Purification of a Factor Inhibiting Differentiation from Conditioned Medium of Nondifferentiating Mouse Myeloid Leukemia Cells*

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Mouse myeloid leukemia M1 cells are induced to differentiate by various differentiation inducers. Activity for inhibition of induction of differentiation of M1 cells (I-factor activity) was detected in conditioned medium of variant M1 cell clones that were resistant to differentiation inducers, and this I-factor activity was shown to be closely associated with resistance of the cells to differentiation inducers. In this work, the I-factor was purified to apparent homogeneity from conditioned medium of resistant M1 cells. The purification procedure consisted of ammonium sulfate precipitation, CM-Sepharose CL-6B, Sephadex G-200, reverse-phase high performance liquid chromatography on a C18 hydrophobic support, and high-performance liquid chromatography on a gel filtration column. The factor was analyzed by radiiodination, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography. The purified factor gave a single band of protein with a molecular weight of 68,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis which coincided with its biological activity. The concentration of I-factor required for 50% inhibition of dexamethasone-induced differentiation of M1 cells was 24 pm. At its effective concentration it had no effect on cell proliferation, and even at 1.2 nm it did not inhibit colony formation of normal bone marrow cells, suggesting that it was distinct from the inhibitor of normal precursors of macrophages and/or granulocytes.

The abbreviations used are: I-factor, factor inhibiting differentiation; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; CM, conditioned medium.

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Activity of Differentiated Cells

Phagocytic Activity—M1 cells (2 × 10^6/ml) were treated with various inducers for 3 days. The cells were harvested by centrifugation and incubated for 4 h with a suspension of polystyrene latex particles (0.2% in serum-free medium; average diameter, 1 μm; Dow Chemical Co., Indianapolis, IN). Then the cells were thoroughly washed 3 times with PBS, and cells containing more than five latex particles were scored as phagocytic cells. The percentage of phagocytic cells among at least 200 viable cells was calculated as described previously (9). For assay of the phagocytic activity of W6/32-B4 cells, the cells were incubated for 4 h with a suspension of polystyrene latex particles in medium containing 2% fetal calf serum.

Lysozyme Activity—Lysozyme activity in the cells was determined by the lysosyme method described previously (11). The activity, in micrograms equivalents of hen egg white lysozyme, was calculated from a standard curve prepared using purified hen egg white lysozyme (Sigma). The results are expressed as amounts of lysozyme in microgram equivalents/mg of cell protein.

Fc Receptors—Fc receptors were measured by counting rosetting forming cells. The percentage of cells with a rosette was measured quantitatively by mixing 2 × 10^6 IgG-coated ox erythrocytes with 10^6 M1 cells in a volume of 10 μl and incubating for 30 min at 37°C. IgG-coated ox erythrocytes were purchased from Immuno Biological Laboratories (Tokyo, Japan).

Morphological Change—The percentage of cells that were morphologically similar to macrophages was determined in cytospun smears stained with May-Gruenwald-Giemsa, and the percentage of mature cells was scored on 600 cells.

Adherence—Cells were plated at 2 × 10^5 cells/35-mm dish in 1 ml of medium in the presence of differentiation inducer. After 3 days nonadherent cells were removed, and dishes were rinsed twice with 0.5-ml volumes of PBS. Cells were suspended in the medium, and washes were pooled and counted on a Model ZBI Coulter counter.

Preparation of Conditioned Medium of R-1 Cells

R-1 cells (5–6 × 10^6/ml) were cultured in serum-free medium in spinner culture for 2 days. The medium was collected and centrifuged (5000 × g, 20 min) to remove cells and cell debris, and the supernatant fluid was stored at −10°C.

