Role of Immune Interferon in the Monocytic Differentiation of Human Promyelocytic Cell Lines Induced by Leukocyte Conditioned Medium

By Elahe Talieh Dayton, Michiko Matsumoto-Kobayashi, Bice Perussia, and Giorgio Trinchieri

Conditioned medium (CM) from lectin-stimulated human leukocytes contains factors that induce human promyelocytic cell lines to differentiate along the monocytic pathway. In this report, we show that human promyelocytic cell lines are also induced to differentiate along this pathway by immune interferon (IFNγ). Various preparations of IFNα tested did not induce this differentiation. In cultures containing IFNγ, the cells are induced to coordinately express monocyte markers and functions such as monocyte-specific surface antigens, HLA-DR antigens, nonspecific esterase, receptors for the Fc fragment of IgG, and the ability to mediate antibody-dependent cell-mediated cytotoxicity. Our data indicate that differentiation induced by IFNγ is not secondary to an arrest of growth of promyelocytic cell lines, but rather that a proportion of cells is induced along a programmed pathway of terminal differentiation similar to that of normal monocytes. CM contains IFNγ, but its ability to induce differentiation is greater than expected on the basis of its content of IFNγ. Treatments at 56 °C or at pH 2.0, which abolish IFNγ activity, abrogate the differentiation ability of CM. The antiviral activity and the differentiation activity contained in the CM are coeluted from gel filtration and reverse-phase columns. Monoclonal antibodies anti-IFNγ, which completely abrogate the differentiation ability of IFNγ and the antiviral activity in the CM, completely suppress the induction of some monocyte markers by CM, but only reduce the expression of others. When IFNγ is added to CM, promyelocytic cell lines are induced to differentiate to a much greater extent than that induced by either IFNγ or IFNγ-depleted CM alone. These results show that the differentiation activity of leukocyte CM is due to the synergistic effect of IFNγ and other factors not yet identified.

SINCE the original observation by Elias et al., several studies have shown that induction of promyelocytic cell lines along the monocytic pathway can be readily demonstrated by culturing the cells in the presence of medium conditioned by lectin-stimulated leukocytes. Most cells from human promyelocytic cell lines, such as HL-60 or ML3, cultured in the presence of conditioned medium from phytohemagglutinin-stimulated lymphocytes (PHA-CM) acquire morphological characteristics of differentiated monocytemacrophages, express enzymatic and phenotypic characteristics typical of monocytes, and lose the ability to proliferate in culture. We have demonstrated that promyelocytic cells cultured in the presence of PHA-CM express, among other markers, monocyte-specific and HLA-DR antigens absent from the parental cells and become potent effectors of antibody-dependent cell-mediated cytotoxicity (Ab-CMC) against erythrocytes and tumor-derived cells. In the same cultures, an equilibrium between proliferation and differentiation is established, and two cell populations can be separated on the basis of expression of differentiation surface markers. A population of differentiated monocyte-like cells originate from a proportion of the proliferating promyelocytes that respond to the differentiation inducers contained in the CM. These cells have a differentiated morphology, express nonspecific esterase activity and the phenotype of differentiated monocytes, mediate Ab-CMC, and have a limited ability to proliferate. A second population retains the phenotype of undifferentiated promyelocytes and continues to proliferate.

Lectin-stimulated lymphocytes secrete a variety of soluble factors, including colony-stimulating factors (CSFs), interferons (IFNs), and T cell growth factor (interleukin 2 [IL 2]). The differentiation-inducing factor contained in the CM has not yet been identified. Metcalf et al., using the M1 or WEHI-3B murine cell lines, suggested that granulocytemacrophage CSF might be the differentiation factor. However, no such evidence is available in the human system. The differentiation along the myeloid lineage observed with the murine cell lines appears to differ from the monocytic differentiation induced by PHA-CM on human cells. These findings suggest that two discrete mechanisms of induction and two different inducing factors might be operative in the human and murine systems. Olsson et al. reported that concanavalin A-induced CM obtained from human lymphocytes contains two molecular species of differentiation-inducing factors: a 25,000-dalton species that co-elutes with the colony-stimulating activity and a 40,000-dalton species not associated with this activity.

PHA-CM has also been shown to induce partial monocytic differentiation in cells freshly obtained from patients with acute myelogenous leukemia (AML). We have originally shown that immature myeloid cells from normal bone marrow or peripheral blood of patients with chronic myelogenous leukemia can be induced to differentiate to monocyte-like cells by PHA-CM and by immune interferon (IFNγ). We have identified IFNγ as the predominant differentiation factor contained in the CM. Purified or recombinant IFNγ, but not various preparations of IFNα or β, can induce monocytic differentiation in myeloid cells. In cultures containing conditioned medium, the cells fail to continue myeloid maturation and are induced to express monocyte markers and functions such as monocyte-specific surface antigens, HLA-DR antigens, Fc receptors (FcR) for monomeric immunoglobulins, nonspecific esterase, and the ability to mediate Ab-CMC. Even myeloid cells as mature as metamyelocytes or band cells are induced by relatively low doses of IFNγ to undergo monocyte differentiation, but monocyte-specific or HLA-DR antigens are not induced in...
mature neutrophils. IFNγ rapidly induces or enhances the expression of high-affinity FcR for monomeric immunoglobulins on immature myeloid cells and on mature monocytes and neutrophils. These findings revealed a previously unknown specific function of human IFNγ and offered new insights to the regulation of monocyte recruitment and differentiation during, for example, virus infection or immune response, cases in which IFNγ is produced. The finding that IFNγ induces monocyte differentiation and antibody-dependent cytotoxic ability in myeloid cells has been confirmed and extended by other authors using human myeloid cell lines, including U937 and HL-60.

