Retinoic Acid Cooperates With Tumor Necrosis Factor and Immune Interferon in Inducing Differentiation and Growth Inhibition of the Human Promyelocytic Leukemic Cell Line HL-60

By Giorgio Trinchieri, Mark Rosen, and Bice Perussia

In this study, we analyzed the effect of tumor necrosis factor (TNF) on retinoic acid (RA)-induced myeloid differentiation of the promyelocytic HL-60 leukemia cell line. We show that low concentrations of the two substances, almost inactive in inducing differentiation when used separately, induce differentiation when added simultaneously to the cell cultures. Cells simultaneously expressing both monocyte/macrophage phenotype (typically induced by TNF) and granulocyte characteristics (typically induced by RA) are induced by a combination of the two factors, indicating that TNF and RA potentiate each other’s activity. The results obtained using immune interferon (IFN-γ) in combination with the two inducers suggest that the mechanism of action of TNF and IFN-γ are possibly different. The inhibitory effect of RA on the expression of HLA class I antigens and of the high-affinity Fc receptor is potentiated by TNF but completely reversed by rIFN-γ.

EUKEMIA-DERIVED human myeloid cell lines, such as the promyelocytic HL-60, are often used as models to study terminal differentiation of myelomonocytic cells. Dimethylsulfoxide (DMSO), retinoic acid (RA), and other substances induce HL-60 cells to differentiate along the myeloid lineage, whereas 12-O-tetradecanoyl phorbol-13-acetate (TPA), 1,25-dihydroxyvitamin D₃, and leukocyte products contained in medium conditioned from phytohemagglutinin (PHA)-activated leukocytes (PHA-CM) induce them to differentiate into cells with characteristics of monocytes/macrophages. We demonstrated that PHA-CM in which immune interferon (IFN-γ) is neutralized with specific antibodies or antibodies from which IFN-γ has been removed by immune affinity chromatography is less potent than untreated PHA-CM in inducing differentiation of human myeloid cell lines and fresh myeloid leukemia cells. IFN-γ, however, exists in PHA-CM in concentrations insufficient to account for its differentiation-inducing activity, and other factors have been detected in IFN-γ-depleted PHA-CM that synergize with IFN-γ to induce differentiation.

Recently, purification and cloning of the genes for tumor necrosis factor (TNF) and lymphotoxin (LT) have allowed more detailed studies of the biology of these cytotoxins. The two cytotoxins also appear to mediate regulatory effects on various cell types, e.g., they act as growth factors on fibroblasts and regulate proliferation, differentiation, and functions of myelomonocytic cells at all stages of differentiation. In particular, we demonstrated that a soluble factor that inhibits myeloid and erythroid colony formation in vitro is produced by natural killer (NK) cells and has characteristics of TNF, and that LT and IFN-γ, produced by mitogen-activated T cells, act synergistically to block colony formation. We also showed that cytotoxins TNF or LT at concentrations on the order of 10⁻¹⁴ mol/L induce monocytic differentiation of human myeloid cell lines and act synergistically with IFN-γ.

The possibility of using inducers of differentiation for the therapy of leukemias or other tumors has recently received considerable interest. Of the many agents that induce differentiation of HL-60 cells, RA has until now been considered one of the inducers of possible therapeutic use because it is active in vitro at physiologic concentrations, induces differentiation of fresh leukemia cells in primary cultures, and is effective in vivo on patients with acute promyelocytic leukemia. Cooperation between suboptimal concentrations of RA and the differentiation inducer(s) contained in PHA-CM in inducing differentiation of HL-60 cells have been reported. LT and low levels of TNF (G. Trinchieri, M. Rosen, and B. Perussia, unpublished observations) are contained in PHA-CM and, at least in part, are responsible for its differentiation-inducing activity, synergistic with IFN-γ, present in the PHA-CM. In the present study, we analyzed the combined effect of TNF, LT, and RA on the differentiation of HL-60 cells. Concentrations of the two substances, almost inactive by themselves, induced most HL-60 cells to differentiate when added simultaneously to the cultures. The combined effect of the two substances powerfully induced cells simultaneously expressing both monocyte/macrophage markers, typically induced by TNF, and granulocyte characteristics, typically induced by RA.

