A differentiation-inducing factor (DIF) for the promyelocytic HL-60 cell line is constitutively produced by the malignant T lymphocyte HUT-i 02-conditioned media by means of diethylaminoethanol (DEAE)-chromatography, gel chromatography, and high-resolution, ion-exchange chromatography on a MonoQ column and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition to inducing differentiation of wild-type HL-60 cells, resulting in secondary inhibition of growth, DIF, at a tenfold lower concentration, inhibited the growth of some clones of the monoblastic U-937 cell line as well as that of subclones of HL-60. The latter effect was most likely a primary growth inhibition and not secondary to differentiation; 50% inhibition of clonogenic growth in agar was seen at ~1.0 pmol/L of DIF. In addition, the clonogenic growth of fresh leukemia cells from 10 of 12 patients with acute myeloid leukemia (AML) was inhibited with 50% inhibition at ~10 pmol/L of DIF. The growth of normal granulocyte-macrophage colonies was inhibited at a similar concentration, whereas early erythroid colonies were much more resistant. DIF and interferon-γ (γ-IFN) were shown to be separate molecules inasmuch as a neutralizing antibody for γ-IFN did not abolish the DIF effect. The differentiation effect on wild-type HL-60 and the proliferation inhibitory effect on leukemic and normal myeloid cells chromatographed through all purification steps suggest that both activities are exhibited by identical polypeptides. DIF may have a role in regulating normal hemopoiesis. The growth inhibitory effect of DIF and the ability to induce differentiation of some leukemia cells may suggest a clinical utility in the treatment of leukemia.

**T Lymphocyte-Derived Differentiation-Inducing Factor Inhibits Proliferation of Leukemic and Normal Hemopoietic Cells**

By Urban Gullberg, Eva Nilsson, Mangalasseril G. Sarngadharan, and Inge Olsson

A CUTE MYELOID leukemia (AML) is characterized by an accumulation of immature cells, which may preserve normally transient phenotypes. The block in cell differentiation is not necessarily irreversible, as was shown originally using the murine M-1 myeloid leukemia cell line. Agents that induce differentiation of M-1 cells inhibited leukemia development in mice, indicating that such agents can act in vivo. Furthermore, injection of M-1 cells into embryos can result in the appearance in adult mice of normal granulocytes partially derived from the leukemic clone, indicating that the malignant cells respond to normal differentiation control in the embryo. We others demonstrated that mitogen-stimulated human mononuclear blood cells release polypeptides called differentiation-inducing factors, DIFs, which can induce the promyelocytic HL-60 line to mature into monocyte-like cells. Subsequently, we showed that the T lymphocyte leukemic cell line HUT-102, which releases human T-cell leukemia virus (HTLV), displayed constitutive production of DIF. DIF has been partly characterized; it has a mol wt of ~50,000. It acts synergistically with retinoic acid to induce differentiation of HL-60.

Now, we report that DIF, in addition to its differentiation effect on HL-60 cells, has a potent growth inhibitory effect on other myeloid cell lines and on fresh clonogenic cells from patients with AML. Normal granulocyte-macrophage clonogenic precursors (CFU-GM) and erythroid progenitors (BFU-E) are also susceptible. Thus, the ability of the same agent both to inhibit self-renewal and to induce the process of differentiation of some leukemic cells is emphasized.

**MATERIALS AND METHODS**

**Chemicals**

12-O-Tetradecanoyl phorbol 13-acetate (TPA) and Nitroblue tetrazolium (NBT), were from Sigma Chemical Co (St Louis). Acrylamide/bis-acrylamide (29:1) was from Bio-Rad (Richmond, CA). The Bolton-Hunter reagent, [125I]I-4, hydroxyphenylpropionic acid was from New England Nuclear, Dreieich, FRG. Diethylaminoethanol (DEAE)-Sephacel, Sephadex G-75, MonoQ columns, and Superose columns were from Pharmacia Fine Chemicals (Uppsala, Sweden). Luxol fast blue was from T.T. Gurr, High Wycombe, Bucks, England.

