A Combination of a T Cell–Derived Lymphokine Differentiation-Inducing Activity and a Physiologic Concentration of Retinoic Acid Induces HL-60 to Differentiate to Cells With Functional Chemotactic Peptide Receptors

By Masue Imaizumi and Theodore R. Breitman

The human acute promyelocytic leukemia cell line HL-60 is induced by retinoic acid (RA) and N,N-dimethylformamide (DMF) to differentiate into cells having many of the functional and morphologic characteristics of mature granulocytes. With normal human phagocytic cells there is both superoxide anion (O$_2^-$) production and chemotaxis in response to chemoattractants such as N-formyl-methionyl-leucyl-phenylalanine (FMLP). We have now found that although HL-60 cells induced with RA alone produce O$_2^-$ in response to 12-O-tetradecanoyl-phorbol-13-acetate (TPA) they are deficient in FMLP-stimulated O$_2^-$ production and chemotaxis. In contrast, HL-60 induced either with DMF or with a combination of 10 mM/L RA and a T cell–derived lymphokine, differentiation-inducing activity (DIA), produce O$_2^-$ and exhibit chemotaxis in response to FMLP. The basis for these results appears to be the concentration of cell surface chemotactic peptide receptors. Thus, untreated HL-60 and HL-60 induced with either RA alone or DIA alone do not have measurable levels of FMLP receptors, whereas HL-60 induced with a combination of RA and DIA has 5,400 receptors per cell. HL-60 induced with RA and DIA plus 1 μmol/L dexamethasone have 25,000 receptors per cell and have greater chemotactic activity than HL-60 induced with the combination of RA and DIA. Thus, differentiation of HL-60 to cells with many properties of normal phagocytes can be induced in vitro by physiologic substances.

 MATERIALS AND METHODS

**Materials.** RA, DEX, FMLP, nitroblue tetrazolium (NBT), bovine insulin, human transferrin, and cytochalasin B were obtained from Sigma Chemical Co (St Louis, MO). N,N-dimethylformamide (DMF), human T cell line (HTCL), human T cell–derived lymphokine (DIA), human transferrin, insulin, NADPH oxidase, NADPH oxidase, NADPH oxidase, NADPH oxidase, and TPA are not the same even though the same respiratory enzyme, NADPH oxidase, is activated. Thus, the reaction pathways for O$_2^-$ production in response to FMLP and TPA are not the same even though the same respiratory enzyme, NADPH oxidase, is activated. Thus, the reaction pathways for O$_2^-$ production in response to FMLP and TPA are not the same even though the same respiratory enzyme, NADPH oxidase, is activated.

Chemotaxis, the directed migration of cells along a chemical gradient, is another important function of phagocytic cells and is required for the accumulation of effector cells at sites of inflammation. Phagocytic cells exhibit directed migration towards a variety of chemoattractants including N-formyl peptides, and the presence of surface chemotactic peptide receptors is essential for this activity. Thus two functions of phagocytic cells, O$_2^-$ production and chemotaxis, can be dependent on chemotactic peptide receptors.

Earlier studies showed increases in both the chemotactic peptide levels and chemotaxis of HL-60 induced with either DMF or DMSO and that the glucocorticoid dexamethasone (DEX) increases chemotactic peptide receptor expression on differentiating HL-60 cells. It was also shown that HL-60 cells induced by RA, with or without DEX, had an increase in the number of FMLP receptors and that RA blocked the induction of these receptors by DMF or DMSO. These latter findings were of major interest because RA, of the many agents that induce differentiation of HL-60, has probably the most promise of being of use in the clinic. This is because it is active in vitro at physiologic concentrations, induces differentiation of fresh human leukemia cells in primary culture, and has been effective on patients with acute promyelocytic leukemia (APL). The prospects that RA may produce mature phagocytes deficient in receptor-mediated O$_2^-$ production and chemotaxis prompted the present investigation on the expression and functional activity of the chemotactic peptide receptors in HL-60 induced with a combination of a physiologic concentration of RA and the lymphokine DIA.
from Sigma Chemical Co, St Louis, Mo. Cytochalasin B was stored at -20°C in ethanol at a concentration of 10 mg/mL. DMF was from Aldrich Chemical Co, Milwaukee, Wis. TPA was obtained from Pharmacia P-L Biochemicals, Inc, Milwaukee, Wis, and was stored at -20°C in acetone at a concentration of 200 μg/mL. FMLP (phenylalanine-ring-2-3-4H(N)) (48.3 Ci/mmol) was from NEN, Boston.

