Isolation and Characterization of a T Lymphocyte-Derived Differentiation Inducing Factor for the Myeloid Leukemic Cell Line HL-60

By Inge L. Olsson, Mangalasseril G. Sarngadharan, Theodore R. Breitman, and Robert C. Gallo

Mitogen-stimulated mononuclear blood cells produce differentiation inducing factors (DIFs) for the promyelocytic cell line HL-60. We report that DIF is produced constitutively by a malignant T lymphocyte line HUT-102. DIF was purified 7,000-fold from HUT-102 conditioned media by utilizing ion-exchange chromatography with DEAE-Sepharose, gel chromatography, Blue-Sepharose chromatography, and preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The final preparation is susceptible to protease treatment, has a molecular weight of 46,000, as determined by SDS-PAGE and approximately 55,000 by gel filtration, has an isoelectric point of approximately 5.2, does not adhere to lectin-Sepharose and is resistant to periodate oxidation, and is free of colony-stimulating factor. DIF induced maturation of HL-60 into phagocytizing nitro blue tetrazolium reducing cells with the morphological characteristics of myelomonocytic or monocyte-like cells. An activity, cochromatographing with DIF, acts synergistically with retinoic acid to induce maturation not only of HL-60, but also of the monoblast-like cell line U-937 (measured as percentage of cells reducing NBT).

**MATERIALS AND METHODS**

**Chemicals**

All-trans-RA was obtained from Sigma (St. Louis, MO). 12-0-Tetradecanoylphorbol 13-acetate (TPA) was obtained from P-L Biochemicals (Milwaukee, WI) and stored at −20°C in acetone at 200 µg/ml. Nitroblue tetrazolium (NBT) was from Aldrich (Milwaukee, WI), DEAE-Sepharose, G-75 Sephadex Superfine and Blue Sepharose were obtained from Pharmacia (Uppsala, Sweden). Lectin-Sepharoses were purchased from P-L Biochemicals. Acrylamide for gel electrophoresis was obtained from Fisher (Fair Lawn, NJ). Trypsin, protease (Streptomyces griseus, type VI), ribonuclease (bovine pancreas type II-A), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Neuraminidase was from Behringwerke (Marburg, FRG).

**Cells**

The HL-60 and U-937 cells (the latter provided by Dr. K. Nilsson, Uppsala, Sweden) were grown in Corning polystyrene tissue culture flasks containing equal volumes of Dulbecco’s modified Eagle’s minimal essential medium (MEM) and Ham’s F-12 supplemented with 1.2 g NaHCO₃/liter, 15 mM HEPES, and 10% (v/v) fetal bovine serum (FBS) (Flow Laboratories, McLean, VA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cell counts were determined with a Coulter counter. Viability was assessed by trypan blue exclusion. Cytospin slide preparations were stained with Wright-Giemsa for morphological assessment.

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*From the Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD, and the Department of Internal Medicine, Lund Hospital, Lund, Sweden.

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Address reprint requests to Dr. Inge L. Olsson, Research Department 2, E-Blocket, Lund Hospital, S-221 85 Lund, Sweden.
Exponentially growing cells were used for differentiation assays. All such studies were performed in a serum-free medium supplemented with bovine insulin (5 μg/ml) and human transferrin (5 μg/ml). In addition, the medium for U-937 contained bovine serum albumin (600 μg/ml) to prevent adherence of these cells to the plastic surface.

**Assay for DIF**

Various amounts of DIF-containing fractions were added to test cells (3 x 10⁸/ml) in 35-mm tissue culture dishes in a final volume of 3 ml. After 4 days, the cell number, viability, morphology, and capacity to reduce NBT were determined. NBT reduction was carried out as described. Approximately 2 x 10⁶ cells/ml medium with 20% FBS was incubated for 25 min at 37°C with an equal volume of 0.2% NBT dissolved in PBS containing 20 ng TPA/ml. Cytospin slides were stained with Wright-Giemsa, and the number of cells containing formazan deposits were counted. One unit of DIF is defined as the amount necessary to increase by 10% the number of NBT-positive HL-60 cells. Stains were also performed for α-naphthyl butyrate (nonspecific) esterase.