MATERIALS AND METHODS

Monoclonal antibodies. Monoclonal antibodies B9.8, B52.1, B33.1, and B137.17 were produced and characterized in our laboratory; OKM1, 5E9, W6.32, and BBM1 were produced from cell lines obtained from the American Tissue Culture Collection (Rockville, MD); 3G8 was kindly donated by Dr. J. Unkeless (Mount Sinai School of Medicine, New York), and KuFc79 was a gift of Dr. P. Gambel (Medical College of Virginia). Antibodies B9.8 (IgM, a) and OKM1 (IgG2b, anti-C3b receptor, b) react with two distinct antigens expressed on all differentiated neutrophilic granulocytes and monocytes and start to be expressed, during differentiation, at the myelocytic and promyelocytic stages, respectively. Antibody B52.1 (IgM, a) reacts with all peripheral blood monocytes and all

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α-naphthyl acetate esterase (ANAE)-positive cells in the bone marrow, and cross-competes for binding with antibody Mo2. Antibody W6.32 (IgG2a) reacts with β2-microglobulin, and antibody B33.1 (IgG2a) reacts with HLA class II antigens. Antibody SE9 (IgG1) reacts with the transferrin receptor. Antibody 3G8 (IgG3) reacts with a surface molecule of mol wt 50,000 to 72,000 identified as the low-affinity receptor for aggregated IgG on neutrophilic granulocytes, NK cells, and macrophages. The mouse monoclonal IgG2a B137.17 is an Ig of unknown specificity selected on the basis of its high-affinity binding, in monomeric form, on neutrophilic granulocytes, NK cells, and macrophages. The binding of B137.17 to cells is Fc fragment dependent, and is identified as the low-affinity receptor for aggregated IgG on neutrophilic granulocytes, NK cells, and macrophages. The mouse monoclonal IgG2a B137.17 is an Ig of unknown specificity selected on the basis of its high-affinity binding, in monomeric form, to the IFN-γ-inducible FcR on monocyte and myeloid cell lines.34 The binding of B137.17 to cells is Fc fragment dependent, and is blocked by monomeric human IgG1 and IgG3 and murine IgG2a and IgG3. Sepharose-linked antibody B137.17 precipitates from human monocytes and myeloid cell lines a molecule of mol wt 70,000, identified as the high-affinity FcR for monomeric IgG. Antibody KuFc79 (IgG2b) reacts with a 42,000-dalton FcR species present on monocytes, granulocytes, and B cells.

Cytokine preparations. Purified human recombinant IFN-γ (rIFN-γ) from Escherichia coli was kindly supplied by Dr H. M. Shepard (Genentech, South San Francisco) and has an antiviral activity titer of 7 × 10^7 U/mg of HeLa cells. Two preparations of human recombinant TNF (rTNF) were used in this study with identical results: one (90% pure, 10^7 U/mg as tested on L-929, a subline, cells) was kindly supplied by Dr J. S. Price (Cetus, Emeryville, CA), and the other (electrophoretically homogeneous, 5 × 10^7 U/mg) was supplied by Dr H. M. Shepard (Genentech).

Induction of myeloid cell lines. HL-60 cells were seeded at 1.5 × 10^6 cells/mL in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 15% fetal bovine serum (FBS). The various inducers of differentiation were present throughout the culture period. All-trans-RA (Sigma) was dissolved in ethanol (0.3 mg/mL).

Cytchemistry. Staining for ANAE was according to Platt. Ability of the cells to reduce nitroblue tetrazolium (NBT, Sigma) was determined by mixing on a microscope slide 1 vol of cells in medium (5 × 10^6 cells/mL) and 1 vol of 1 mg/mL of NBT in NaCl 0.15 mol/L. After a 30-minute incubation at 37°C in a humidified atmosphere, slides were dried, stained with Wright’s-Giemsa, and the percentage of cells containing blue-black formazan deposits was scored from at least 200 cells.