In the present paper, we compare the effect of IFNγ and PHA-CM on the induction of differentiation in human promyelocytic cell lines and analyze the role of the IFNγ contained in the PHA-CM on the induction of differentiation.

**MATERIALS AND METHODS**

**Cell lines.** The human promyelocytic cell lines HL-60 and ML3, mouse mastocytoma P815, the rhodomyosarcoma line (RDMC), the B lymphoid cell line Daudi, and the Detroit 532 fibroblasts were grown in our laboratory in RPMI 1640 medium (Flow Laboratories, Rockville, Md) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), HL-60 cells at low passage number were used.

**Monoclonal antibodies.** Mouse anti-human leukocyte monoclonal antibodies used in this study were produced and characterized in our laboratory. The IgM antibodies B44.1, B52.1, and B77.1 specifically react with all peripheral blood monocytes and all α-naphthylacetate esterase (α-NAE)-positive cells in the bone marrow. Antibodies B44.1 and B52.1 cross-react with the antimonocyte antibody Mo2 for binding sites on monocytes. On the basis of the competition experiments, antibody B44.1 appears to have the highest binding affinity; B52.1, intermediate; and Mo2, the lowest. Because antibody B52.1 does not bind significantly to uninduced HL-60 and ML3, it was used as a marker for monocyte differentiation in most experiments. Antibody B77.1 does not cross-react with any of the other antibodies and is presumably directed against a separate antigenic determinant expressed on mature monocytes (unpublished observations, 1983). The IgG2a antibody B33.1,2,19 reacts with a nonpolymorphic determinant of the HLA-DR antigen. The F(ab')2 fragment of B33.1, prepared by pepsin digestion, was used throughout this study.

**Conditioned medium.** Human peripheral blood mononuclear cells (separated on a Ficoll-Hypaque [F-H] gradient) were irradiated (20 Gy) and incubated (10^7/ml) in RPMI 1640 medium supplemented with 1% PHA-M (Wellcome Research Laboratories, Beckenham, England) and 1% FBS for 72 hours at 37 °C in 5% CO₂ in air. Cell-free supernatant was collected and used as a crude PHA-CM preparation. Most preparations of PHA-CM contained between 100 and 1,000 U/ml of IFNγ.

**Fractionation of PHA-CM.** PHA-CM was concentrated 50-fold by vacuum dialysis and fractionated by gel filtration or reverse-phase chromatography using a fast pressure liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Uppsala, Sweden). For gel filtration, 100-μl samples were repeatedly applied to a TSK G3000 SW column (Pharmacia Fine Chemicals) and eluted with 0.1% polyethylene glycol 8000 in phosphate-buffered saline (PBS), pH 7.4, at a flow rate of 0.8 ml/min. Fractions of 0.5 ml were collected. For reverse-phase chromatography, a 2-ml sample was applied to a Pro RPC HR 5/10 column (Pharmacia) and eluted with a linear gradient from 0% to 60% of ethylene glycol in PBS at a flow rate of 0.3 ml/min. One-milliliter fractions were collected. Fractions were dialyzed against the medium and sterilized by filtration.

**IFN preparation.** Purified human IFNα (10^6 antiviral U/mg protein) was obtained from Interferon Sciences, Inc (New Brunswick, N.J.). Human recombinant IFNγ (rIFNγ) from Escherichia coli, with a titer of approximately 10^6 antiviral U/mg, was kindly supplied by Dr C.G. Sevastoopoulos (Genentech, Inc, San Francisco). The antiviral titer of the rIFNγ preparation was periodically analyzed as described below. Partially purified IFNα (Leucoferon, 10^6 antiviral U/mg) was obtained from Biotechnologies, Inc (East Hartford, Conn). Recombinant types A and D IFNα from E coli (rIFNα 2 × 10^6 U/mg and rIFNαD 5.3 × 10^5 U/mg) were kindly supplied by Hoffmann-La Roche Inc (Nutley, NJ). Monoclonal antibody B133.3 (IgG1), produced in our laboratory, reacts specifically with human IFNγ and with neither IFNα nor β. In the present study, the tissue culture supernatant fluid from B133.3 cells was used that neutralized 32,400 units of the NIH IFNγ standard per milliliter. An immunofluorescence assay for removal of IFNγ from the supernatant was prepared by coupling 5 mg/ml of purified B133.3 IgG1 to CNBr-activated Sepharose 4B (Pharmacia). Monoclonal antibody B132.6 (IgG1) is specific for human IL 2.

**Myeloid cell lines induction.** HL-60 and ML3 cells (10^5 cells per milliliter) were cultured for the indicated periods of time in 24-well flat-bottom plates in the presence or absence of PHA-CM and different species of IFN at the concentrations indicated. At the end of the culture period, cells were washed and used in the different assays. Viability (higher than 85%) and the number of cells did not differ significantly among samples. In some experiments, cells were centrifuged on an F-H gradient (1.077 g/ml) before immunofluorescence assay by cytofluorometry in order to avoid the nonspecific background due to dead cells.

**IFN tests.** Antiviral activity in the IFN preparations or in the CM was tested by inhibition of the cytopathic effect of vesicular stomatitis virus (VSV) on human fibroblast strain Detroit 532, derived from a subject with trisomy 21. The IFN concentration inducing 50% protection of the cytopathic effect on Detroit 532 cells was determined for each preparation.