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Preparation Procedures

I-factor was precipitated from 135 liters of CM with 90% saturation of ammonium sulfate. The precipitate was suspended in 0.14 M NaCl in 0.06 M sodium acetate buffer, pH 5.0, dialyzed against the same buffer, and chromatographed on a column (1.9 × 35 cm) of CM-Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) at 4°C. Fractions with I-factor activity, eluted with 0.27–0.4 M NaCl, were concentrated, dialyzed against PBS, and subjected to gel filtration in PBS on a column (1.9 × 86 cm) of Sephadex G-200 (Pharmacia Fine Chemicals) at 4°C. Fractions with activity from four columns of Sephadex G-200 were pooled, concentrated, and dialyzed against PBS, and the solution was adjusted to 20% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid, clarified by centrifugation, and applied to a reverse-phase HPLC column. The HPLC system (Waters Associates, Milford, MA) consisted of two M-6000 A pumps, an M-660 solvent programmer, a Model 440 fixed wavelength detector, and a reverse-phase HPLC column. The HPLC system (Waters Associates, Milford, MA) consisted of two M-6000 A pumps, an M-660 solvent programmer, a Model 440 fixed wavelength detector, and a reverse-phase HPLC column.
I-factor was concentrated from the CM by precipitation with 90% saturation of ammonium sulfate. The precipitation material was subjected to chromatography on a CM-Sepharose CL-6B column which was equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 0.14 M NaCl. Under these conditions, the I-factor activity was eluted with buffer containing 0.27-0.4 M NaCl. Then the I-factor preparation was chromatographed on a Sephadex G-200 column. Most of the I-factor activity was eluted in fractions corresponding to an apparent Mr of 20,000-90,000 (Fig. 1, fraction 35-48). The fractions indicated by a bar were pooled as partially purified I-factor.

This partially purified I-factor was next chromatographed, on a C18 hydrophobic support column equilibrated with 20% acetonitrile. On development with 30-55% acetonitrile, the I-factor activity was eluted with 30-35% (fractions 38-41) and 45-50% (fractions 69-79) acetonitrile (Fig. 2). The I-factor activity in the latter fraction was purified further, because its specific activity was 30 times that of the former fraction (data not shown). The fractions indicated by a bar were pooled for further purification. The final step of purification was high performance gel filtration chromatography on TSK G3000-SW columns. The volatile solvent 45% acetonitrile in 0.1 M trifluoroacetic acid and was used. On gel filtration chromatography, several protein species were resolved, and I-factor activity was eluted as a single peak. This step was performed three times, and Fig. 3 showed the result on the third chromatography. A single protein peak was coeluted with I-factor activity for the TSK G3000SW column. The steps of purification of I-factor are summarized in Table I.

The partially purified I-factor by radioiodination, SDS-polyacrylamide gel electrophoresis, and autoradiography. Fig. 4 shows the homogeneity of the labeled preparation. The apparent Mr of the I-factor ranged from 60,000 to 70,000 (mean = 68,000). Assuming that Mr of the I-factor is 68,000, its concentration at step 5 (3rd) for 50% inhibition of induction of differentiation of M1 cells was approximately 24 pM (1.6 ng/ml) (Fig. 5B). This concentration of the I-factor did not affect the growth (Fig. 5A) or viability of M1 cells (data not shown).

Effect of Purified I-factor on Differentiation of M1 Cells—M1 cells can be induced by various substances, including dexamethasone, 1α,25-dihydroxyvitamin D₃, and CM from various cell cultures (1). We next examined whether the differentiation of M1 cells by other known inducing agents was also inhibited by the purified I-factor. As shown in Fig. 5, the purified I-factor significantly inhibited the induction of phagocytic activity of M1 cells induced by 1α,25-dihydroxyvitamin D₃ (48 h) or CM from peritoneum (30%). These results suggest that the I-factor inhibits differentiation of M1 cells induced by various types of differentiation agents.

In the process of purification of I-factor, I-factor was assayed by measuring inhibition of induction of phagocytic activity in M1 cells induced by dexamethasone, because induction of phagocytic activity in the cells was associated with the induction of other phenotypic markers of cell differentiation, such as morphological differentiation and lysozyme activity. We next examined whether other markers of differentiation of M1 cells were altered in the presence of the purified I-factor. Dexamethasone and CM of peritoneum induced (i) adherence of M1 cells to tissue culture plastic dishes, (ii) Fc receptors, (iii) lysozyme activity, and (iv) changes in cell morphology (1). The purified I-factor, at the concentrations

![Fig. 2. Chromatography of I-factor by reverse-phase HPLC.](image)

![Fig. 3. High performance gel filtration chromatography of I-factor on TSK G3000SW (3rd).](image)

### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>I-factor*</th>
<th>Purification</th>
<th>Total* I-factor</th>
<th>Yield</th>
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<td>R-1 cell-conditioned medium (135 liters)</td>
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<tr>
<td>1. 90% (NH₄)₂SO₄ precipitate</td>
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<td>6,000</td>
<td>1</td>
<td>204,000</td>
<td>100</td>
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<tr>
<td>2. CM-Sepharose CL-6B</td>
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<td>680</td>
<td>9</td>
<td>54,882</td>
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<tr>
<td>3. Sephadex G-200</td>
<td>8.38</td>
<td>230</td>
<td>26</td>
<td>36,435</td>
<td>17.9</td>
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<tr>
<td>4. µBondapak C₁₈</td>
<td>0.734</td>
<td>82.5</td>
<td>73</td>
<td>8,897</td>
<td>4.4</td>
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<tr>
<td>5. TSK G3000SW</td>
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<td>2,000</td>
<td>1.0</td>
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<tr>
<td>1st.</td>
<td>0.0016</td>
<td>1.6</td>
<td>3,750</td>
<td>1,000</td>
<td>0.5</td>
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</table>

* I-factor (nanograms of protein) required for 50% I-factor activity.
* Total protein (nanograms)/I-factor.

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Factor Inhibiting Differentiation of Myeloid Leukemic Cells
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Effect of purified I-factor on various markers of differentiation of M1 cells

<table>
<thead>
<tr>
<th>Markers of differentiation</th>
<th>Purified I-factor</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml</td>
<td>−Inducer</td>
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<tr>
<td>Phagocytic activity (%)</td>
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<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>Lysozyme activity (units/mg protein)</td>
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<td>3.9</td>
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<tr>
<td>Cell morphology (macrophage %)</td>
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<td>Adherence to plastic dish (%)</td>
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<tr>
<td></td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>33.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.3 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Reconstitution of resistance of R-1 cells to differentiation inducer. Results are for untreated R-1 cells (column 1) and R-1 cells treated for 2 days with 5 ng/ml of actinomycin D (column 2), 4 μM dexamethasone (column 3), actinomycin D plus dexamethasone (column 4), or actinomycin D plus dexamethasone plus purified I-factor (4 ng/ml, column 5). Columns and bars show mean values ± S.D. for four independent samples.

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified I-factor. A, SDS-polyacrylamide gel electrophoresis of 125I-labeled purified I-factor. B, the gel was sliced and I-factor activity was eluted overnight with serum-free medium containing 0.02% Tween 20. The molecular mass markers used were: phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), and carbonic anhydrase (31K).

Fig. 5. Effect of purified I-factor on induction of differentiation of M1 cells induced by several differentiation agents. M1 (S-2) cells were incubated with various concentrations of the purified I-factor in the presence of dexamethasone, 1α,25-dihydroxyvitamin D3, or CM from peritoneum of SL strain mice containing a protein inducer of differentiation of M1 cells for 2 days. The inhibition of induction of their phagocytic activity (B) and the number of cells (A) were assayed. ○, dexamethasone (2 μM); □, 1α,25-dihydroxyvitamin D3 (48 nM); ×, CM from peritoneum (30%). The numbers of cells (A) in control cultures in the presence of dexamethasone, 1α,25-dihydroxyvitamin D3, and CM from peritoneum were 9.3 ± 0.4 × 10^5/ml (n = 4, S.D.), 8.0 ± 0.8 × 10^5/ml (n = 4, S.D.), and 9.2 ± 0.7/ml (n = 4, S.D.), respectively. The phagocytic activities (B) of control cells in the presence of dexamethasone, 1α,25-dihydroxyvitamin D3, and CM from peritoneum were 68.9 ± 4.3% (n = 4, S.D.), 33.4 ± 9.2% (n = 4, S.D.), and 54.9 ± 6.6% (n = 4, S.D.), respectively.

which blocked the induction of phagocytic activity, also inhibited the induction of Fc receptors, lysozyme activity, and changes in cell morphology (Table II). The adherence to plastic dishes was slightly inhibited (Table II). These results suggest that the purified I-factor inhibits multiple aspects of differentiation of M1 cells by the differentiation inducers.