**Cell Lines**

The HL-60, U-937, K-562, and KG1 cell lines were maintained in suspension culture in RPMI 1640 medium containing 10% fetal bovine serum (FBS). The same medium was used for agar cultures. Exponentially growing cells were used for assays. Subclones of cell lines were obtained by picking individual colonies from agar cultures and expanding the cells by incubation in suspension culture.

**Assay for DIF**

The differentiation effect was assayed as previously described. In brief, cells were exposed to DIF in suspension culture for 4 days, and the number of unresistant esterase-positive and NBT-positive cells was determined. One unit of DIF is defined as the amount necessary to increase by 10% the number of NBT-positive cells. The effect of DIF on growth of cell lines was determined both in suspension and in agar culture. In the latter case, 2,000 cells were seeded in 0.3% agar, and colonies were counted after 7 days.

**Assay for CFU-GM**

Human marrow cells (10^5) (density <1.077 g/mL) obtained by centrifugation in Isopaque-Ficoll were cultured in 1 mL of 0.3% agar on top of 1 mL of 0.5% agar in McCoy's medium with 15% FBS in

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35-mm tissue culture dishes. As a source of colony-stimulating activity (CSA), human placenta conditioned medium (HPCM) was used after purification by chromatography on Sephadex G-75. For colony morphology studies, agar gels were fixed in 5% glutaraldehyde and dried; they were then stained with 0.1% Luxol fast blue and counterstained with Harris hematoxylin.

**Assay for BFU-E**

Mononuclear blood cells of four healthy subjects were isolated from heparinized blood by centrifugation on Ficoll-Hypaque and cultured at 2 x 10^6/mL in 0.8% methyl cellulose in Iscove's modification of Dulbecco's medium, supplemented with 30% FBS, 20 mmol/L of glutamine, 50 μmol/L of β-mercaptoethanol, 1.7% bovine serum albumin (BSA), 1.6 μmol/L of FeCl3, and 4.5 μmol/L of transferrin. Ten percent conditioned medium from the Mo cell line was used as CSA together with 1 U/mL of human urinary erythropoietin, kindly provided by Dr David Golde of the Department of Oncology-Hematology, University of California at Los Angeles, and Dr Miloslav Beran, M.D. Anderson Hospital, Houston, respectively. The BFU-E were counted after 20 days. Control incubations without DIF contained 41 ± 25 (SD) BFU-E per dish.

**Interferon-γ (γ-IFN) and Anti-γ-IFN**

Recombinant γ-IFN (Ernst-Boehringer Institut für Arzneimittel-forschung) contained 2 x 10^5 IFN U/mg of protein. A murine IgG monoclonal antibody that neutralized the antiviral and anticytotoxic effects of γ-IFN was obtained from the same source. Neutralizing anti-γ-IFN antibody was incubated with DIF overnight before testing.

**Production of Conditioned Medium**

Roller bottle cultures of HUT-102, clone B2, grown in RPMI, 10% FBS, were harvested at 96 hours postseeding. Supernatant was clarified by centrifugation at 1,600 rpm for 20 minutes and run through a continuous flow Model K ultracentrifuge (Electro-Nucleonics, Rockville, MD) at 35,000 rpm at a rate of 10 to 11 L/h on a sucrose step gradient (20% to 60% in 10 mmol/L of Tris, pH 7.2, 0.1 mol/L of NaCl, 1 mmol/L of EDTA). The virus-free effluent (LyCM) was collected and stored at 4°C.

**Isolation of DIF**

DIF was isolated from 40 L of conditioned medium, which was concentrated by ultrafiltration using a Pellicon casette (Millipore, Bedford, MA) with a filter of a cut-off point at a mol wt of 10,000. Dialysis was carried out against 10 mmol/L of Tris-HCl buffer, pH 8.0, containing 0.1 mmol/L of phenylmethylsulfonyl fluoride (PMSF), 0.01% polyethylene glycol (PEG) 6,000, and 1 mmol/L of dithiothreitol (buffer A). The concentrated material was incubated with 5% FBS in 0.15 mol/L of NaCl, dialyzed against 0.15 mol/L of NaCl, buffer A, and assayed for biological activity.