**Cell morphology.** Cell morphology was examined by cytocentrifuge preparations (cytopsin centrifuge, Shandon Southern Instrument, Inc, Sewickley, Pa) stained by the May-Grünewald-Giemsa method. Staining for α-NAE was carried out according to Platt et al. Detection of receptor for the Fc fragment of IgG. Ox erythrocytes (E) sensitized with rabbit IgG anti-ox-E (Cappel Laboratories, Cochranville, Pa) (EA7S) were used as the indicator system for Fc receptors. Cells and erythrocytes were mixed (1:50) and incubated as pellets for 20 minutes at 4 °C. The proportion of cells forming rosettes with sensitized erythrocytes was determined by scoring at least 200 cells; only cells with more than five erythrocytes bound were considered positive.

**Indirect immunofluorescence studies.** Cells were sequentially incubated with appropriate dilutions of the different monoclonal antibodies and with fluorescein isothiocyanate (FITC)-labeled goat F(ab')2 anti-mouse Ig antibody for 30 minutes at 0 °C as described.

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The cells were then washed at 4 °C with PBS containing 5% normal human serum. The use of F(abc) fragments and the presence of human serum completely exclude the possibility that binding of the antibodies to the cells is due to binding through FcR. The proportion of cells reactive with the different antibodies was scored on the Ortho Cytofluorograf 50H (Ortho Diagnostics, Westwood, Mass) connected to a Data General MP/200 microprocessor. The threshold fluorescence intensity was used at which 99% of the cell population treated with control supernatant from the parental myeloma and FITC-anti-mouse Ig antibodies were negative. Mean fluorescence intensity is reported in arbitrary units, ranging from 1 to 200, and corresponds to the mean fluorescence channel of the positive cells. Fluorescence intensity allows the comparison of results only within each experiment: in order to allow determination on a sufficient number of cells, fluorescence intensity is reported only when the percentage of positive cells is >5%.

Cell-mediated cytotoxicity assay. Lytic activity was tested in a three-hour 51Cr-release assay by incubating various numbers of effectors in microtiter plates at 37 °C with a constant number of either 51Cr-labeled chicken erythrocyte target cells (5 x 10^6) sensitized with a 1:25 dilution of rabbit anti-chicken erythrocytes IgG (Cappel) or 51Cr-labeled P815y cells (10^6) sensitized with a 1:1,000 dilution of a rabbit anti-P815y antiserum. Supernatants were harvested and the percentage of specific cytotoxicity was calculated as previously reported.

Thymidine incorporation and simultaneous detection of thymidine incorporation and surface antigen expression. HL-60, ML3, and Daudi cells were cultured (10^5 cells per milliliter; 200 μL) in the wells of a flat-bottom microtiter plate in the presence or absence of various IFN dilutions. After 66 hours of culture, 3H-TdR (1 μCi per well; New England Nuclear, Boston) was added to the wells. After a six-hour incubation, the cells were collected on glass fiber filters using an automatic cell harvester (Skatron, Sterling, Va) and the incorporated 3H-TdR was assayed by liquid scintillation. For the simultaneous identification of cells incorporating 3H-TdR and cells expressing the monocyte differentiation antigen (Mo-Ag) defined by monoclonal antibody B52.1, cells were cultured as above for five days, but the 3H-TdR pulse was limited to one hour. Cells were then washed, sensitized with antibody B52.1, and allowed to react with CrCl2-treated anti-mouse immunoglobulin-coated sheep erythrocytes according to the technique of indirect rosetting previously described. Cyto centrifuge smears were prepared, fixed with absolute methanol for 30 minutes and covered with NTB-2 emulsion (Eastman Kodak, Rochester, NY) for a two-day exposure. B52.1 (+) cells were identifiable on the slide by formation of rosettes with the sheep erythrocytes. The proportion of cells incorporating 3H-TdR and that of the cells positive or negative with the monoclonal antibody were then scored by counting at least 300 cells.

**Results**

Induction of differentiation of promyelocytic cell lines by PHA-CM and different IFN species. HL-60 and ML3 cells (10^5 per milliliter) were cultured for five days in the absence or presence of PHA-CM and of different species of IFN. At the end of the culture, cells were still in the growth phase (50% to 60% of the cells incorporating 3H-TdR in a one-hour pulse) and had reached a concentration of 5 to 8 x 10^5 per milliliter. In most experiments, viability was >95%. The cells were then washed and analyzed for the presence of various markers of differentiation. The markers analyzed are those whose expression was shown in our previous studies to correlate with cell differentiation in the presence of PHA-CM. Markers enabling a semiquantitative evaluation (surface antigens and receptors, enzymatic activity and cytotoxic activity) were selected rather than the more subjective traits such as morphology and adherence capability evaluated in