Cells. HL-60 was maintained in suspension culture in RPMI 1640 (Advanced Biotechnologies, Inc., Silver Spring, Md) supplemented with 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) and 10% (vol/vol) fetal bovine serum (GIBCO, Grand Island, NY). The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air and split every week. Cell counts were determined by a Coulter Counter (Coulter Electronics, Hialeah, Fla), and viability was estimated by trypan blue dye exclusion. The HL-60 cell line was negative in tests for mycoplasma infection.

Induction of differentiation of HL-60. HL-60 was induced to differentiate with RA, DMF, and a T-cell-derived lymphokine (DIA). HL-60 cells growing exponentially were harvested by centrifugation and resuspended at a density of 2.5 to 3.0 x 10^6/mL in a serum-free nutrient medium supplemented with 5 μg of insulin/mL and 5 μg of transferrin/mL. After resuspension of HL-60 in this defined medium, induction of differentiation was initiated by adding various concentrations of RA, DMF, and DIA in the presence or absence of DEX. After incubation for four to five days as indicated, the capacity of the cells to reduce NBT in response to TPA or FMLP, expression of FMLP receptors, and chemotactic activity were studied. RA and DEX were dissolved in ethanol and diluted into the culture medium so that the final concentration of ethanol was less than 0.1%.

Assay for NBT reduction. The capacity of HL-60 to reduce NBT was assessed and expressed in two different ways: quantitatively as nanomoles of formazan produced per 10^6 cells and qualitatively as the percentage of formazan-positive cells. Viable cells (10^6) were harvested by centrifugation (400 g, seven minutes) and resuspended in 0.5 mL of RPMI 1640 containing 20% fetal bovine serum. This cell suspension was mixed with an equal volume of phosphate-buffered saline (PBS, 1.5 mmol/L KH2PO4, 8.1 mmol/L Na2HPO4, 136.9 mmol/L NaCl, pH 7.2) containing 1 mg of NBT/mL and either 200 ng of TPA/mL or various concentrations of FMLP. The reaction mixture was incubated at 37°C and the reaction was terminated by cooling in an ice water bath. A 0.5-mL portion of the reaction mixture was applied to a microsample filtration device with 1.5 mmol/L MgCl2, 0.5 mmol/L CaCl2, 0.5 mg bovine serum albumin/mL, 1 mmol/L Na2HPO4, and 50 μmol/L L-phenylalanine) at 25°C for 45 minutes. At the end of the incubation period the reaction mixtures were cooled in an ice water bath, and 0.3 mL of cold reaction buffer was added to each reaction mixture. The reaction mixtures were filtered through glass fiber filters, and the filters were washed thoroughly with cold reaction buffer. The filters were dried and then counted in a β-liquid scintillation counter. All results are expressed as specific binding, defined as the difference between 3H-FMLP binding in the absence and in the presence of 10 μmol/L unlabeled FMLP. Scatchard plot analysis of 3H-FMLP binding was performed with the LIGAND computer program.27

Chemotaxis. Chemotaxis was assayed as described3 with a 48-well microchemotaxis chamber (Neuro Probe, Inc, Cabin John, Md) and with polyvinylpyrrolidone-free polycarbonate filters (10 μm thick, 5-μm pores) (Nucleopore Corp, Pleasanton, Calif). The lower compartments of the wells were filled with 35 μL of chemotaxis buffer (Hanks' balanced salt solution supplemented with 10 mmol/L HEPES) containing various concentrations of FMLP. The upper compartments of the wells were filled with 50 μL of a cell suspension (2 x 10^6 viable cells/mL of chemotaxis buffer). The upper and lower compartments were separated by the filter. The chamber was incubated for 45 minutes at 37°C in a humidified atmosphere of 5% CO2 in air. After incubation, the cells on the upper surface of the filter (nonmigrated side) were removed by gently scraping the filter with a wiper blade. The filters were fixed in methanol and stained with Wright-Giemsa. The number of cells that migrated to the underside of the filter was evaluated with an Optomax System 4 Image Analyzer (Optomax, Inc, Hollis, NH).

