Stimulation by Interferon of Induction of Differentiation of Mouse Myeloid Leukemic Cells

Mikio Tomida,2 Yuri Yamamoto, and Motoo Hozumi

ABSTRACT

Mouse myeloid leukemic M1 cells were induced to differentiate in vitro into macrophages and granulocytes by various inducers, such as differentiation stimulating factor (D-factor) in conditioned medium of mouse peritoneal macrophages and mouse embryo cells, dexamethasone, lipopolysaccharide, and polyinosinic acid. Interferon, which was prepared from M1 cells treated with copolymer of polyinosinic and polycytidylic acids and purified by anti-interferon antibody column chromatography, did not itself induce differentiation of M1 cells, but it enhanced the induction of differentiation by D-factor, lipopolysaccharide, or polyinosinic acid. Interferon did not stimulate differentiation of the cells by another inducer, dexamethasone. Interferon alone could induce lysozyme activity in M1 cells. The effects of interferon and D-factor or dexamethasone on induction of the lysozyme activity were synergistic.

The sera from mice given injections of the copolymer of polyinosinic and polycytidylic acids, containing interferon and D-factor, induced the differentiation of all M1 clone cells, including R-4 and DR-3 clone cells that cannot be induced to differentiate by D-factor alone. The degree of inhibition of cell growth by interferon varied in different clones of cells. Growth of three clones, clones R-1, R-4, and DR-3, that are resistant to inducers of differentiation, was inhibited more than was that of three other clones, S-1, T-22, and B-24, that respond to inducers.

INTRODUCTION

The mouse myeloid leukemic cell line M1 can be induced to differentiate into macrophages and granulocytes in vitro by a protein inducer, D-factor, in conditioned medium of mouse embryo cells or macrophages, in ascitic fluids from mice or rats, or by glucocorticoid hormones, lipopolysaccharide, poly(l)-poly(C), or various other compounds. Differentiation of the cells is accompanied by induction of phagocytic and locomotive activities, Fc and C3 receptors in the cells, and activities of lysosomal enzymes.

We have previously shown that synthetic double-stranded RNA's such as poly(l)-poly(C) induced interferon in M1 cells and enhanced induction of differentiation of the cells by D-factor and that an anti-interferon serum blocked the enhancing effect of poly(l)-poly(C). These results suggested that the action of the double-stranded RNA's on M1 cells was mediated by the induced interferon.

In the present work, we examined the effect of interferon from M1 cells treated with poly(l)-poly(C) or from L-cells infected with NDV and interferon from sera of mice given injections of poly(l)-poly(C) on induction of differentiation of M1 cells by various inducers. We found that interferon itself enhanced induction of differentiation of the cells by inducers such as D-factor.

MATERIALS AND METHODS

Cell and Cell Culture

Myeloid leukemic M1 cells were originally obtained from a spontaneous myeloid leukemia in an SL mouse. Subclones of M1 cells, clone T-22, S-1, and B-4 cells can be induced to differentiate into macrophages and granulocytes by various inducers. Clone R-1, R-4 (20), and DR-3 (3) cells, which cannot be induced to differentiate in vitro by inducers alone, were also isolated from M1 cells. The cells were cultured in Eagle's minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with double the usual concentrations of amino acids and vitamins and with 10% heat-inactivated bovine calf serum, at 37° under 5% CO2.

Interferon Preparations

M1 Cell Interferon. Clone T-22 cells (7 x 10^5/ml) were treated with poly(l)-poly(C) (2 μg/ml) for 2 days. Culture fluid containing 1000 IU of interferon per ml was applied to a column of anti-interferon antibody, prepared by Iwakura et al. (13) of the Institute for Virus Research, Kyoto University. The anti-interferon serum was prepared by injecting into a rabbit L-cell interferon (3.4 x 10^7 IU/mg protein) which had been purified by Yamamoto and Kawade (30). In order to obtain interferon-specific antisera, the serum was extensively absorbed with various possible impurities such as calf serum, NDV, normal L-cell culture fluid, and L-cell extract according to the method described previously (13). The serum obtained by this procedure precipitated only interferon by polyacrylamide gel electrophoresis of the immune precipitate. M1 interferon was eluted from the column with 0.1 M citrate buffer, pH 2.5, containing 560 μg of bovine serum albumin per ml, and dialyzed against PBS. The pooled fraction contained 95,000 IU of interferon per ml and 370 μg of protein per ml, which mainly consisted of the added bovine serum albumin. We confirmed that D-factor passed through the affinity column and was separable from interferon.