<p>| Table 1. Effect of PHA-CM and Different Types of IFN on the Induction of Monocyte Differentiation Markers on Promyelocytic Cell Lines |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment*</th>
<th>HLA-DR (B83.1)</th>
<th>Mo-Ag (B84.1)</th>
<th>Mo-Ag (B52.1)</th>
<th>Mo-Ag (B77.1)</th>
<th>α-NAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>None</td>
<td>1†</td>
<td>9† (88)‡</td>
<td>4†</td>
<td>5†</td>
<td>15†</td>
</tr>
<tr>
<td></td>
<td>PHA-CM</td>
<td>2</td>
<td>48 (124)</td>
<td>41 (136)</td>
<td>21 (117)</td>
<td>30</td>
</tr>
<tr>
<td>IFNy</td>
<td>1</td>
<td>37 (137)</td>
<td>38 (139)</td>
<td>26 (117)</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>rIFNy</td>
<td>2</td>
<td>33 (133)</td>
<td>15 (124)</td>
<td>6 (110)</td>
<td>32</td>
<td>35</td>
</tr>
<tr>
<td>rIFNβ</td>
<td>1</td>
<td>7 (81)</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>rIFNα</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>rIFND</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>ML3</td>
<td>None</td>
<td>3</td>
<td>7 (48)</td>
<td>1</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PHA-CM</td>
<td>19 (75)</td>
<td>42 (82)</td>
<td>23 (120)</td>
<td>13 (125)</td>
<td>ND</td>
</tr>
<tr>
<td>IFNy</td>
<td>34 (91)</td>
<td>39 (69)</td>
<td>16 (79)</td>
<td>6 (96)</td>
<td>ND</td>
<td>37</td>
</tr>
<tr>
<td>rIFNy</td>
<td>52 (84)</td>
<td>34 (71)</td>
<td>8 (81)</td>
<td>2</td>
<td>ND</td>
<td>37</td>
</tr>
<tr>
<td>rIFNβ</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>rIFNα</td>
<td>3</td>
<td>6 (52)</td>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>rIFND</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

ND, not determined.

*HL-60 and ML3 cells were incubated for five days in culture medium or in the presence of PHA-CM (50% and 20% final concentrations, respectively) or with different IFN types (10^6 U/mL).

†Percentage of cells that express the antigen detected by the monoclonal antibodies indicated, as analyzed by indirect immunofluorescence (flow cytofluorometry).

‡Mean intensity of fluorescence (arbitrary units ranging from 1 to 200).

§Percentage of cells positive for α-NAE activity.

¶Percentage of specific 51Cr release (three-hour assay) induced by HL-60 or ML3 cells on IgG antibody-sensitized chicken erythrocytes (effector-to-target ratio, 10:1).
our previous analyses. The results reported in Table 1 are obtained from a single experiment and are representative of at least five independent determinations for each condition tested. As previously reported, \( \text{PHA-CM} \) always induced HLA-DR antigens on ML3 cells, whereas these antigens were usually absent or induced at very low levels on HL-60 cells (Table 1). Similar results were obtained using IFN\( \gamma \). A proportion of cells of both lines also expressed the other differentiation markers tested (one or more Mo-Ag, \( \alpha \)-NAE, and Ab-CMC) upon incubation with PHA-CM or with the two preparations of purified and recombinant (r)IFN\( \gamma \). Unlike the observation with HLA-DR antigens, monocyte antigens and Ab-CMC were more readily inducible on HL-60 than on ML3 cells. As previously reported in detail for PHA-CM, induction of Mo-Ag and Ab-CMC in ML3 cells by IFN\( \gamma \) and rIFN\( \gamma \) was variable and, in few cases, not significant. Expression of the same differentiation markers was never induced on cells treated with partially purified IFN\( \alpha \) or the two species of rIFN\( \alpha \) or rIFN\( \gamma \).

The kinetics of induction of three of the differentiation markers analyzed, ie, HLA-DR, a monocyte antigen, and FcR, were compared on HL-60 and ML3 cells cultured in the presence of PHA-CM or rIFN\( \gamma \) (not shown). In all cases FcR was induced on the cells with very fast kinetics, with maximum expression usually on day 1 of induction. The kinetics of induction of HLA-DR and of the monocyte antigen on both cell types was similar for rIFN\( \gamma \) and PHA-CM; HLA-DR and monocyte antigen appeared on day 3 of induction on ML3 cells, and the monocyte antigen appeared on day 5 on HL-60 cells. IFN\( \alpha \) preparations did not induce differentiation markers at any time of incubation.

The effect of different antiviral doses of the various IFN species on the expression of a monocyte antigen and on the cytotoxic ability of the cells in Ab-CMC was tested on HL-60 cells (Fig 1). The effects of purified IFN\( \gamma \) and rIFN\( \gamma \) were dose-dependent, whereas purified IFN\( \alpha \), rIFN-A, and rIFN-D were inactive at all concentrations used. At doses of IFN\( \gamma \) <20 U/mL, a significant effect was usually not observed; doses of IFN\( \gamma \) >1,000 U/mL (up to 10,000 U/mL) did not induce significantly more effect than did 1,000 U/mL (not shown). A similar dose-dependent effect of the two IFN\( \gamma \) preparations was observed when the expression of the other differentiation markers was analyzed (not shown).

The relative ability of the different IFN preparations to inhibit VSV replication in HL-60 and ML3 cells was determined (Fig 2). Both cell lines were relatively insensitive to the antiviral effect of all types of IFN, and high doses were required to obtain a significant decrease in VSV replication. Comparable results were obtained with the IFN\( \alpha \) and IFN\( \gamma \) preparations, using equivalent numbers of NIH IFN units.

**Effect of IFN on the proliferative ability of human promyelocytic cell lines.** The ability of different doses of the IFN preparations to affect proliferation of HL-60 and ML3 cells was analyzed by measuring \( ^{3} \text{H}-\text{TDR} \) incorporation after three days of culture (not shown). The three IFN\( \alpha \) preparations induced a slight decrease in thymidine incorporation by HL-60 cells, whereas no significant effect, or a slight stimulation, was observed with IFN\( \gamma \) preparations. No inhibition was observed on ML3 cells with any IFN preparation, whereas the three IFN\( \alpha \), but not the two IFN\( \gamma \) preparations, almost completely inhibited thymidine incorporation in the human B lymphoid cell line, Daudi, used as the control.