DIA. Olsson et al.30,32 reported that mitogen-stimulated peripheral blood human mononuclear cells released polypeptides (differentiation inducing factor(s)) that induce the maturation of HL-60. Breitman et al.33 proposed the more general term, differentiation inducing activity or DIA, because more than one polypeptide has this activity.34 DIA was prepared from the conditioned medium of the T lymphocyte cell line HUT-102 and purified through the diethyl aminoethyl (DEAE) ion-exchange chromatography step essentially as described.6 This preparation had a specific activity of approximately 14,000 U/mg protein and is devoid of other lymphokines such as colony-stimulating activity, interleukin 2, and γ-interferon.8,35 One unit of DIA is defined as an amount per milliliter of serum-free defined medium containing 10 mmol/L RA that causes an increase of one in the percentage of NBT-positive HL-60 cells during a four-day incubation.

RESULTS

Growth and adhesiveness of HL-60 during induction with RA plus DIA. HL-60 cells growing in the presence of both RA and DIA change morphologically from round cells to cells with irregular shapes. This change is time dependent and is observed as early as day 1. By day 2 adhesive cells are observed. At day 4, in cultures grown in the presence of RA and DIA, 35% of the cells are adherent (Table 1). Untreated

Table 1. The combination of RA and DIA Promotes Adhesion of HL-60 Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Cells (x 10^6/mL)</th>
<th>Attached Cells (x 10^6/mL)</th>
<th>Percentage of Attached Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>10.9</td>
<td>0.18</td>
<td>1.6</td>
</tr>
<tr>
<td>10 mmol/L RA</td>
<td>10.3</td>
<td>0.19</td>
<td>1.9</td>
</tr>
<tr>
<td>DIA, 80 U/mL</td>
<td>12.7</td>
<td>0.24</td>
<td>1.9</td>
</tr>
<tr>
<td>10 mmol/L RA + DIA, 80 U/mL</td>
<td>8.4</td>
<td>2.94</td>
<td>35.0</td>
</tr>
</tbody>
</table>

Cells (3 x 10^6/mL), in a total volume of 2.5 mL of defined medium, were grown in wells of a 6-well dish for four days. The medium containing the suspended cells was removed, and the surface of the dish was washed gently with PBS. Attached cells were removed with a cell scraper.
cells and cells grown in the presence of RA alone or DIA alone are not adherent.

**FMLP- and TPA-stimulated NBT reduction of HL-60 induced by either RA, DMF, or RA plus DIA.** The report of Skubitz et al. showed that HL-60 induced with DMF had an increased concentration of FMLP receptors whereas HL-60 induced with RA had no increase and in fact may have had a decrease. In normal human granulocytes both FMLP and TPA stimulate oxidative metabolism with the production of $O_2^-$ as measured by the reduction of ferricytochrome c. We reasoned that FMLP-stimulated $O_2^-$ production, as measured by the reduction of NBT, would correlate with the concentration of FMLP receptors on HL-60 induced under different conditions. DMF, in a dose-dependent manner, induces HL-60 to differentiate to cells that reduce NBT in response to either TPA or FMLP (Fig 1). There is a greater stimulation with TPA than with either 100 nmol/L or 1 mmol/L FMLP. A greater stimulation of oxidative metabolism by TPA than by FMLP has also been observed for normal human granulocytes. In contrast to DMF, only a small percentage of RA-induced cells reduce NBT in response to FMLP even though the population is quite active in response to TPA (Fig 2). A peak response to FMLP occurs with cells induced with approximately 100 nmol/L RA; concentrations of RA greater than 100 nmol/L are inhibitory.