Indirect immunofluorescence, cell cycle analysis, and 3H-thymidine (3H-TdR) incorporation. Indirect immunofluorescence (flow cytometry), cell cycle analysis by staining of DNA using propidium iodide, and 3H-TdR incorporation, were performed as previously described in detail.30

RESULTS

Morphological changes in HL-60 cells cultured with RA and TNF. When cultured in 300 nmol/L of RA for five days, most HL-60 cells acquired the morphology of mature myeloid cells, from myelocytes to polymorphonuclear cells (Fig 1C). Little or no morphological differentiation was observed with 10 nmol/L of RA (Fig 1B). Treatment of HL-60 cells with rTNF (10 U/mL) resulted in minimal morphological changes: TNF-treated HL-60 cells (Fig 1D) became smaller and more irregular, and the proportion of cells with nuclei and primary granules was lower than in control untreated cultures. In cultures with 10 nmol/L of RA and 10 U/mL of rTNF, with or without rIFN-γ, a large proportion of HL-60 cells appeared to have mature myeloid morphology, including polymorphonucleated cells (Fig 1E). Addition of 10 U/mL of rTNF to HL-60 cell cultures in the presence of 300 nmol/L enhanced the formation of cells with mature myeloid morphology: Almost all HL-60 cells on day 5 of culture acquired the morphology of polymorphonuclear granulocytes (Fig 1F).

Induction of differentiation of HL-60 cells by TNF and RA. As previously shown,20 a proportion of HL-60 cells cultured for five days in the presence of TNF expressed the monococyte-specific antigen recognized by antibody B52.1, the myelomonocytic antigen OKM1 at higher density than untreated cells, ANAE activity, and ability to reduce NBT (Fig 2 and Table I). The expression of the myelomonocytic antigen recognized by antibody B9.8 was also increased (not shown), whereas the proportion of cells bearing transferrin-

![Fig 1. Morphology of HL-60 cells cultured in the presence of retinoic acid (RA) and/or recombinant tumor necrosis factor (rTNF).](image-url)
Fig 2. Expression of surface antigens on HL-60 cells cultured with retinoic acid (RA) and/or recombinant tumor necrosis factor (rTNF). The phenotype of HL-60 cells, cultured for five days with the addition of the indicated inducers, was studied using the indicated antibodies. On the histograms, the x axis represents the intensity of green fluorescence on an exponential scale; the y axis represents the number of cells; the thin line histograms represent the fluorescence of control cells in the absence of the monoclonal antibodies; the thick line histograms represent the fluorescence of the cells stained with the indicated monoclonal antibodies. The values in the top right corner of each histogram are the percentages of positive cells, calculated as described in the Materials and Methods section. Results are representative of six experiments with comparable results.

receptor, as detected using antibody 5E9, was slightly decreased (Fig 3). rIFN-γ alone (100 U/mL) had only a minimal effect on the expression of monocytic or myelomonocytic antigens and on ANAE or NBT reduction activities (Figs 2 and 3B, Table 1). Addition of rIFN-γ to TNF potentiated its ability to induce differentiation markers in HL-60 cells; previously reported experiments demonstrated that the two factors act synergistically. Most HL-60 cells were induced by 300 nmol/L of RA to express the myelomonocytic antigens recognized by antibodies OKM1 (Fig 2) and B9.8 (not shown); no induction was observed using 10 nmol/L of RA; however, 10 nmol/L of RA potentiated the ability of rTNF, alone or in combination with rIFN-γ, to induce the two antigens. The number of HL-60 cells reducing NBT was modestly increased by 10 and 300 nmol/L of RA; RA, at both concentrations, acted synergistically with rTNF and rIFN-γ in inducing NBT reduction activity in

Table 1. Induction of ANAE Activity and NBT Reduction Activity in HL-60 Cells Cultured with rTNF, rIFN-γ, and RA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ANAE Positive Cells</th>
<th>NBT Reduction Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (nmol/L)</td>
<td>rIFN-γ (U/mL)</td>
<td>rTNF (U/mL)</td>
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<tr>
<td>---</td>
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<tr>
<td>0</td>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>100</td>
<td>0</td>
</tr>
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</table>

ANAE, α-naphthyl acetate esterase; rTNF, recombinant tumor necrosis factor; rIFN-γ, human recombinant interferon-γ; RA, retinoic acid; NBT, nitroblue tetrazolium.

HL-60 cells were cultured for five days with the indicated inducers and analyzed for ANAE and NBT reduction activities as described. Results are from one representative experiment of four experiments performed with similar results.