After five days of culture, a slight decrease in the percentage of HL-60 cells incorporating thymidine, as analyzed by autoradiography, was observed in the cells treated with PHA-CM, IFN\( \alpha \), or IFN\( \gamma \) as compared to untreated cells (Table 2). Simultaneous scoring of the cells incorporating thymidine and those expressing the B52.1-defined monocyte antigen revealed that the majority of the cells expressing the monocyte antigen after induction with either PHA-CM or IFN\( \gamma \) were unable to incorporate thymidine. By contrast, the proportion of the cells that do not express the antigen but that incorporate thymidine was only slightly lower than in the control untreated cells (Table 2).
Role of IFNγ in the differentiation induced by PHA-CM. The effect of heating (56 °C for one hour) and of low pH (pH 2.0 for 24 hours) treatment on the ability of purified IFNγ, rIFNγ and PHA-CM to induce differentiation was tested. Table 3 shows one representative experiment of three performed. Heating drastically reduced the differentiation-inducing activity of all three inducers, and pH 2.0 treatment virtually destroyed it. When concentrated PHA-CM was fractionated by gel filtration on an FPLC system (Fig 3), most of the antiviral activity of IFNγ was eluted in a broad peak of mol/wt from 30,000 to 40,000. Antiviral activity of IFNγ preparations was eluted in fractions corresponding to a slightly higher mol wt (40,000 to 50,000 daltons) on a G100 column (not shown), probably because of different hydrophobicity of the matrix in the two columns. Most of the colony-stimulating activity was eluted in a discrete peak of higher molecular weight than IFNγ (Fig 3). The factors able to induce FcR, Ab-CMC, and B52.1 monocyte antigen on HL-60 cells (Fig 3) and HLA-DR on ML3 and on a melanoma cell line, eluted in the same fractions containing antiviral activity, but not colony-stimulating activity. Most of the proteins of PHA-CM were not retained on a reverse-phase column (Fig 4) after eluted with PBS, but antiviral, colony-stimulating, and differentiation-inducing activities were retained on the column. When the column was eluted with a gradient of ethylene glycol, the antiviral activity, the colony-stimulating activity, and the differentiation activity (induction on HL-60 of FcR, cytotoxic activity, B52.1 monocyte antigen, and α-NAE) were co-eluted in a broad peak starting at 20% ethylene glycol. The same fractions contained the factors inducing HLA-DR antigens on ML3 and on a melanoma cell line.

The addition of the monoclonal anti-IFNγ antibody B133.3 (supernatant, 10% final concentration in culture) completely prevented the rIFNγ-dependent induction of the expression of the different markers on HL-60 and ML3 cells, whereas no inhibition was observed when an anti-IL 2 monoclonal antibody of the same isotype (1G11) was added. Anti-IFNγ antibody also completely blocked the induction

Table 3. Effect of Heating and pH 2.0 Treatment on the Differentiation-Inducing Activity of IFNγ and PHA-CM

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>HL-60 Cells</th>
<th>ML3 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-DR</td>
<td>Mo-Ag</td>
</tr>
<tr>
<td>None</td>
<td>1²</td>
<td>5² (61)²</td>
</tr>
<tr>
<td>IFNγ</td>
<td>1 (38) (94)</td>
<td>22 (43) (45)</td>
</tr>
<tr>
<td>IFNγ (56 °C)</td>
<td>1 (18) (66)</td>
<td>19 (37) (37)</td>
</tr>
<tr>
<td>IFNγ (pH 2.0)</td>
<td>1 (4)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>rIFNγ</td>
<td>1 (13 (83))</td>
<td>22 (54) (54)</td>
</tr>
<tr>
<td>rIFNγ (56 °C)</td>
<td>1 (6 (72))</td>
<td>4 (46) (46)</td>
</tr>
<tr>
<td>rIFNγ (pH 2.0)</td>
<td>1 (5 (67))</td>
<td>9 (39) (39)</td>
</tr>
<tr>
<td>PHA-CM</td>
<td>3 (35 (99))</td>
<td>20 (32) (41)</td>
</tr>
<tr>
<td>PHA-CM (56 °C)</td>
<td>1 (19 (87))</td>
<td>13 (35) (35)</td>
</tr>
<tr>
<td>PHA-CM (pH 2.0)</td>
<td>1 (4)</td>
<td>6 (7)</td>
</tr>
</tbody>
</table>

*HL-60 and ML3 cells were incubated for five days in culture medium or in the presence of IFNγ (500 U/mL), rIFNγ (500 U/mL), or PHA-CM (50% final concentration) that had been or had not been treated at 56 °C for one hour or at pH 2.0 for 24 hours.

†Percentage of cells expressing the antigen detected by the indicated monoclonal antibodies, as analyzed by indirect immunofluorescence (flow cytometry).

‡Mean fluorescence intensity (arbitrary units ranging from 1 to 200).

§Percentage of specific 51Cr release, induced by the HL-60 or ML3 cells, at an effector-to-target cell ratio of 10:1, on antibody-sensitized chicken erythrocytes, three-hour assay.