**Assay for Retinoic Acid Promoting Activity (RAPA)**

These assays were carried out as described above for DIF, except that lower concentrations of DIF were used and RA was added to a final concentration of 10 nM for HL-60 and 100 nM for U-937. RA was dissolved in 100% ethanol and diluted 1,000-fold into the growth medium such that the final concentration of ethanol was no higher than 0.1%. RAPA is expressed as the potentiated effect of added DIF on RA-induced differentiation after subtraction of the results with RA and DIF alone.

**Assay for Colony-Stimulating Factor (CSF)**

Human marrow cells (10⁷) (density <1.077 g/ml obtained by centrifugation in Isopaque-Ficoll) were cultured in 1 ml of 0.3% agar medium such that the final concentration of ethanol was no higher than 0.1%. CSF was dissolved in 1 ml of 0.3% agar and incubated for 25 min at 37°C with an equal volume of 0.2% NBT dissolved in PBS containing 20 ng TPA/ml. Cytospin slides were stained with Wright-Giemsa, and the number of cells containing formazan deposits were counted. One unit of DIF was dissolved in 1 ml of medium with 20% FBS was incubated for 25 min at 37°C with an equal volume of 0.2% NBT dissolved in PBS containing 20 ng TPA/ml. Cytospin slides were stained with Wright-Giemsa, and the number of cells containing formazan deposits were counted. One unit of DIF is defined as the amount necessary to increase by 10% the number of NBT-positive HL-60 cells. Stains were also performed for α-naphthyl butyrate (nonspecific) esterase.

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**Production of Conditioned Medium From the T Lymphocyte Line**

Roller bottle cultures of HUT-102, clone B2, were harvested at 96 hr postseedling. Supernatant was clarified by centrifugation at 1,600 rpm for 20 min and run through a continuous flow Model K ultracentrifuge (Electro-Nucleonics) at 35,000 rpm at a rate of 10–11 liter/hr on a sucrose step gradient (20%–60% in 10 mM Tris, pH 7.2, 0.1 M NaCl, 1 mM EDTA). The virus-free effluent (LyCM) was collected and stored at 4°C.

**Methods for Isolation of DIF**

**Step 1. Ammonium sulfate precipitation.** All purification steps, except for SDS-PAGE, were performed at 4°C. The virus-free LyCM collected from the continuous flow centrifugation step was filtered through Nalgene 0.45-μ filters. Ammonium sulfate in a quantity to produce a 70% saturated solution was slowly added to 2.5 liter of LyCM, and after stirring for 1 hr, the precipitate was collected by centrifugation at 15,000 g for 15 min. The precipitate was dissolved in 10 mM Tris·HCl buffer, pH 8.0, containing 0.1 mM PMSF, 0.01% polyethylene glycol (PEG) (6,000), and 1.0 mM dithiothreitol (buffer A). Dialysis was carried out against 10 vol of buffer A (without PEG) with several changes of dialysate.

**Step 2. DEAE-Sepharose chromatography and gel chromatography.** The material obtained in step 1 was applied on a 180-ml column of DEAE-Sepharose equilibrated with buffer A. Elution was performed with a 400-ml NaCl/buffer A gradient, ranging from 0 to 0.3 M NaCl. Fraction volume was 7 ml. DIF-containing material eluted from the DEAE-Sepharose column was analyzed by gel chromatography on a Sephadex G-75 column (2.5 x 90 cm) eluted with 0.15 M NaCl, 10 mM HEPES, pH 7.4. Four milliliters of pooled DIF-containing material was concentrated by lyophilizing to a volume of 2 ml and applied to the Sephadex column.