**RESULTS**

**Purification of DIF from HUT-102 Conditioned Medium**

The purification steps are modifications of procedures used in a previous report on the biochemical characterization of DIF. Especially high-resolution ion-exchange chromatography of the present work represented a powerful improvement. Table 1 summarizes data on the yield and specific activities from each step of purification. DIF-containing fractions obtained by MonoQ chromatography (step 3) showed a 37,000-fold purification with an overall

![Table 1](image-url)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (g)</th>
<th>Total Activity (U x 10^5)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification (Fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUT-102 medium</td>
<td>195</td>
<td>80</td>
<td>0.41</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>6.25</td>
<td>64.4</td>
<td>10.3</td>
<td>25</td>
<td>81</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>0.44</td>
<td>48</td>
<td>109.1</td>
<td>266</td>
<td>60</td>
</tr>
<tr>
<td>MonoQ I</td>
<td>64</td>
<td>40.8</td>
<td>637.5</td>
<td>37,195</td>
<td>31</td>
</tr>
<tr>
<td>MonoQ II*</td>
<td>1.6</td>
<td>24.4</td>
<td>15,250</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

DIF, differentiation-inducing factor; HUT-102, malignant T lymphocyte cell line; DEAE, diethylaminoethyl; SDS-PAGE, sodium dodeyl sulfate-polyacrylamide gel electrophoresis.

*MonoQ II refers to rechromatography on MonoQ followed by collection of DIF eluted at <0.08 mol/L of NaCl to increase the specific activity.

†The protein content of fractions eluted from the gel was too low to be determined.
yield of 31% and were used in experiments described below unless otherwise stated. Concentrations of DIF are given as total protein added of this fraction. From experiments with radiiodination using the Bolton-Hunter reagent followed by SDS-PAGE (Fig 1) and elution of DIF from the gel ~5% of the radiiodinated protein was calculated to be associated with DIF activity. Estimations of molar concentrations of DIF which follow are based on these data and a mol wt of 50,000.

**Proliferation Inhibitory Effects on Myeloid Leukemia Cell Lines**

Induction of ~50% of wild-type HL-60 into NBT-positive, α-naphthyl butyrate esterase-positive monocytelike cells was achieved with ~50 pmol/L of DIF. This represents the characteristic differentiation-inducing effect of DIF reported earlier. The results reported here demonstrate that at a tenfold lower concentration, DIF inhibited the growth of some subclones of both U-937 and HL-60 without the production of mature NBT-positive cells. Figure 2 shows a dose-dependent inhibitory effect of DIF on proliferation of a subclone of U-937 in suspension culture (top panel). The antiproliferative effect was partly reversible; when the cells were exposed to DIF for 3 days, washed, and resuspended in fresh medium, a slow recovery of growth occurred, indicating that at least a fraction of the cells had survived (Fig 2, middle panel). The growth inhibitory effect (like the differentiation effect) was rather heat stable but was destroyed by boiling (Fig 2, lower panel). The growth of a subclone of HL-60 (HL-60-10) was inhibited at similar concentrations (data not shown). In contrast to wild-type HL-60, the subclone was α-naphthyl butyrate esterase-positive and thus similar to the monoblastlike U-937.

The growth inhibitory effect of DIF was more pronounced using a clonogenic assay instead of suspension culture. The growth inhibitory effect of DIF on the clonogenic growth in agar of various myeloid leukemic cell lines was compared (Table 2). HL-60-10, U-937, and KG-1 showed a similar susceptibility to DIF, whereas the K-562 cell line showed a tenfold lower susceptibility.