L-Cell Interferon. L-cells were infected with NDV at 10 multiplicity of infection units. Then the NDV was washed off, and the L-cells were cultured for 24 hr in Eagle's medium.
containing 2% calf serum. The culture fluid obtained was adjusted to pH 2 with HCl, was left to stand for 4 days at 4°C to inactivate the NDV, and then was neutralized and assayed for interferon. It was found to contain 3250 IU of interferon per ml. Mock interferon was prepared in the same way but without virus infection.

**Mouse Serum Interferon.** SL mice were given i.p. injections of 200 µg of poly(I)-poly(C) (Yamasa Shoyu, Choshi, Japan). Blood was collected for preparation of serum 3 hr later, when interferon and D-factor activity were maximally induced. The serum contained 8000 IU of interferon per ml.

**Anti-Interferon Serum**

Anti-interferon serum was kindly donated by Dr. Yoshimi Kawade and Dr. Yoko Yamamoto, Department of Cellular and Molecular Virology, Institute for Virus Research, Kyoto University. Antiserum against L-cell interferon (3.4 × 10^7 IU/mg protein), which had been purified by Yamamoto and Kawade (30), was prepared in a rabbit as described by Iwakura et al. (13). It neutralized 10 IU of either L-cell or M1 cell interferon or mouse serum interferon at a dilution of 2000. We previously confirmed that the antiserum did not inhibit the activity of D-factor (31).

**Assay of Interferon**

Interferon was assayed by 2 methods: a plaque reduction method using L-cells and VSV; and a method for determination of synthesis of VSV RNA (15). In this work, titers of interferon were measured with NIH reference standard mouse interferon (Catalog No. G002-904-511) as a standard and were expressed in international reference units (IU). One unit in our system was equal to 5 NIH IU.

**Source of D-Factor**

Peritoneal macrophages were obtained from ICR mice as described in the preceding paper (25). Macrophages were cultured in serum-free Eagle's minimal essential medium for 2 days and then the culture fluid was harvested. The conditioned medium of macrophages was used as a source of D-factor in most experiments. In an experiment on lysozyme induction, conditioned medium of mouse embryo cells was used as D-factor.

**Assay for Properties of Differentiated Cells**

**Phagocytic activity.** M1 cells (5 to 7 × 10^5/ml) were incubated with various inducers and interferon for 2 days. The cells were harvested by centrifugation, suspended in serum-free Eagle's medium containing 0.2% of a suspension of polystyrene latex particles (average diameter, 1.099 µm; Dow Chemical Co., Indianapolis, Ind.) and incubated for 4 hr at 37°C. Then the cells were thoroughly washed 3 times with PBS, and the percentage of phagocytic cells among more than 400 viable cells was calculated. The cells containing more than 10 latex particles were scored as positive cells.

**Fc Receptors.** Fc receptors were measured by counting rosette-forming cells by the method of Lotem and Sachs (17). Sheep erythrocytes and rabbit antiserum to sheep erythrocytes were mixed and incubated at 37°C for 30 min in PBS. The antibody-coated erythrocytes were washed 3 times with PBS and resuspended in Eagle's medium without bicarbonate, pH 7.0. The percentage of cells with a rosette was measured quantitatively by mixing 10^6 antibody-coated erythrocytes with 10^6 M1 cells in a volume of 1 ml, centrifuging the suspension, and incubating the precipitated cells for 30 min at 37°C.

**Lysozyme Activity.** Lysozyme activity in the cells was determined by the lysoplate method of Osserman and Lawlor (21) as modified by Kasukabe et al. (14) and described previously (26). The activity, in µg equivalents of hen egg white lysozyme, was calculated from a standard curve prepared using purified hen egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.). The results are expressed as amounts of lysozyme in µg equivalents per mg of cell protein.