**Step 3. Blue-Sepharose chromatography.** The active fractions from step 2 were pooled and dialyzed against buffer A. The material was subsequently applied on a 40-ml column of Blue-Sepharose that was previously equilibrated with buffer A. The column was washed with buffer to eliminate unbound protein and then eluted in 4-ml fractions with a 300-ml NaCl/buffer A gradient, ranging from 0 to 0.6 M NaCl.

**Step 4. Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** SDS-PAGE was carried out essentially as described by Bonner et al. The slab gel, 1.5-mm thick, consisted of a 20-cm long resolving gradient gel (5%–20% acrylamide), with a stacking gel of 3.5% acrylamide on top of the resolving gel. DIF-containing material (50 ml) obtained by Blue-Sepharose chromatography (see Fig. 4) was concentrated to 1.3 ml in a dialysis bag placed inside a heap of solid PEG-20,000. It was brought to electrophoresis sample buffer, and the mixture was incubated at room temperature for 1 hr. The mixture contained 50 mM Tris·HCl buffer, pH 6.8, 2% SDS, 2% mercuricacetat etol, 1 mM PMSF, and 20% glycerol. Electrophoresis was performed overnight at a constant current of 20 mA. Molecular weight markers were coelectrophoresed. The gel matrix was subsequently sliced into 1.0-cm horizontal strips after removing cuts with the molecular weight markers and a median longitudinal cut for staining. The slices were extracted overnight with 6 ml buffer A containing 0.15 M NaCl. The SDS was removed by passing the extract over 0.5-ml columns of AG-1 x 8 resin prepared in Pasteur pipettes, and the fractions were filter sterilized and assayed for DIF.

**Biochemical Characterization of DIF**

DIF obtained by DEAE chromatography was used for determination of the isoelectric point. Ten milliliters of pooled material, corresponding to approximately 450 U of DIF, was mixed with 2% ampholines (pH range 3.5–10) and focused overnight at 8 W using an LKB Multiphor unit. The Ultradex gel was sliced, and the pH of the individual slices determined. The gel slices (7 mm) were incubated with 6 ml buffer A containing 0.15 M NaCl. The SDS was removed by passing the extract over 0.5-ml columns of AG-1 x 8 resin prepared in Pasteur pipettes, and the fractions were filter sterilized and assayed for DIF.

**Periodate oxidation.** DIF-containing fractions were incubated with 1 mM sodium metaperiodate in the dark at room temperature for 10 min. The reaction was terminated by the addition of 20 mM glyceraldehyde, and the samples were dialyzed extensively before assay.

**Enzyme treatments.** Fractions were incubated with the following enzymes: 0.5 mg trypsin/ml, 0.5 mg protease/ml, 1.0 mg ribonuclease-A/ml, and 0.5 U neuraminidase/ml. Incubations were at 37°C for 30 min, and the samples were assayed for DIF activity. In control experiments, cells were treated with enzymes alone at their final concentrations in the cell suspension, and no effects on the target cells were seen.

**Lectin-Sepharose chromatography.** Lentil-lectin-Sepharose and concanavalin-A-Sepharose, equilibrated with 0.2 M NaCl/10 mM KH₂PO₄, pH 6.5, containing 1 mM CaCl₂, 1 mM MgCl₂, and...
0.2 mM MnCl₂, were used to determine the binding properties of DIF to glycosyl binding substances. Elution of lentil-lectin column was with buffer alone and with 2 M α-methyl mannoside in buffer. Elution of the concanavalin-A-Sepharose was with buffer alone and with 50 mM α-methyl-D-glucoside in buffer. Fractions were dialyzed extensively in 0.15 M NaCl in buffer A before assay for DIF.

RESULTS

Isolation of DIF

Step 1 of the purification scheme consisted of ammonium sulfate precipitation of HUT-102 supernatant followed by chromatography of DEAE-Sepharose (step 2). DIF and RAPA were eluted at an ionic strength of 0.05–0.10 M NaCl (Fig. 1). CSF eluted at an ionic strength of 0.15–0.20 M NaCl, clearly separated from DIF. DIF-containing fractions were pooled and such material was used for gel chromatography and isoelectric focusing. When subjected to gel chromatography on Sephadex G-75 (Fig. 2), DIF and RAPA appeared in one position corresponding to an apparent molecular weight of approximately 55,000. When subjected to isoelectric focusing (Fig. 3), DIF focused as a single peak at a pH of approximately 5.2.