The addition of 4,000 neutralizing units of anti–γ-IFN antibody did not neutralize the proliferation inhibitory effect...
Table 2. The Growth Inhibitory Effect of DIF on Clonogenic Growth in Agar of Myeloid Leukemic Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>50% Growth Inhibition (ng/mL DIF)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60 (wild type)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HL-60-10</td>
<td>1</td>
</tr>
<tr>
<td>HL-937</td>
<td>1</td>
</tr>
<tr>
<td>KG-1</td>
<td>2</td>
</tr>
<tr>
<td>K-562</td>
<td>11</td>
</tr>
</tbody>
</table>

*Differntiation-inducing factor (DIF) from step 3 (MonoQ chromatography) was used for these assays.

of DIF (Fig 3). Identical results were also obtained when immunoprecipitates, which might have formed with the antibody, were removed by adsorption to protein A-Sepharose prior to testing. Furthermore, γ-IFN 100 to 4,000 U/mL, unlike DIF, did not induce any maturation of wild-type HL-60 as judged by lack of induction of NBT-positive cells (data not shown). Treatment at pH 2 only partially reduced the DIF activity (Fig 3). All these results indicate that DIF and γ-IFN are nonidentical.

Proliferation Inhibitory Effects on Normal Hemopoietic Clonogenic Cells and on Fresh AML Cells

Figure 4 shows that normal CFU-GM and BFU-E as well as clonogenic fresh AML-cells from 10 of 12 patients responded with growth inhibition to the addition of DIF. CFU-GM counted on day 14 were more susceptible than were CFU-GM counted on day 10, whereas cluster-forming cells counted on day 7 were even less susceptible. Only a 20% inhibition of growth of BFU-E was documented over a wide range of DIF concentrations. Actually, a significant stimulation of the growth of BFU-E was documented over a wide range of DIF concentrations. Effects on CFU-GMs at such low concentrations of DIF were not investigated. A 50% growth inhibition of both CFU-GM (day 10) and AML cells was seen at ~10 pmol/L of DIF (Fig 4). Treatment with DIF did not change the commitment of CFU-GM inasmuch as the distribution of macrophages and granulocytes in residual colonies did not differ from that of control cultures (data not shown). Like the antiproliferative effect of DIF on leukemic cell lines, the effect on normal CFU-GM was rather heat resistant, resistant to a neutralizing antibody against γ-IFN, and only partially susceptible to low pH (Fig 5).

Fig 3. Effect of low pH and a neutralizing anti-γ-IFN antibody on the proliferation inhibitory effect of DIF on U-937. DIF was treated at pH 2 or with 4,000 neutralizing units of antibody overnight prior to testing. Control shows growth of cells without added DIF.

Fig 4. Effects of DIF on clonogenic growth of bone marrow cells from ten patients with acute myeloid leukemia (AML), normal CFU-GM of marrow cells, and normal BFU-E of mononuclear blood cells. DIF from step 3 (MonoQ chromatography) was used. The plating efficiency of marrow cells from the AML patients varied between 0.3% and 2.0%. Cells from two patients with AML were resistant to DIF, and results for these were excluded. Growth of clonogenic AML cells was read at day 7. Cluster-forming units (CFU) were read at day 7. CFU-GM were read at days 10 and 14, and BFU-E were read at day 20. Bars, SEM.

Fig 5. Effect of heat treatment, low pH, and a neutralizing anti-γ-IFN antibody on the proliferation inhibitory effect of DIF on normal CFU-GM. Control incubation with DIF (△). DIF was treated at pH 2 (○) or with 4,000 neutralizing units of the antibody (★) overnight prior to testing. Heat treatment was for 20 minutes at 70 °C and 100 °C (℃).
COPURIFICATION OF DIFFERENTIATION-INDUCING EFFECT AND PROLIFERATION INHIBITORY EFFECT OF DIF

Both activities cochromatographed exactly on DEAE chromatography (step 1). When material from step 3 was subjected to rechromatography on a MonoQ column or on a Superose column, the activities copurified (Fig 6). Some molecular heterogeneity is indicated by the elution pattern from the MonoQ column. This is consistent with results from chromatofocusing demonstrating that the isoelectric point varied between pH 5.4 and 5.9 (data not shown). In addition, when the material from step 3 was subjected to SDS-PAGE followed by gel slices, both activities were found in the same fractions (Fig 1) and a mol wt of ~50,000 was calculated. By addition of some ^125^I-labeled DIF prior to electrophoresis, it was estimated that 5% of the protein content of the MonoQ fraction may consist of DIF.