Fig 3. Synergistic effect of recombinant tumor necrosis factor (rTNF), human recombinant interferon-γ (rIFN-γ), and retinoic acid (RA) on the expression of the transferrin receptor, detected by antibody 5E9 (A), and of the monocyte-specific antigen detected by antibody B52.1 (B). HL-60 cells were cultured for five days with the indicated concentrations of rTNF in medium containing or not containing RA and rIFN-γ, washed, and tested by indirect immunofluorescence (flow cytometry) for expression of the indicated surface markers. HL-60 cells were cultured in medium containing: neither RA nor rIFN-γ (○); 100 U/mL of rIFN-γ (■); 10 nmol/L of RA (□); 10 nmol/L of RA and 100 U/mL of rIFN-γ (▲); 300 nmol/L of RA (△); and 300 nmol/L of RA and 100 U/mL of rIFN-γ (▲).
HL-60 cells (Table 1). The expression of transferrin receptor as detected by antibody 5E9 (Fig 3A) was decreased by RA, and, more efficiently, by RA and rTNF; no synergism between rIFN-γ and RA in inhibiting transferrin receptor expression was observed. Finally, two monocytic markers, i.e., the antigen recognized by antibody B52.1 (Fig 2) and ANAE activity (Table 1), were not induced by RA alone, but RA, at both 10 and 300 nmol/L, strongly potentiated the ability of rTNF, alone or in combination with rIFN-γ, to induce these markers in HL-60 cells. rTNF, at doses as low as 1 U/mL, significantly potentiated the effect of RA in reducing expression of transferrin receptor (Fig 3A), whereas the effect on the expression of B52.1 antigens was observed at rTNF doses ≥3 U/mL (Fig 3B). The effect on the expression of the two antigens was dose-dependent up to the highest concentration tested (200 U/mL of rTNF) and was potentiated by the presence of 100 U/mL of rIFN-γ (Fig 3). The analysis of these quantitative data demonstrated that the effects of the combination of RA and TNF on surface antigens expression are synergistic. A similar conclusion was derived from dose–response experiments (not shown) on the induction of ANAE activity and NBT reduction activity.

**Cooperation between RA and rTNF in inhibiting proliferation of HL-60 cells.** RA at 300 nmol/L almost completely suppressed ³H-TdR incorporation by HL-60 cells cultured for five days (Table 2). At 10 nmol/L of RA, the inhibition was partial but became almost complete in the presence of rTNF. rIFN-γ did not potentiate the ability of RA to suppress ³H-TdR incorporation in these cultures. Cell cycle analysis of proliferation revealed that almost no HL-60 cells were in S phase on day 5 of culture in the presence of 300 nmol/L of RA, rTNF, and rIFN-γ, but that a proportion of cells was in G2 phase after culture in the presence of 300 nmol/L of RA. At 10 nmol/L of RA, only a modest effect was observed on the distribution of the cells in the cell cycle, but addition of rTNF produced a profound decrease of cells in S phase, with some cells remaining in G2 phase, as was observed at higher RA concentrations.

**Expression of different FcR types and HLA class I antigens on HL-60 cells induced with rTNF, rIFN-γ, and RA.** The results of the analysis of FcR expression on HL-60 cells during differentiation are shown in Table 3. The low-affinity FcR of neutrophils, NK cells, and macrophages recognized by antibody 3G8 was poorly induced by rTNF, rIFN-γ, or RA alone, but was significantly induced by the combination of RA with rTNF or rIFN-γ. When HL-60 cells were cultured in the presence of the three inducers together, 3G8 antigen was induced in almost 50% of the cells. The expression of the 42,000-dalton FcR of neutrophils, monocytes, and B cells recognized by antibody KuFc79 was not significantly affected by rTNF and rIFN-γ, but the proportion of positive cells was strongly reduced in cultures with 10 or 300 nmol/L of RA. The inhibition of KuFc79 antigen expression by RA was partially reversed when either rTNF or rIFN-γ was present in the cultures, and the reversal was even greater when both were present. Expression of the high-affinity FcR for monomeric IgG, detected by binding of monoclonal IgG2a B137.17, was strongly enhanced by rIFN-γ but not by rTNF (Table 3). rTNF, however, potentiated the effect of rIFN-γ in inducing the receptor. RA, at 10 or 300 nmol/L, strongly suppressed the expression of the high-affinity FcR; rTNF potentiated the inhibitory effect of RA on induction of this FcR, whereas rIFN-γ completely reversed it.