Step 3 consisted of chromatography on Blue-Sepharose (Fig. 4). DIF and RAPA eluted in a broad peak corresponding to an ionic strength of 0.3–0.5 M NaCl. Pooled DIF fractions revealed no CSF activity at all when tested on human marrow cells.

Preparative SDS-electrophoresis constituted step 4 of the purification scheme (Fig. 5). Biologic DIF activity could be eluted from the gel that corresponding to a molecular weight of 46,000.

Table I shows the yield and specific activities from each step of purification. To compute the yields and specific activities from each step of purification, serial dilutions of pooled fractions were assayed (Table 1). As RAPA cochromatographed with DIF throughout all purification steps, it seems to be identical with DIF. DEAE chromatography gave a very high yield, and gel chromatography could also be carried out with a high yield (Fig. 2). However, considerable losses in activity were experienced in steps where very diluted protein solutions were handled, e.g., SDS-PAGE.

Biochemical Properties of DIF

The DIF-containing fractions obtained by Blue-Sepharose chromatography (Fig. 4) were used for biochemical characterization. DIF was found to be resistant to the action of ribonuclease, deoxyribonuclease, and neuraminidase. Protease destroyed 75% and trypsin 34% of the DIF activity under the incubation conditions described in Materials and Methods. Therefore, DIF is presumably a polypeptide. Treatment with 1 mM PMSF or 10 mM dithiothreitol did not affect the DIF activity.

DIF failed to adhere to the lectin Sepharoses tested (lentil-Sepharose and concanavalin-A-Sepharose), which is consistent with the absence of significant carbohydrate on the DIF molecule. Furthermore, periodate oxidation did not destroy the activity, indicating that the biologic activity does not depend on intact carbohydrate moieties.

Stability of DIF

DIF activity is highly stable at −20°C, except at very low protein concentrations. Loss of activity could be prevented by addition of 0.01% PEG, which was included in all buffers used for chromatography. PMSF was used to minimize proteolytic degradation. DIF is also stable during lyophilization. Heating to
60°C for 15 min did not decrease DIF activity, whereas 70°C for 15 min partially destroyed activity. Heating to 80°C for 15 min completely destroyed DIF activity.

**DIF-Induced Morphological Changes of HL-60**

When HL-60 cells were treated for 4–6 days with DIF, a dose-dependent production of NBT-positive cells occurred, indicating functional myeloid maturation. There was a corresponding increase in the capacity for phagocytosis (data not shown). However, only a fraction of cells (less than 50%) matured under these conditions. The growth rate of the cells was not significantly inhibited by DIF during a 4-day incubation. When HL-60 cells, treated for 4 days with DIF, were washed and then incubated with fresh medium, they continued to proliferate, and obviously, cells unaffected by DIF outgrew the cells that matured in its presence. However, if DIF was added repeatedly every second day (and the cells diluted in fresh medium with DIF every fourth day) growth ceased after 8–10 days. After 4 days of treatment with DIF, a fraction of cells accumulated with decreased nuclear diameter, increased cytoplasmic area, and disappearance of azurophil granules (Fig. 6). These cells reduced NBT. They also showed nonspecific activity with α-naphthyl butyrate substrate. Therefore, they are most likely...
monocytic cells. With multiple additions of DIF, the dominating cell appearing after 8–10 days is a nonspecific esterase-positive monocytic cell with abundant cytoplasm and folded nuclei (Fig. 6). This cell has a tendency to adhere slightly to the surface of the culture dish.