**Morphological Change.** The percentage of cells that were morphologically similar to granulocytes and macrophages was determined in smear preparations stained with May-Grünwald-Giemsa solution.

**RESULTS**

**Stimulation by Interferon of Induction of Phagocytic Activity in M1 Cells.** We examined the effect of interferon on induction of phagocytic activity, a marker of differentiation of M1 cells. Interferon was prepared from M1 cells by treatment with poly(I)-poly(C) and purified by anti-interferon column chromatography. Interferon alone did not induce phagocytic activity in M1 cells, but it enhanced induction of phagocytic activity by D-factor in conditioned medium of mouse peritoneal macrophages as shown in Chart 1. The stimulating effect of interferon was completely blocked by simultaneous treatment of the M1 cells with anti-interferon serum. It was previously confirmed that the anti-interferon serum did not inhibit the action of D-factor and that it neutralized M1 cell interferon and L-cell interferon (31).

Next, the effect of another preparation of interferon was examined. L-cell interferon was induced by infection of NDV. After inactivation of the virus, the crude interferon preparation could induce phagocytic activity in M1 cells (Chart 2). Simultaneous treatment of M1 cells with anti-interferon serum par-

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4 M. Tomida, Y. Yamamoto, and M. Hozumi. Inhibition of leukemogenicity of myeloid leukemic cells in mice and in vivo induction of normal differentiation of the cells by poly(I)-poly(C), Gann., in press 1980.
tially suppressed the induction of phagocytic activity in the cells by the interferon preparation. Mock interferon also induced phagocytic activity in the cells at higher concentrations; 50% conditioned medium of uninfected L-cells induced 40 to 50% of phagocytic cells (data not shown). These results indicate the presence of D-factor in the conditioned medium of L-cells and the L-cell interferon preparation. Chart 2 shows that a 5% interferon solution containing 150 IU of interferon per ml was less effective for induction of phagocytic activity in M1 cells than were 100 IU of M1 interferon plus 5% conditioned medium of macrophages per ml. However, in the presence of the conditioned medium of macrophages, the differentiation-stimulating activity of L-cell interferon was similar to that of M1 cell interferon (data not shown).

Interferon has been reported to enhance the phagocytic activity of normal macrophages (8, 11). Therefore, we examined the direct effect of interferon on assays of phagocytic activity of M1 cells. M1 cells were treated with various concentrations of D-factor for 2 days, and interferon (250 IU/ml) was added when the phagocytic activity of the cells was assayed. Results showed that it had no significant effect on the phagocytic activity. These results indicate that interferon potentiated the differentiation-inducing action of D-factor on M1 cells but does not induce phagocytic activity itself.

Pretreatment experiments further support that interferon was sensitizing M1 cells rather than supplying some factors necessary for induction of differentiation (Table 1). The cells became sensitive to the D-factor when they were incubated with interferon alone for more than 1 hr.

Besides the protein inducer D-factor, various other chemicals are known to induce differentiation of M1 cells. Therefore, we examined the effects of interferon on the actions of several other inducers. Interferon enhanced the induction of phagocytic activity in T-22 clone cells by lipopolysaccharide or poly(I) but not by dexamethasone (Table 2). Similar results were obtained with clone B-24 cells.

**Effect of Interferon on Induction of Fc Receptors and Morphological Differentiation of M1 Cells.** Both D-factor and dexamethasone induced Fc receptors and morphological differentiation of M1 cells. Interferon alone did not induce Fc receptors or morphological differentiation but did enhance their induction by D-factor. However, interferon did not enhance their induction by dexamethasone (Table 3).

**Induction of Lysozyme Activity by Interferon in M1 Cells.** Lysozyme activity was induced by treatment of M1 cells with inducers such as D-factor and dexamethasone for 4 days. Unexpectedly, interferon itself induced lysozyme as well as enhancing the induction of the enzyme by D-factor or dexamethasone (Table 4).