Table 2. Thymidine Incorporation and Monocyte Antigen (B52.1) Expression by HL-60 Cells Induced With PHA-CM and With Different Types of IFN

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Total HL-60 Cells</th>
<th>B52.1 (+)</th>
<th>B52.1 (+)</th>
<th>B52.1 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>²H-TdR (+)</td>
<td>²H-TdR (+)</td>
<td>²H-TdR (+)</td>
<td>²H-TdR (+)</td>
</tr>
<tr>
<td>None</td>
<td>50</td>
<td>0</td>
<td>NA</td>
<td>50</td>
</tr>
<tr>
<td>PHA-CM 50%</td>
<td>26 (31) (31)</td>
<td>2 (37)</td>
<td>2 (37)</td>
<td></td>
</tr>
<tr>
<td>IFNγ 10⁵ U/mL</td>
<td>23 (25) (25)</td>
<td>2 (30)</td>
<td>2 (30)</td>
<td></td>
</tr>
<tr>
<td>IFNγ 10⁶ U/mL</td>
<td>37 (37) (37)</td>
<td>NA</td>
<td>37 (37)</td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable [no B52.1 (+) cells had been scored].

*HL-60 cells were cultured in the presence or absence of PHA-CM or purified IFNγ or α for five days. Cells were then labeled with ²H-TdR (one-hour pulse, 1 μCi/mL), and cytotoxicity smears prepared after indirect rosetting of the cells to identify those reactive with B52.1 antibody.

†The percentage of cells incorporating ²H-TdR and of B52.1 (+) cells was evaluated by autoradiography and morphological identification of rosetted cells, respectively. The percentage of cells incorporating ²H-TdR was determined on total cells, on B52.1 positive (rosetting), and on B52.1 negative (nonrosetting) cells.
of HLA-DR, α-NAE, and the Ab-CMC potential of both HL-60 and ML3 cells. Induction of the B52.1 monocyte antigen (as judged from proportions of positive cells and intensity of fluorescence on the positive ones) was partially but consistently reduced. Anti-IFNγ antibody reduced induction of FcR by rIFNγ partially at day 5 (Fig 5) and completely at day 1 (not shown and reference 14).

To test whether factors other than IFNγ are contained in the PHA-CM and act together with IFNγ to induce differentiation, a preparation of PHA-CM containing 320 IFNγ U/mL was passed over a column of anti-IFNγ antibody B133.3 linked to Sepharose 4B. The IFNγ titer in the absorbed PHA-CM, as detected by radioimmunoassay, was less than 0.5 IFNγ U/mL. The absorbed PHA-CM had a reduced ability to induce differentiation markers, analogous to the data obtained after direct addition of anti-IFNγ antibody to the culture (Figs 6 and 7). Addition of 2,000 U/mL of rIFNγ to the cultures containing absorbed PHA-CM restored the differentiation-inducing effect of PHA-CM, often to levels higher than those induced by rIFNγ alone (Figs 6 and 7). Addition of rIFNγ to the absorbed PHA-CM or even to untreated PHA-CM determined induction of certain traits (eg, B52.1 monocyte antigen [Fig 8B] and Ab-CMC [Fig 7] on ML3 cells) to much higher levels than those induced by either rIFNγ or PHA-CM alone. Note that, although untreated PHA-CM contained IFNγ activity, the quantity of rIFNγ added in this experiment represents an IFN concentration ten times higher than that present in the PHA-CM. PHA, either alone or in combination with IFNγ, was unable to induce differentiation of HL-60 and ML3 cells (not shown). To better analyze the observed synergistic effect, both IFNγ-depleted PHA-CM and rIFNγ were titrated in combination for their ability to induce Ab-CMC by HL-60 and ML3 cells (Fig 8).

In this series of experiments, a PHA-CM with an initial titer of 800 U/mL of IFNγ was used. Less than 0.5 U/mL of IFNγ was present in the PHA-CM absorbed on the anti-IFNγ column. The synergistic effect of rIFNγ was already observed with 2 U/mL and reached a plateau between 20 and 2,000 U/mL. At all rIFNγ concentrations tested, the differentiation effect of the IFNγ-depleted PHA-CM was dose-dependent, with a significant effect at concentrations >5%. A plateau was not reached at the highest concentration of PHA-CM tested (50%).

DISCUSSION

All IFN types, in addition to exerting antiviral activity, modulate cellular functions. Most of these anticytoplasmic effects are inhibitory, with marked suppression of proliferation and cellular DNA and protein synthesis. However, IFNs can also stimulate the functions of effector cells involved in nonadaptive immunity, ie, natural killer cells and monocyte-macrophages,22,24 efficiently enhancing nonspecific effector cell activity.25 Mouse IFNs can inhibit or stimulate terminal differentiation of Friend erythroleukemia cells, depending on the dose.26,27 Mouse L cell IFN inhibits differentiation in various cellular systems.28-30 Human IFNγ has an inhibitory effect on granulopoietic maturation of colonies grown in vitro, inducing an arrest in maturation at the level of small clusters.31 Purified mouse IFN enhances macrophages CM-or polyinosinic acid-induced differentiation of M1 mouse myeloid leukemic cells to monocyte-macrophages.32 IFNα, IFNβ, and (25')oligo-isoadenylate can induce the human macrophage cell line U937, but not the more immature HL-60 cell line, to mediate Ab-CMC.33 IFN can induce or enhance the expression of Ia antigens on a variety of cell types,34 and recently it has been shown that IFNγ is much more potent than either IFNα or IFNβ in this induction.35 IFNγ, but not IFNα enhances or induces expression of Ia antigens on melanoma cells,36 monocyte-macrophages,34-38 immature myeloid cells,31,38,40 and endothelial cells.41

Studies by us13,14 and others15,16 have shown that IFNγ induces monocyte differentiation in normal and leukemic myeloid cells and in cell lines derived from myeloid leuke-
Fig 4. Reverse-phase chromatography of PHA-CM. PHA-CM concentrated 50 times was fractionated on an FPLC system using a RPC HR 5/10 column as described in Materials and Methods, and fractions were tested for (A) optical density at 280 nm; (B) antiviral activity (○) and colony-stimulating activity (●); (C) induction of FcR (△) and antibody-dependent cytotoxic ability (△) in HL-60 cells; and (D) induction of BS21 antigen (■) and α-NAE activity (●) in HL-60 cells.