The response of DMF-induced and RA-induced HL-60 to FMLP stimulation of NBT reduction appeared at this stage of our study to correlate qualitatively with the reported relative levels of chemotactic peptide receptors. The finding that HL-60 induced with a combination of RA and DIA actively reduced NBT when stimulated with FMLP indicated that these cells had increased concentrations of FMLP receptors (Fig 3). In the presence of a physiologic concentration of RA (10 nmol/L) there is a DIA concentration-dependent increase in the percentage of cells reducing NBT in response to either TPA or FMLP, with the response to TPA being greater than to FMLP. Cells incubated with DIA alone or RA alone were essentially inactive to stimulation by FMLP. Cells incubated with DIA alone responded to TPA with values for differentiation of approximately 8% (Fig 3).

**Quantitation of formazan production.** In the aforementioned experiments, a qualitative NBT assay was used that determines the percentage of cells in a population that have a microscopically determined level of cell-associated formazan. This assay does not give information on how much formazan is produced except in subjective terms. To obtain this information we have recently adapted (unpublished observations, 1984) the quantitative NBT test of Baehner and Nathan to measure the quantity of NBT that is reduced. This assay, as with the more commonly used reduction of ferricytochrome c, gives information on the activity of the total population of cells. The data in Fig 4 show that HL-60 grown for four days in the presence of a combination of 10 nmol/L RA and various concentrations of DIA have a greater capacity to reduce NBT in response to stimulation by FMLP than either control cells, cells incubated with 10 nmol/L RA alone, or cells grown with DIA alone. In addition, exposure of these cells to cytochalasin B
Expression of FMLP receptors. The increased response to FMLP of HL-60 cultured in the presence of RA and DIA suggested that these cells had increased concentrations of FMLP receptors. Measurements of the specific binding of FMLP show that HL-60 grown for four days in the presence of RA and DIA have a much greater capacity to bind FMLP than untreated cells or cells grown with 300 nmol/L RA (Fig 5). In separate experiments incubation with DIA alone did not result in an increase of FMLP binding (data not shown). In agreement with the findings of Brandt et al. and Skubitz et al. for DMF- and DMSO-induced cells, the addition of 1 μmol/L DEX to the induction medium results in a marked increase of specifically bound FMLP (Fig 5). From a Scatchard plot analysis (Fig 6) there are 5,400 receptors per cell for cells induced with RA plus DIA and an increase of approximately fivefold to 25,000 receptors per cell for cells induced with added DEX. The calculated K_D values are 19.73 nmol/L and 11.37 nmol/L, respectively. Thus, treatment with DEX results in an increased number of chemotactic peptide receptors with an increased affinity for FMLP.

It has been reported that DEX has little effect on DMF-induced or DMSO-induced differentiation of HL-60 assessed by cell morphology or by the ability of the cells to reduce NBT when stimulated by TPA. However, FMLP-stimulated NBT reduction was not examined. NBT reduction in response to FMLP was measured in HL-60 cells induced for four days in the presence of 10 nmol/L RA and...
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Fig 6. Scatchard plot analysis of FMLP binding to HL-60 cells. Data from Fig 5 for the conditions of growth in the presence of 10 nmol/L RA and DIA (80 U/mL) with () and without (O) DEX were analyzed with the LIGAND computer program. The lines were drawn from the computed values for Kd and receptor concentration. The insert is an enlargement of the data for the RA plus DIA condition.

100 U DIA/mL with and without 1 μmol/L DEX. It was found that at FMLP concentrations greater than 100 nmol/L only approximately 1.2-fold more formazan was produced by cells induced in the presence of DEX (data not shown). In a two-tailed t test this difference had a P value of .05 and is not statistically significant. These results may indicate that a minimum receptor concentration of less than 5,400 per cell is required for maximum chemotactic peptide stimulation of O2 production.

Chemotaxis of HL-60. HL-60 induced by RA plus DIA exhibits chemotactic peptide concentration-dependent migration (Fig 7). Cells induced in the presence of DEX are more active. The percentage of total cells migrating in response to the optimal concentration of 100 nmol/L FMLP increased from 26% for cells induced in the absence of DEX to 47% for cells induced in the presence of 1 μmol/L DEX. This difference has a P value of .01 and is considered significant. However, most if not all of the increased response in DEX-treated cells is probably a result of the increased nondirected migration observed in the absence of FMLP (Fig 7). Untreated cells, cells induced with RA alone (Fig 7), and cells cultured with DIA alone (data not shown) were essentially unresponsive to FMLP.