Effect of Purified I-factor on R-1 Cells Sensitized by Actinomycin D—R-1 cells could not be induced to differentiate even by a higher concentration of the inducer. As described above, the R-1 cells produced the I-factor. We found previously that the production of I-factor by R-1 cells could be inhibited by treating the cells with a low concentration of actinomycin D and after this treatment the cells became sensitive to the inducer both in vitro and in vivo (7-9). Then we examined whether the restoration of the sensitivity of R-1 cells by actinomycin D, which inhibited the production of I-factor, could be overcome by exogenous addition of purified I-factor. As shown in Fig. 6, in the presence of actinomycin D, R-1 cells were induced to differentiate by dexamethasone. However, the induction of differentiation was inhibited by exogenous addition of the purified I-factor. Four ng of I-factor/ml suppressed 64% of the induction of differentiation of R-1 cells by actinomycin D (5 ng/ml) and dexamethasone (4 μM). Fig. 7B also shows that the purified I-factor caused concentration-dependent inhibition of the induction of differentiation of R-1 cells. These results suggest that the I-factor in R-1 cells is closely associated with the resistance of these cells to the inducer.

Effect of Purified I-factor on Normal Bone Marrow Cells and Various Leukemic Line Cells—We examined whether the partially purified I-factor (step 3 preparation, 0.05-5 μg/ml), had any effect on the induction of differentiation of various leukemic line cells by various differentiation inducers. We used (i) human leukemic line cells, HL-60, ML-1, and K562 cells, and (ii) mouse leukemic line cells, Friend, and WEHI-3B-D+ cells. HL-60 and ML-1 cells were treated with 12-O-tetradecanoylphorbol 13-acetate (0.5 ng/ml) and retinoic acid (1 μM) for 6 days in the absence and presence of I-factor, and then their ability of reducing nitro blue tetrazolium dye, as a marker of differentiation, was tested. K562 cells were treated
with hemin (50 μM), 1-β-D-arabinofuranosylcytosine (0.72 μM), or sodium butyrate (0.5 mM), and Friend cells were treated with dimethyl sulfoxide (2%) or actinomycin D (5 ng/ml) for 4 days, and then the induction of benzidine-positive cells, as a marker of erythroid differentiation, was assayed. WEHI-3B-D+ cells were treated with retinoic acid (40 μM) for 2 days, and then the induction of phagocytic activity was assayed. The partially purified I-factor even at a high concentration (5 μg/ml) did not affect the induction of differentiation of HL-60, ML-1, K562, Friend cells by the above inducing agents (data not shown). But the induction of phagocytic activity of WEHI-3B-D+ cells was inhibited by the partially purified I-factor at a higher concentration (5 pg/ml) than that used for M1 cells (0.05-0.5 μg/ml). Therefore, we examined the effect of the purified I-factor on WEHI-3B-D+ cells. The purified I-factor (8-32 ng/ml) also inhibited the induction by retinoic acid of phagocytic activity in WEHI-3B-D+ cells (Fig. 7B). At its effective concentrations, the purified I-factor slightly suppressed the proliferation of WEHI-3B-D+ cells (50% inhibition at most). However, at the same concentrations, it did not inhibit growth of M1 cells (Fig. 7A). The sensitivity of M1 (S-2) cells to the purified I-factor was six to eight times that of WEHI-3B-D+ cells.

We have reported that the CM of R-1 cells also contains activity to inhibit colony formation of normal bone marrow cells induced by GM-CSF (12, 17). This inhibitory activity in the CM to bone marrow cells also decreased on treatment of R-1 cells with a low concentration of actinomycin D. Therefore, we examined whether the purified I-factor could inhibit the colony formation of bone marrow cells. The partially purified I-factor in the same concentration range (0.05-0.5 μg/ml) as that used for M1 cells, inhibited colony formation of normal mouse bone marrow cells induced by GM-CSF or M-CSF (data not shown), but the purified I-factor was not inhibitory even at a higher concentration than that used for M1 cells (Fig. 7C). These results suggest that the I-factor for M1 cells was distinct from the inhibitor of normal hematopoietic precursor cells.

**DISCUSSION**

In this work we purified an inhibitory protein factor, I-factor, to homogeneity, which could inhibit induction of differentiation of sensitive M1 cells by various differentiation inducers. This purified I-factor from the CM of R-1 cells gave a single band of protein with an Mr of 68,000 on SDS-polyacrylamide gel electrophoresis and inhibited dexamethasone-induced differentiation of M1 cells when added at a concentration of 24 pm.