**Differences in Inhibitions of Cell Growth by Interferon and Poly(I)-Poly(C) in Different Clones of M1 Cells.** During examination of the effect of interferon on the differentiation of M1 cells, we observed that it caused different extents of inhibition of growth of different clones. As shown in Chart 3, all 3 clones, clones R-1, R-4, and DR-3, that were resistant to inducers of differentiation alone were more sensitive to growth inhibition by interferon than were clones that were sensitive to inducers (clones T-22, B-24, and S-1). Treatment with 325 IU of interferon per ml for 2 days caused complete inhibition of growth of R-4 and DR-3 cells but only 40% inhibition of growth of the 3 sensitive clones. Cytolysis was induced in clones R-1, R-4, and DR-3 but not in clones T-22, B-24, and S-1.

On the contrary, the degree of susceptibility to growth inhibition by poly(I)-poly(C) was associated with the susceptibility...
Effect of interferon on induction of Fc receptors and morphological differentiation of M1 cells

T-22 cells were incubated with inducers with or without 200 IU of M1 cell interferon per ml for 3 days.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Interferon</th>
<th>Fc rosettes (%)</th>
<th>Blasts</th>
<th>Intermediate</th>
<th>Macrophages and granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>1.3 ± 1.0</td>
<td>98 ± 1</td>
<td>2 ± 1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (10^-8 M)</td>
<td>–</td>
<td>0.4 ± 0.4</td>
<td>94 ± 2</td>
<td>6 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>0.7 ± 0.7</td>
<td>91 ± 3</td>
<td>9 ± 3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (10^-6 M)</td>
<td>–</td>
<td>10.3 ± 2.5</td>
<td>28 ± 5</td>
<td>70 ± 6</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>+</td>
<td>7.8 ± 0.1</td>
<td>24 ± 4</td>
<td>73 ± 5</td>
<td>3 ± 1</td>
<td></td>
</tr>
<tr>
<td>D-factor (5%)</td>
<td>–</td>
<td>0.3 ± 0.3</td>
<td>95 ± 2</td>
<td>5 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>19.3 ± 0.6</td>
<td>60 ± 6</td>
<td>37 ± 6</td>
<td>3 ± 0</td>
<td></td>
</tr>
<tr>
<td>D-factor (50%)</td>
<td>–</td>
<td>18.3 ± 2.5</td>
<td>34 ± 6</td>
<td>57 ± 11</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>+</td>
<td>52.4 ± 0.5</td>
<td>11 ± 5</td>
<td>78 ± 7</td>
<td>11 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

* The percentage of cells with a rosette was measured as described in "Materials and Methods." More than 200 viable cells were counted from each sample.

* Morphology of the cells was examined by staining the cells with May-Grünwald-Giemsa. More than 200 cells were analyzed from each sample.

* Mean ± S.E. of duplicate determinations.

Effect of interferon on induction of lysozyme in M1 cells

T-22 cells were incubated with inducers with or without 200 IU of M1 cell interferon per ml for 4 days.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Interferon</th>
<th>Lysozyme (µg equivalents/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>5.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (10^-8 M)</td>
<td>–</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>+</td>
<td>22.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>D-factor (10%)</td>
<td>–</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>+</td>
<td>17.2 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

* Lysozyme activity in the cells was determined as described in "Materials and Methods." Activities are expressed as amounts of lysozyme in µg equivalents of hen egg white lysozyme per mg of cell protein.

* Mean ± S.E. of duplicate determinations.

Interferon alone could not induce differentiation of M1 cells, but it stimulated the differentiation by macromolecular inducers including D-factor, lipopolysaccharide, and poly(I)·poly(C). Differentiation of M1 cells by another inducer of lower molecular weight, dexamethasone, was confirmed by the finding that phagocytic activity was induced in T-22 cells by serum neutralized with anti-interferon serum. On the other hand, anti-interferon rabbit serum abolished the actions of the mouse serum on R-4 and DR-3 cells, such as the inductions of phagocytic activity and growth inhibition. These results indicate that interferon in the serum caused inhibition of cell growth and sensitization of the cells to D-factor.

DISCUSSION

Interferon alone could not induce differentiation of M1 cells, but it stimulated the differentiation by macromolecular inducers including D-factor, lipopolysaccharide, and poly(I)·poly(C).
plasma membrane rather rapidly, was not stimulated by interferon. The actual mechanisms of action of interferon on M1 cells are not yet known, but its primary effect may be modification of the surface membrane as reported previously (1,16).