DISCUSSION

RA induces HL-60 to differentiate to cells with granulocytelike morphology and with the ability to express reactions associated with the respiratory burst, such as O2 production, NBT reduction, and an increased hexose mono-phosphate shunt (HMPS) activity. However, although these HL-60 cells actively produce O2 in response to TPA (measured by NBT reduction), they are relatively inactive in response to FMLP, a potent chemotactic peptide (Fig 2). In contrast, HL-60 induced to differentiate with DMF is active in reducing NBT in response to either TPA or FMLP (Fig 1). This difference in the response of HL-60 to FMLP was expected from previous studies showing that the number of chemotactic peptide receptors on HL-60 is increased by treatment with DMF or DMSO and does not change or is decreased by RA treatment.

A lower response to FMLP than to TPA is observed for HL-60 induced with increasing concentrations of DMF (Fig 1). A similar difference in the response to these soluble mediators has been found for normal human granulocytes for which evidence has been presented that it is a consequence of different transductional mechanisms operating under different controls and is not due to an activation of different respiratory enzymes. The low response of RA-induced HL-60 to FMLP (Fig 2) is explained by the very low levels of FMLP binding to these cells (Fig 5). These results are in agreement with Skubitz et al who, in addition, found that RA concentrations greater than 10 nmol/L blocked induction by DMF of FMLP receptors. Therefore, the increase followed by a decrease in the percentage of cells reducing NBT in response to 100 nmol/L and 1 μmol/L FMLP (Fig 2) may be the sum of inductive and inhibitory effects of various RA concentrations. At low concentrations of RA (10 nmol/L) there is primarily induction, whereas at higher concentrations (>100 nmol/L) the inhibitory effect dominates. This inhibitory effect of RA appears to be confined to FMLP receptor-mediated O2 production since TPA-stimulation was quite active (Fig 2). Thus, it appears that HL-60 induced with RA develops the transductional...
mechanism that can be stimulated by TPA but does not express the transductional mechanism that is stimulated by FMLP because of a deficiency of chemotactic peptide receptors. In a related study, Stendahl et al. 22 have reported recently that RA-induced HL-60, compared with DMSO-induced HL-60, is deficient in receptors for FMLP and in both FMLP-stimulated calcium uptake and FMLP-stimulated O₂⁻ production measured by ferricytochrome c reduction. In addition, these workers found that 500 nmol/L RA inhibited DMSO-induced increases in these parameters. IgG-mediated phagocytosis and leukotriene B₃-stimulated calcium uptake were not affected by RA, and C₃b-mediated phagocytosis was partially decreased by RA. Thus, pharmacologic concentrations of RA result in a maturation of HL-60 to cells that are deficient in some characteristics of mature normal phagocytes. The mechanism by which RA inhibits the development of FMLP receptors is not known.

Although induction of HL-60 with RA alone yields cells that are deficient in chemotactic peptide receptor concentration and function, our results indicate that the combination of a T cell–derived lymphokine (DIA) and a physiologic concentration of RA (10 nmol/L) induces HL-60 to differentiate to cells with an increased number of FMLP receptors (Figs 5 and 6). These receptors are functional as shown by FMLP-dependent O₂⁻ generation (Figs 3 and 4) and FMLP-directed migration (Fig 7). Pretreatment of these cells with cytochalasin B increased FMLP-stimulated NBT reduction by approximately 1.5-fold (Fig 4). This effect is seen also with normal human granulocytes. 36

There have been two reports on the effect of DEX on the properties of FMLP receptors expressed on HL-60 induced by DMF or DMSO. 5,21 These studies indicate that DEX induces a three- to fourfold increase in receptor number without a significant change of affinity. Our results on RA plus DIA agree with these reports regarding receptor number but indicate that there is a twofold increase in the affinity of the chemotactic peptide receptor induced in the presence of DEX (Fig 6). In normal human monocytes there are two populations of monocytes in regard to chemotactic activity and expression of FMLP receptors; one subpopulation migrates to FMLP and has saturable binding sites for FMLP. The other subpopulation does not migrate and exhibits little chemotactic peptide binding. 23 In contrast to monocytes, migrating and nonmigrating populations of normal human granulocytes show identical binding characteristics for FMLP. 33 We have not studied whether there are subpopulations with heterogeneous chemotactic activity and receptor expression in the monocytic/macrophagic HL-60 cells induced by RA plus DIA.