In this report, we have compared the effect of IFN-γ and PHA-CM on human promyelocytic cell lines.

IFN-γ, like PHA-CM, induces expression on HL-60 and ML3 cells of surface antigens, enzymes, and functional activities characteristic of cells differentiating along the monocytic pathway. By contrast, neither IFNα nor β at the same concentration is able to induce monocytic differentiation. The inability of IFNα to induce differentiation was not due to unresponsiveness of the cell lines to IFNα because IFNα and γ preparations were equally effective in preventing VSV replication in these cells. However, homogeneous preparations of recombinant IFNγ have a maximum specific activity of 5 to 8 × 10^7 antiviral U/mg protein, whereas homogeneous recombinant IFNα preparations have a specific activity ten times higher or more. Therefore, at equivalent antiviral activity, the protein concentration of IFNγ added was much higher than that of IFNα. On the other hand, purified or recombinant IFNα does not induce differentiation even when used at concentrations of antiviral activity 100-fold higher than the lowest concentration of IFNγ still able to induce differentiation. Thus, our data indicate that the ability to induce differentiation is restricted to IFNγ.

The effect of IFN on the proliferation of promyelocytic cell lines in the first three days of culture was marginal, with IFNα preparations showing a more detectable growth-inhibiting effect than IFNγ on HL-60 cells. These results are analogous to those previously reported for PHA-CM, showing a significant growth inhibition only at day 4 or later. Proliferation of the lymphoblastoid cell line Daudi was almost completely blocked by low concentrations of IFNα, but was much more resistant to the antigrowth effect of IFNγ, as previously reported by de Ley et al. Those authors also showed that proliferation of the T cell line Molt 4 is inhibited by IFNγ more than by IFNα or β. Thus, different cell types may show a differential relative susceptibility to the growth inhibitory effect of various IFN types, and the original assumption of Blalock et al that immune IFN has a more potent antacellular activity than viral-induced leukocyte or fibroblast IFN may not extend to all cell types. When the ability to incorporate thymidine in the cultures induced with IFNγ was analyzed on the cells expressing a monocye
differentiation marker, we observed that the differentiated cells did not incorporate thymidine, but that the proportion of thymidine-incorporating cells among the nondifferentiated cells in the same culture was only slightly lower than in the control uninduced cultures. The results with the human myeloid cell lines indicate that arrest of growth of the cells only occurs after induction of differentiation. These results are analogous to those we and others reported for PHA-CM–induced cultures and suggest that the cells induced to terminal monocytic differentiation by IFNγ eventually lose their proliferative ability. On the contrary, another inducer of monocyte differentiation, the phorbol ester phorbol-12-myristate-13-acetate (PMA) induces complete inhibition of proliferation in all cells within 24 hours, whereas the markers of monocyte differentiation appear only after 48 to 72 hours.

In this study, we used the appearance of a series of differentiation markers (monocyte antigens, HLA-DR antigens, α-NAE, FcR, ability to mediate Ab-CMC) that are coordinately expressed on a proportion of cells induced by PHA-CM as criteria for monocytic differentiation in HL-60 and ML3 promyelocytic cell lines. Accordingly, PHA-CM and IFNγ seem to induce a proportion of the cells along a programmed pathway of terminal differentiation similar to that of normal monocytes rather than inducing a nonspecific derepression of genes encoding some differentiation markers of the myelomonocytic lineage. Consistent with the findings using PHA-CM, expression of HLA-DR is always induced on IFNγ-treated ML3 cells, but only occasionally on HL-60 cells. In the experiments reported in Table 1, for example, no surface expression of HLA-DR antigen on HL-60 cells could be demonstrated. However, preliminary experiments have shown that, although no HLA-DR antigen could be observed on the membrane of HL-60 cells, HLA-DR are present in the cell extract and in the supernatant from IFNγ-induced HL-60 cells at the same level as in those of ML3 cells.

IFNγ is produced by human lymphocytes upon stimulation with PHA, and it is contained in the PHA-CM preparations. When the differentiation-inducing ability of rIFNγ, purified IFNγ, or crude PHA-CM at equivalent concentrations of IFNγ was compared to that of rIFNγ in the differentiation of HL-60 and ML3 cells, we observed that the ability of IFNγ to induce monocytic differentiation was not as high as that of PHA-CM. This difference in the differentiation-inducing ability of IFNγ and PHA-CM is likely due to the presence of other cytokines in PHA-CM preparations that may enhance the differentiation of HL-60 and ML3 cells. These results suggest that PHA-CM contains factors in addition to IFNγ that are required for the induction of monocytic differentiation in HL-60 and ML3 cells.