DISCUSSION

DIF induces the differentiation of wild-type HL-60 along the monocyte-macrophage pathway, resulting in secondary inhibition of growth. In addition, results from this report show that, at a tenfold lower concentration, DIF inhibits growth of subclones of both HL-60 and U-937. The latter effect was expressed without visible differentiation, as judged by the lack of production of NBT-positive cells characteristic of the mature differentiation product. Even if phenotypic modulation may have occurred, it is unlikely that the rapid antiproliferative effect observed was secondary to induction of differentiation in this case. Thus, DIF appears to display different actions on clones from the same cell line. Target cells that are resistant to a growth inhibitory effect at a low concentration may respond with differentiation at a higher concentration of DIF as wild-type HL-60 cells do. In both cases, the final result will be inhibition of the self-renewal capacity of the leukemic cells.

In a cell line, a large fraction of cells has a capacity for clonal self-renewal. Among fresh leukemia cells from patients with AML only a minority of cells are clonogenic, however. Therapeutic efforts must be directed toward the clonogenic leukemia cells because nonclonogenic cells will disappear anyway. The growth inhibition by DIF of marrow cells from patients with AML using a clonal assay may suggest a utility of DIF in the treatment of leukemia. The effect on AML cells may be a direct inhibition of proliferation of leukemic clonogenic cells or may be secondary to induced differentiation. The plating efficiency in agar of marrow cells from patients with AML varied considerably, and it was a parameter of prognosis inasmuch as a high plating efficiency indicated a less favorable prognosis. For natural reasons, the antiproliferative effect of DIF could only be significantly evaluated on AML cells with a relatively high plating efficiency. Therefore, the patient material investigated became selected to include patients with a less favorable prognosis preferentially. On the other hand, in such cases with high plating efficiency one can be confident that the majority of clonogenic cells really represents leukemic and not normal cells.

The growth inhibitory effect on normal CFU-GM suggests that DIF may be involved in a physiological regulation of hemopoiesis. It is important to determine if DIF also has effects on multipotent (mixed) progenitors (CFU-GEMM), especially if DIF is considered as a treatment in leukemia. Assaying the effects of DIF on multipotent progenitors was difficult, however, because daughter cells of such cells are CFU-GM, which are susceptible to the growth inhibitory effects of DIF.

As far as we can judge, DIF is not identical with previously described lymphokines. Interferons affect many cell functions in addition to their inhibitory action on virus replication. Among these effects are proliferation inhibitory effects on hemopoietic cells in vitro and differentiation-inducing effects on leukemic cells. An identity between DIF and γ-IFN was ruled out, however, because a neutralizing anti-
body to γ-IFN did not interfere with the proliferation inhibitory effect of DIF. Furthermore, in contrast to DIF, recombinant γ-IFN did not induce differentiation of wild-type HL-60 judged by lack of production of NBT-positive cells. Whether DIF is a unique differentiation and cytotoxic protein produced by lymphocytes or is related to the family of polypeptides called lymphotoxins remains to be investigated.22

That both the differentiation and the antiproliferative effects are exhibited by the same polypeptide(s) is strongly suggested since both activities cochromatographed during all purification steps. Furthermore, the yield of both activities from various purification procedures was similar (data not shown). A purification to homogeneity has not been achieved, however, and it has not been shown that completely purified DIF displays both activities.

Constitutive production of DIF by the HUT-102 line is a valuable source for large-scale production and for isolation and cloning of the gene that codes for DIF. An agent like DIF that can both inhibit self-renewal and induce differentiation of some leukemic cells is of potential utility in the treatment of leukemia.

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T lymphocyte-derived differentiation-inducing factor inhibits proliferation of leukemic and normal hemopoietic cells

U Gullberg, E Nilsson, MG Sarngadharan and I Olsson