Chemotaxis is an integrated function of phagocytic cells, consisting of various properties such as adhesiveness, orientation, presence of chemotractrant receptors, and directed mobility. Several groups have shown that HL-60 induced by DMF and DMSO exhibits an increase of chemotactic activity. 15,18,19,24 In this study we have shown that HL-60 cells induced by RA in combination with DIA exhibit not only expression of FMLP receptors but also chemotactic activity (Fig 7). Furthermore, induction in the presence of DEX increases both the number of receptors and chemotactic activity (Figs 6 and 7). Alteri and Leonard 35 found that normal human peripheral monocytes exhibit a maximal chemotactic response at concentrations of 10 nmol/L to 100 nmol/L FMLP. In contrast, the highest chemotactic activity of normal granulocytes is at 1 μmol/L FMLP 33 a concentration that markedly inhibits chemotaxis of monocytes. HL-60 induced by RA plus DIA, with and without DEX, exhibited maximal chemotaxis at concentrations of 10 nmol/L to 100 nmol/L FMLP, and 1 μmol/L FMLP was inhibitory (Fig 7).

Recently, there has been interest in the possibility that differentiation inducers may have use in the treatment of some malignancies. This concept is predicated on the belief that some malignancies are a result of a block in differentiation that if relieved would result in a more differentiated and therefore more benign malignancy. As a concept for therapy this approach holds the further promise that induction of differentiation could not only relieve the tumor burden but also increase the number of functional cells, the absence of which is, at least for some malignancies, a major complication. HL-60 has been a useful model system in the search for substances that are active as inducers of differentiation. Of the many compounds that induce differentiation of HL-60, RA has probably the most promise of being of use in the clinic. This is because it is active at physiologic concentration in vitro, 3 has been shown to induce differentiation of fresh human leukemia cells in primary culture, 33,23,36,37 and has been reported to be effective on patients with APL. 24,35 However, while RA as a sole agent and at pharmacologic concentrations of approximately 1 μmol/L may be a good therapeutic agent, experiments in vitro indicate that the combination of RA and either cyclic adenosine monophosphate–inducing agents or lymphokines act synergistically to induce differentiation of HL-60 as well as other myelomonocytic cell lines. 3,8 In addition, combinations of RA and DIA synergistically induce differentiation of fresh human leukemia cells in primary culture. 4 To the extent that it is possible, results in vitro should suggest treatments in vivo. To this end we have been interested in determining what induction conditions result in the most normal mature HL-60.

Previous studies have indicated that combinations of RA and the lymphokines DIA or γ-interferon differentiate HL-60 to monocytelike cells. 33 These cells exhibit immunophagocytosis, increases in F₇ receptors, reduction of NBT, 5'-nucleotidase activity, nonspecific esterase activity, and antibody-dependent cellular cytotoxicity. One of the more important functions of the normal phagocytic cell is its ability to seek out and kill invading organisms. Our results show that HL-60 induced with a combination of 10 nmol/L RA and DIA mature to cells that show adherence (Table 1) and increases in FMLP receptors that are functional for both O₂⁻ production and directed migration. We feel that it is important that this differentiation occurs in response to two physiologic substances and that one of the components of this combination, RA, is at the physiologic plasma concentration of approximately 10 nmol/L. 7 The physiologic concentration of DIA is unknown, but it is probable that this substance plays a role, along with RA and other factors, in normal hematopoiesis.
ACKNOWLEDGMENT

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A combination of a T cell-derived lymphokine differentiation-inducing activity and a physiologic concentration of retinoic acid induces HL-60 to differentiate to cells with functional chemotactic peptide receptors

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