**Synergistic Effects Between DIF and RA**

Various concentrations of DIF and RA have been used in combination (Fig. 7). For example, 50 μl of DIF alone induces maturation of 30% of cells. When combined with 3 nM RA, 75% NBT-positive cells occurred, when only 40% NBT-positive cells were expected if the action of the inducers was additive. Thus, DIF is synergistic with RA in inducing HL-60 to mature into NBT-positive cells. RA induces myeloid differentiation and DIF induces monocytic differentiation in HL-60. The synergistic effect was seen for induction of NBT-positive cells but not for nonspecific esterase-positive cells (data not shown). Thus, the nature of the cell induced by a combination of RA and DIF may be myeloid, but more detailed studies are necessary to clarify this problem.

DIF alone did not induce maturation of U-937 into NBT-positive cells. However, it acted synergistically with RA to induce maturation of U-937 (Fig. 8). Thus, in the presence of DIF, the RA effect was potentiated approximately ten-fold.

**DISCUSSION**

The present study has demonstrated that the T lymphocyte line, HUT-102, which also releases a type C human retrovirus called HTLV, produces a differentiation inducing factor (DIF) for the human promyelocytic cell line HL-60. In addition, this factor acts synergistically with RA to induce maturation of both HL-60 and the monoblastic line U-937. The HUT-102 line is a constitutive producer of DIF, providing a rich and convenient source for purification of this lymphokine, which may have a biologic role, although yet unproven, in myeloid differentiation. HL-60 cells in a serum-free medium supplemented with insulin and transferrin were used routinely for assay of DIF. However, identical differentiation results were also seen when cells were incubated in a serum-containing medium.

Several methods of protein purification were explored. Ion-exchange chromatography on DEAE, gel filtration, and Blue-Sepharose chromatography
turned out to be useful separation steps. The yield of “purified” DIF was only 5%. The losses occurred primarily when protein concentration was low, e.g., with Blue-Sepharose chromatography and with the final preparative SDS-PAGE. The stability of the biologic activity was increased by addition of 0.01% PEG, which probably acted by preventing undesired hydrophobic interaction between DIF and tube surfaces. More efficient final purification steps still have to be applied for the large-scale production of pure DIF.

HUT-102-produced DIF is apparently a homogeneous molecular species by ion-exchange chromatography and gel filtration. This is consistent with the finding of only one peak of activity on isoelectric focusing. However, the molecular weight, as determined by preparative SDS-PAGE, is lower (46,000) than that predicted by gel filtration (approximately 55,000). Based on its failure to adhere to lectin-Sepharoses, it may not be glycosylated. The resistance to periodate oxidation also indicated that the activity is not dependent on carbohydrate moieties.

DIF was originally described as a product of mitogen-stimulated human mononuclear blood cells. Such cells produced two species of DIF with apparent molecular weights of 40,000 and 25,000, depending on the type of mitogen employed. At least the 40,000 molecular weight DIF was distinct from the CSF produced simultaneously. Although the molecular weights differ somewhat, the 40,000 mol wt species of DIF produced by mitogen-stimulated human mononuclear blood cells has biochemical features in common with constitutively produced DIF of HUT-102. The

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**Table 1. Purification of DIF**

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Yield (%)</th>
<th>Specific Activity</th>
<th>Purification (Fold)</th>
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<tr>
<td>Crude LCM</td>
<td>12.550</td>
<td>5,383</td>
<td>100</td>
<td>0.43</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>11.615</td>
<td>5,010</td>
<td>93</td>
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<tr>
<td>DEAE-Sepharose</td>
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<td>76</td>
<td>73</td>
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<tr>
<td>Blue-Sepharose</td>
<td>2.9</td>
<td>965</td>
<td>18</td>
<td>332</td>
</tr>
<tr>
<td>Preparative SDS-PAGE</td>
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<td>275</td>
<td>5</td>
<td>3,056</td>
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</tbody>
</table>

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**Fig. 6.** Morphological changes. Uninduced HL-60 (A). HL-60 treated with DIF for 4 days (B), and HL-60 treated with DIF for 3 days (C) with the addition of DIF on days 0, 4, and 6 of incubation and with change of medium at day 4. Bar = 10 μm.

