% granulocyte-colonies, and 17.0% mixed colonies. IFN inhibited colony formation of macrophages more than that of granulocytes in GM-CSF-stimulated cultures (Fig. 4B). Although, in liquid culture, a low concentration of IFN (50–100 IU/ml) enhanced production of macrophages by GM-CSF, it decreased the number of macrophage colonies in agar culture.

**DISCUSSION**

IFN has been reported to inhibit the formation of granulocyte and/or macrophage colonies from bone marrow cells in semisolid agar cultures containing CSF. In the present study, using liquid suspension cultures, we confirmed the inhibitory effect of IFN on the growth of mouse bone marrow cells stimulated by M-CSF and GM-CSF. Our study further demonstrated that, in both semisolid agar cultures and liquid suspension cultures, IFN inhibited the growth of mouse bone marrow cells stimulated by M-CSF more than that of cells stimulated by GM-CSF (Fig. 3 and Fig. 4A). Although two different types of CSF stimulate different target cells among bone marrow cells and mechanisms of action of CSF still remain unknown, our study suggests that IFN preferentially inhibited growth of precursor cells of macrophages. In fact, IFN inhibited markedly colony formation of macrophages, but not of granulocytes, from bone marrow cells stimulated with GM-CSF in a similar manner to that of cells stimulated with M-CSF (Fig. 4B). However, the number of macrophages was significantly increased by IFN when the bone marrow cells were stimulated with GM-CSF in liquid cultures (Table 1). The reason for this discrepancy is unknown, but these results suggest that IFN has two actions on growth and differentiation of mouse bone marrow cells: one is inhibition of the growth of early precursor...
cells of macrophages, and the other is stimulation of the later stage of growth and differentiation of macrophages. In agar cultures, we observed mainly growth of early precursor cells of macrophages because a single cell formed a colony by clonal extension. In liquid cultures, in addition to the growing of early precursor cells of macrophages, we could observe both cell differentiation without cell division and differentiation with a few cell divisions at the late stage of macrophage differentiation. It is interesting in this context that we previously showed the stimulatory effect of IFN on the induction of differentiation of mouse and human myeloid leukemic cell lines into macrophages and granulocytes.1,2 Production of activated macrophages by IFN was also reported.22

On the other hand, Verma et al. recently reported that, in semisolid agar cultures of human bone marrow cells, human leukocyte IFN decreased the number of granulocyte colonies, but increased the number of cluster-size aggregates of predominantly granulopoietic precursors.11 Furthermore, they reported that, in liquid suspension culture, IFN blocked granulopoietic differentiation beyond the myelocyte level and caused accumulation of precursor cells.13 However, we did not detect either an increase in clusters in semisolid agar cultures containing IFN or a block of differentiation into granulocytes. These results suggest that the effect of IFN on granulopoiesis is different in humans and mice. The human system used by Verma et al. did not produce any cells of the lineage of monocyte-macrophages.13

During preparation of this manuscript, Hale and McCarthy reported results similar to ours.23 They showed preferential inhibition by IFN of colony formation by macrophages from mouse bone marrow cells with M-CSF derived from pregnant mouse uterus and GM-CSF derived from mouse lung tissue.

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REFERENCES

Effect of mouse interferon on growth and differentiation of mouse bone marrow cells stimulated by two different types of colony-stimulating factor

Y Yamamoto-Yamaguchi, M Tomida and M Hozumi