Both rTNF and rIFN-γ increased expression of HLA class I antigens (as detected by antibody W6.32) and β₂-microglobulin (as detected by antibody BBM1) on HL-60 cells when used alone; a much better induction was observed in most but not all experiments when a combination of the two cytokines was used (not shown). RA at 300 but not at 10 nmol/L decreased expression of HLA class I antigens on HL-60 cells. rTNF, which by itself increases HLA class I antigen expression on HL-60 cells, potentiated the inhibitory effect of 300 nmol/L of RA and decreased HLA class I antigens expression in the presence of 10 nmol/L of RA. Unlike rTNF, rIFN-γ strongly increased HLA class I antigen expression in the presence of either 10 or 300 nmol/L of RA.

**DISCUSSION**

RA is a potent inducer of myeloid differentiation in vitro of promyelocytic cell lines and of fresh leukemic cells from patients with acute promyelocytic M3 leukemia. The
human histiocytic cell line U937 is also induced to differentiate by RA, but the human erythromyeloid leukemic cell line K562 and the myeloblastic leukemic cell line KG1 are not. RA, however, is a potent inhibitor of clonal growth not only of the HL-60 line, but also of the KG1 cell line that is not induced to differentiate by it, and of leukemic cells not induced to differentiate by RA, but the human histiocytic (CML) or from chronic myelogenous leukemia (CML) or from acute myelogenous leukemia (AML) patients of M1, M2, and M3 stages. The concentrations of RA active in vitro in inducing differentiation of fresh leukemic cells or leukemia-derived cell lines as well as those inducing inhibition of clonal growth are in the order of $10^{-9}$ to $10^{-8}$ mol/L and are obtainable in vivo without prohibitive side effects. In addition, RA is toxic to normal hematopoietic precursor cells, but, on the contrary, significantly enhances the growth of normal hematopoietic colonies. Other potent inducers of differentiation of myeloid cells such as TNF have, unlike RA, high systemic toxicity and are potent inhibitors of proliferation of both normal and leukemic hematopoietic progenitor cells, as well as of other cell types. These activities limit their therapeutic potential for treatment of leukemias or other malignancies. The synergistic interaction of RA with other differentiation-inducing factors offer the promising possibility to induce differentiation and growth inhibition of leukemic cells at concentrations of either factor that might not affect normal precursor cells or have a generalized toxic effect.

RA is strongly synergistic with TNF in inducing differentiation of HL-60 cells, even at concentrations of $10^{-9}$ mol/L which, in the absence of TNF, are ineffective in inducing differentiation. The combination of $10^{-9}$ mol/L of RA and $10^{-7}$ mol/L of TNF halts growth of HL-60 cells and induces expression of differentiation markers in almost all cells. An at least additive effect of IFN-γ and RA in inducing differentiation of U937 cells has been reported by Gulberg and colleagues. The synergy of IFN-γ with RA is not as great as that of TNF and RA in inducing differentiation or growth inhibition of HL-60 cells, but the combination of the three factors often induces the most complete differentiation. When TNF is added to HL-60 cells cultured with doses of RA able to induce myeloid differentiation ($3 \times 10^{-7}$ mol/L), the generation of mature myeloid cell types is not prevented and the induced cells, although expressing specific monocytic markers such as ANAE and B52.1 antigen, have typical mature myeloid morphology. When TNF is added with $10^{-8}$ mol/L of RA, a concentration unable to induce morphological maturation of HL-60 cells, mature myeloid cell types are induced in the cultures. Thus, not only does RA potentiate the monocytic differentiation induced by TNF, but TNF potentiates the myeloid differentiation induced by RA, and cells with intermediate myelomonocytic characteristics are generated. Analogously, B52.1 monocyte-specific antigen expression is induced on HL-60 cells during the myeloid differentiation induced by DMSO but not by RA, and intermediate myelomonocytic characteristics are induced in a subset of chronic myelogenous leukemia cells by PHA-CM and IFN-γ. This lineage ambiguity is not peculiar to leukemic cells, because similar results have been obtained with normal bone marrow immature myeloid cells when forced to differentiate in vitro.