We reported previously that R-1 cells release inhibitory activities affecting normal mouse bone marrow colony formation, in addition to acidic isoferritins (12, 17, 18). In the present work, we found that the purified I-factor was distinct from the other inhibitory activities released from R-1 cells that affected normal mouse bone marrow colony formation induced by colony-stimulating factor. However, during the purification procedure these inhibitory activities for normal bone marrow cells remained associated with the I-factor activity until the last few steps, suggesting that their characteristics are very similar to those of the I-factor. When injected into syngeneic SL mice, the resistant R-1 cells were much more leukemogenic than the sensitive M1 cells (4), so these inhibitory factors may give the resistant R-1 cells leukemogenic advantages by inhibiting both the proliferation of normal hematopoietic cells and the differentiation of the leukemic cells. Further characterization of these inhibitory activities is necessary to clarify their mode of action.

As shown in Fig. 6, R-1 cells became sensitized to inducer when treated with a low concentration of actinomycin D, and this recovery of sensitivity was inhibited by exogenous addition of purified I-factor. However, the inhibition was not complete, and some cells remained sensitive to inducer (Fig. 6). One possible explanation of this is as follows: The I-factor activities in the CM of R-1 cells are heterogeneous (Figs. 1 and 2), and in this work we purified only the major fraction of the activity. Therefore, this purified I-factor, which was one of several fractions of I-factor activity, may not alone be sufficient to reconstitute complete resistance of R-1 cells. Another possibility is that sensitization of R-1 cells by actinomycin D may have been the result of not only decrease in the production of I-factor but also some other mechanism(s).

We found previously that various compounds including nonsteroidal anti-inflammatory agents, phenolic antioxidants, α-tocopherol, tumor promoters, retinoic acid, prostaglandin Fsyn, and some basic proteins could inhibit induction of differentiation of M1 cells (5, 19-24). The inhibition of nonsteroidal anti-inflammatory agents or phenolic antioxidants was suggested to be due to inhibition of synthesis of prostaglandin E2, which is involved in the mechanisms of induction of differentiation of M1 cells (5, 19). On the other hand, the inhibition by retinoic acid or prostaglandin Fsyn was suggested to be due to the production of I-factor-like activity (21, 22). Some basic proteins, such as poly-L-arginine, poly-L-lysine, histone (H2B, H2A, H3), and lactoferrin, also inhibited the induction of differentiation of M1 cells (12, 23, 24). In the present work, the purified I-factor was also found to be a basic protein. However, not all basic proteins are inhibitory.
itors of differentiation (23). The mechanisms of inhibition by the basic proteins that do inhibit differentiation of M1 cells and the structural relationship between these proteins and the purified I-factor remain to be investigated. Moreover, it will be interesting to examine whether the I-factor constitutively produced by R-1 cells is related to the I-factor-like activity induced in M1 cells treated with retinoic acid or prostaglandin F$_2$. 

Mendelsohn et al. (25) reported that HL-60 TR cells, a tetraploid variant of the human acute promyelocytic leukemia cell line HL-60, did not have the ability to develop several common markers of maturation in response to compounds that induced monocytoid differentiation of HL-60 cells. In addition, they released factors that inhibited induction of differentiation of HL-60 cells and also inhibited colony formation by normal mouse bone marrow cells. These findings suggest that, like resistant R-1 cells, resistant HL-60 TR cells have mechanisms for inhibition of growth and differentiation of leukemic cells and normal stem cells. The I-factor purified in the present work did not inhibit induction of differentiation of HL-60 cells by 12-O-tetradecanoylphorbol 13-acetate or retinoic acid, but it inhibited the induction of differentiation of mouse leukemia WEHI-3B-D$^+$ cells by retinoic acid. Thus the I-factor activity may be species specific.

Recently LSCCHD3 chicken erythroleukemia cells, transformed by a temperature-sensitive avian erythroblastosis virus, were reported to secrete a nonmitogenic factor that inhibited the erythropoietin-induced differentiation of untransformed LSCCHD3 cells (at 42 °C) and dimethyl sulfoxide-induced differentiation of Friend murine erythroblastosis cells into hemoglobin-synthesizing erythroid cells (26). The production of this differentiation inhibitor was dependent on the activity of the oncogene product of temperature-sensitive avian erythroblastosis virus v-erbB. On the other hand, constitutive expression of the c-myc oncogene blocked mouse erythroblastosis cell differentiation (27). We do not know how the I-factor blocks the induction of differentiation of M1 cells. It will be interesting to examine whether an oncogene product is involved in its mechanism of action.

REFERENCES