Fig 5. Effect of monoclonal anti-IFNγ antibody on the ability of rIFNγ and PHA-CM to induce differentiation of HL-60 and ML3 cell lines. HL-60 and ML3 cells were cultured in the presence of medium with or without 2,000 U/mL of rIFNγ or 50% PHA-CM. B132.6 (IgG1) anti-IL2 antibody (10% tissue culture supernatant fluid) and B133.3 (IgG1) anti-IFNγ (10% supernatant) were added to the cultures where shown. After five days, cells were washed and tested for: (A) antibody-dependent cytotoxic ability against rabbit antibodies–sensitized P815y cells (50:1 effector-target cell ratio); (B) induction of HLA-DR antigens; (C) induction of α2M antigen; (D) induction of α-NAE; and (E) induction of FcR. ND, not determined.
antiviral activity was compared, PHA-CM was consistently found to be more efficient than IFNγ alone. The ability of both IFNγ and PHA-CM to induce differentiation is drastically reduced by treatment at 56 °C for one hour and abolished by treatment at a pH of 2.0 for 24 hours. According to gel chromatography on Sephadex G100 or on a TSK G3000 SW column, the antiviral activity contained in the PHA-CM coeluted with the differentiation-inducing activity. The fractions containing both activities corresponded to a higher molecular weight on the G100 column (40,000 to 50,000 daltons) than on the TSK column (30,000 to 40,000 daltons). The antiviral and differentiation-inducing activity were clearly separated from the bulk of CSF activity. On a reverse-phase column, antiviral activity, CSF, and differentiation-inducing activity were present together in the fractions eluted at concentrations of ethylene glycol higher than 20%. Monoclonal antibodies against IFNγ completely suppress the differentiation-inducing ability of recombinant or natural IFNγ preparations. When the same antibodies were used together with PHA-CM at concentrations able to completely inactivate the antiviral activity contained in it, an almost complete inhibition of induction of HLA-DR, α-NAE, and Ab-CMC was observed. The induction of B52.1 monocyte antigen and of FcR at day 5 was partially, but consistently, inhibited. The specificity of the inhibition by anti-IFNγ antibody was confirmed by using equivalent amounts of a monoclonal antibody of the same IgG1 subclass and directed against IL 2, a lymphokine that was also present in the PHA-CM preparation used. These results confirm that the IFNγ contained in the PHA-CM is the major factor responsible for differentiation and also that other differentiation-inducing factor(s), acting on promyelocytic cell lines, are present in the CM. These other factors are probably responsible for the higher efficiency of IFNγ-containing PHA-CM than purified IFNγ in inducing differentiation.

To test whether PHA-CM contains factors that act synergistically with IFNγ in inducing differentiation, IFNγ was removed from PHA-CM using a column of anti-IFNγ monoclonal antibodies bound to Sepharose beads. The IFNγ-depleted PHA-CM had a very reduced ability to induce differentiation, in analogy to results obtained when anti-IFNγ antibodies were directly added to the culture. When rIFNγ was added to the IFNγ-depleted PHA-CM, the induction of several of the markers of differentiation was...
Fig 8. Dose dependence of the synergism between PHA-CM and rIFNγ in inducing antibody-dependent cytotoxic activity in HL-60 (A) and ML3 (B) cells. Cells were incubated for five days in the presence of the different inducers, washed, and used as effector cells at a 10:1 effector-to-target-cell ratio against antibody-sensitized P815y cells (10⁵ cells) in a three-hour ⁵¹Cr-release assay. Cell treatment: ○, PHA-CM; □, PHA-CM absorbed on an anti-IFNγ column; ■, IFNγ-depleted PHA-CM + 2 U/mL rIFNγ; △, IFNγ-depleted PHA-CM + 20 U/mL rIFNγ; ▲, IFNγ-depleted PHA-CM + 200 U/mL rIFNγ; and △, IFNγ-depleted PHA-CM + 2,000 U/mL rIFNγ.

often to levels much higher than those observed using either rIFNγ- or IFNγ-depleted PHA-CM alone. These results indicate that there are factors in the CM that synergize the differentiation-inducing effect of IFNγ. These factor(s), in the absence of IFNγ, can induce some of the differentiation markers (eg, B52.1 monocytes antigens, FcR), but not others (α-NAE activity, Ab-CMC, HLA-DR antigens).

Factors contained in PHA-CM, such as CSF, have been reported to act as differentiation inducers in the human and murine systems. It is possible that the monocyte-specific differentiation markers used in the present study would not efficiently reveal differentiation along the myeloid pathway, such as that described, for example, in murine myeloid cell lines induced with CSF. Our preliminary observations have shown that the supernatant of the hepatoma cell line SK-Hep, reported to be a potent inducer of myeloid differentiation in HL-60 cells, is, unlike IFNγ, inefficient in inducing monocyte differentiation markers on HL-60 or ML3 cells. Thus, it is possible that HL-60 and ML3 cells respond to a variety of naturally occurring differentiation inducers by differentiating along either the monocytic or myeloid pathway, as already well documented using synthetic inducers.

The study of the interactions of IFNγ with other substances, such as CSFs, that are involved in induction of proliferation and differentiation of myelomonocytic cells could contribute to the understanding of the processes of regulation of normal and leukemic hematopoiesis.

ACKNOWLEDGMENT
We thank Dr Lois Epstein for helpful discussion, Jeffrey Faust for invaluable assistance at the flow cytometer, Elsa Aglow for histologic staining, Marina Hoffman for editing, and Marion Kaplan for preparing the manuscript.

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IFNγ AND MYELOID CELL DIFFERENTIATION


Role of immune interferon in the monocytic differentiation of human promyelocytic cell lines induced by leukocyte conditioned medium

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