The interplay between TNF, IFN-γ, and RA in regulating expression of differentiation markers on HL-60 cells is best illustrated by the effect on HLA class I antigens and on the different types of Fc receptor. Both TNF and IFN-γ increase expression of HLA class I antigens, as previously described with other cell types. The mechanism of action of the two cytokines is probably different, however, as suggested by results obtained in experiments in which HL-60 cells were tested with RA added. RA alone drastically decreases the expression of HLA class I antigens and TNF potentiates this inhibitory effect, whereas IFN-γ completely reverses it, inducing high expression of HLA class I antigens on most cells. The increased expression of these antigens during monocytic differentiation and their decreased expression during myeloid differentiation parallel the high and low expression of HLA class I antigens on mature monocyte and granulocytes, respectively. The pattern of high-affinity FcR induction on HL-60 cells by the differentiation agents is

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**Table 3. Expression of FcR on HL-60 Cells Treated With rTNF, rIFN-γ, and RA**

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>None</th>
<th>rTNF</th>
<th>rIFN-γ</th>
<th>rTNF + rIFN-γ</th>
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<th>rTNF</th>
<th>rIFN-γ</th>
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<tr>
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<td>RA 10 nmol/L</td>
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<td>3G8</td>
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<tr>
<td>(72)†</td>
<td>8.7</td>
<td>10.7</td>
<td>18.5</td>
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<td>25.1</td>
<td>15.1</td>
<td>40.0</td>
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<tr>
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<td>72.4</td>
<td>77.7</td>
<td>31.5</td>
<td>55.0</td>
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</tr>
<tr>
<td>B137.17</td>
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<td>44</td>
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<td>44</td>
<td>49.1</td>
<td>11.8</td>
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<td></td>
<td>(33)</td>
<td>(13)</td>
<td>(120)</td>
<td>(85)</td>
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*Abbreviations as in Table 1.
†Percentage of positive cells as detected by indirect immunofluorescence (flow cytometry). The threshold fluorescence intensity at which 99% of the cells without first antibody added were negative was determined on HL-60 cells cultured in the absence of differentiation inducers and used for all the samples.
‡Numbers in parentheses indicate average intensity of fluorescence (on an arbitrary linear scale from 1 to 200) of positive cells.
almost identical to that of HLA class I antigens. Again, the induction of high-affinity FcR during monocytic differentiation of HL-60 cells and its decreased expression during myeloid differentiation parallel the distribution of the FcR on normal differentiated cells, i.e., high-level expression on monocytes and lack of expression on granulocytes, although in the latter cell type the high-affinity FcR can be induced by IFN-γ.³³ The low-affinity FcR present on neutrophils but not on monocytes, as detected by antibody 3G8,³¹ is expressed at higher levels on HL-60 cells when the myeloid differentiation induced by RA is potentiated by TNF. Expression of a third type of FcR recognized by antibody KuFc7³² during the differentiation of HL-60 cells is independent of that of both the high-affinity and low-affinity FcR detected with IgG2a B137.17 and with antibody 3G8, respectively.

The synergism of TNF with RA is similar to that reported by Olsson and colleagues for PHA-CM³² and for the differentiation-inducing factor purified from the HUT-102 cell line.⁴² LT and, in some preparations, TNF, are responsible, at least in part, for the differentiation-inducing activity, synergistic with RA, present in PHA-CM,⁴³ whereas the differentiation factor produced by HUT-102 cells seems to be distinct from both LT and TNF on the basis of its biochemical characteristics.⁴⁴ Understanding of the biochemical mechanism of the synergistic effect between TNF and RA might furnish important information on the regulation of growth and differentiation of normal and leukemic hematopoietic cells. To evaluate the therapeutic potential of the combined effect of the two inducers, it will be important to evaluate whether the combined substances affect normal hematopoietic precursor cells in vitro and whether leukemic cell types, not affected by RA or TNF alone, are induced to differentiate and lose their proliferative ability with the combined treatment.

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