**Fig. 7.** Synergistic effects between various concentrations of DIF and 1, 3, or 10 nM RA for induction of differentiation of HL-60. DIF obtained by DEAE chromatography was used (Fig. 1). On the abscissa is given microliters DIF added per 3-mI incubates of HL-60. The protein concentration for the DIF-containing material was 0.8 mg/ml. Differentiation is measured as the production in 4 days of cells capable of reducing NBT.
Fig. 8. Synergistic effects between RA and DIF for induction of differentiation of U-937. Data are given for various concentrations of RA alone (○) and together with DIF-containing material (●) (80 μg protein/3-ml incubates) obtained by DEAE-chromatography (Fig. 1). DIF alone has no measurable differentiation inducing effect on U-937. Differentiation is measured as the production in 4 days of cells capable of reducing NBT.

Data available suggest that DIF represents a family of proteins where the differences between various members may depend on the type of producer cell and type of stimulation. Using the mouse myeloid leukemia models, a naturally occurring polypeptide factor has been shown to induce differentiation. This differentiation-inducing factor (D factor) has been referred to as a special form of macrophage and granulocyte inducer (MGI), MGI-2, which induces differentiation in MGI+ mouse myeloid leukemic cells15 and also the HL-60 cell line.16 The D factor can be at least partially distinguished from GM-CSF.17,18 Mouse spleen cells stimulated with various mitogens produce factors distinct from CSF that induce mouse myeloid leukemia M1 cells to mature into macrophage-like cells.19 These factors may be analogous to DIF produced by human mononuclear blood cells.9

As noted before from studies on mononuclear blood cell-derived DIF,9 the morphological changes induced in HL-60 with DIF are not identical with those induced by DMSO2,3 or RA.4 The latter agents produce mature-appearing granulocytes. On the other hand, phorbol esters induce macrophage-like adherent cells.20 Thus, the promyelocytic HL-60 cell is a precursor for both granulocytes and monocyte-macrophages. DIF-induced cells have some characteristics of monocytic-like cells, but they are only slightly adherent. The monocyte features became more striking after prolonged incubation with multiple additions of DIF.

CSF is regarded as a regulator of myeloid proliferation and differentiation,21 although its role in vivo is as yet not definitely established. It is believed to be required not only for the proliferation and differentiation of early granulopoietic precursor cells, but also for the sequence of late differentiation events.22 The HUT-102 line constitutively produced both CSF and DIF. Our data clearly show that DIF is devoid of CSF activity, as these two factors are completely separated by ion-exchange chromatography. In addition, our most pure DIF did not reveal any CSF activity. It should be mentioned, however, that the low molecular weight species of DIF from mitogen-stimulated mononuclear blood cells cochromatographed with CSF.9 Therefore, it cannot be presently ruled out that some CSFs may have DIF-like activity. The relationship, if any, of HUT-102-produced DIF to other previously described lymphokine factors is not established at present. The physiochemical properties of DIF eliminate its identity with T cell growth factor, produced by the HUT-102 line,23 and the major core protein, p24, of HTLV24 released by HUT-102. In addition, non-virus-producing sublines of HUT-102 still produced DIF.

DIF markedly potentiated RA-induced differentiation of both HL-60 and U-937. A previous study showed that these cell lines can be primed for differentiation by treatment for approximately 1 day with RA, followed by exposure to DIF.25 The reverse sequence was ineffective. Thus, HL-60 could be primed by incubation for less than 20 hr with 10 nM RA to respond by differentiation to the addition of a concentration of DIF, which alone was inactive. It is also possible that there are synergistic effects, for differentiation of leukemic cells, between DIF and chemotherapeutic agents used in leukemia. Thus, inducers may be effective in combinations with chemotherapeutic agents. Additional studies of purified DIF are necessary to define its eventual physiologic role in hemopoiesis and its potential use in leukemia.

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Isolation and characterization of a T lymphocyte-derived differentiation inducing factor for the myeloid leukemic cell line HL-60

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