Lysozyme, one of the markers of differentiation of M1 cells, was induced by interferon alone, but other markers, such as Fc receptors and phagocytic activity, were not. Prostaglandin E or cyclic AMP also induced lysozyme activity but did not induce the other markers of the differentiation of M1 cells (2). Therefore, induction of lysozyme activity in the cells may be under a different control than is induction of other differentiation-associated markers. On the other hand, interferon is reported to increase the levels of prostaglandin E (32) and cyclic AMP (27) in cultured cells. Therefore, induction of lysozyme activity of M1 cells by interferon may be mediated by prostaglandin E or cyclic AMP, although it remains to be established whether interferon increases the levels of prostaglandin E and cyclic AMP in M1 cells.

Lotem and Sachs (18) reported that interferon affects the growth and differentiation of mouse myeloid leukemic cells including clones originating from M1 cells. They showed that interferon has no effect on induction of C3 rosettes, immune phagocytosis, or differentiation to mature macrophages or granulocytes; however, it does enhance the induction of lysozyme by dexamethasone or protein inducer. In contrast, we observed stimulation by interferon of induction of differentiation of our various clones of M1 cells with different sensitivities to inducers. The reasons for this discrepancy between our results and those of Lotem and Sachs (18) are unknown. The effect of interferon on induction of differentiation of myeloid leukemic cells may vary in different cell clones.

We have demonstrated in this report that interferon has different effects on cell growth of different clones of M1 cells: it inhibits the growth of all 3 clones, R-1, R-4, and DR-3, which are resistant to inducers of differentiation alone, more than it inhibits the growth of clones T-22, B-24, and S-1, which are sensitive to inducers. In contrast, poly(I)-poly(C) inhibits the growth of clones T-22, B-24, and S-1 more than that of clones R-1, R-4, and DR-3. The lack of a parallel between sensitivity of cells to poly(I)-poly(C) and sensitivity to interferon suggests that there are different receptors for poly(I)-poly(C) and interferon on the cells. Interferon could be poorly induced by poly(I)-poly(C) in the cells with low sensitivity to poly(I)-poly(C) and high sensitivity to exogenously added interferon. The reverse situation, where the cells are highly sensitive to poly(I)-poly(C) and relatively insensitive to interferon, may be due to a good response of the cells to interferon induction by poly(I)-poly(C) and increase by induced interferon in susceptibility of cells to toxicity of poly(I)-poly(C) (24). Although the mechanisms of anticalcellular action of interferon and poly(I)-poly(C) are still unknown, the difference in the sensitivities of the various clones of M1 cells to interferon and poly(I)-poly(C) may be due to differences in the properties of the surface membranes of the cells. Certain differences have been found in the surface membranes of clones that were sensitive and resistant to induction of differentiation; for example, cap formation by concanavalin A (12), ecto-ATPase activity (28), and the fatty acid composition of the membrane (23) are different in the 2 types of clones.

Inhibition of growth of M1 cells by interferon or poly(I)-poly(C) did not by itself induce differentiation of the cells and did not enhance induction of differentiation by one of the inducers, dexamethasone. Therefore, it seems unlikely that sensitization of M1 cells to D-factor by interferon is merely due to growth inhibition by interferon.

It was shown that differentiated M1 cells in vitro lost both proliferating activity and leukemogenicity (10) and that injection of inducers of normal differentiation of the cells prolonged the survival time of mice inoculated with leukemic cells (4). Therefore, stimulation of in vivo induction of normal differentiation of myeloid leukemic cells is of potential value in leukemia therapy. We have shown that injection of poly(I)-poly(C) into mice induced interferon and D-factor activity in the serum, stimulated induction of differentiation of M1 cells in diffusion chambers transplanted into mice, and prolonged the survival time of mice.
inoculated with the cells. In the present work, we have shown that interferon suppressed growth of M1 cells and sensitized the cells to inducers such as D-factor. Therefore, the antileukemic effect of poly(I)-poly(C) on M1 cells may be attributable in part to the effect of the induced interferon in stimulating differentiation of the cells. Further studies are under way on the mechanisms of action of polyribonucleotides and interferon in inducing differentiation of leukemic cells and on their therapeutic effects on leukemia.

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