

TUMOR NECROSIS FACTOR AND LYMPHOTOXIN INDUCE DIFFERENTIATION OF HUMAN MYELOID CELL LINES IN SYNERGY WITH IMMUNE INTERFERON

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Leukemia-derived human myeloid cell lines, such as the promyelocytic HL-60 (1) and ML3 (2), and the histiocytic U937 (3), are often used as models to study terminal differentiation of myelomonocytic cells. DMSO (4), retinoic acid (RA)¹ (5), and other substances induce HL-60 cells to differentiate along the myeloid lineage, whereas 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) (6), 1,25-dihydroxyvitamin D₃ (7), and leukocyte products contained in medium conditioned from PHA-activated leukocytes (PHA-CM) (8) induce myelomonocytic cells to differentiate into cells with characteristics of monocyte/macrophages. PHA-CM is also a potent inducer of differentiation of bone marrow myeloid cells (9) and of leukemic cells from patients with chronic (CML) (9) or acute (AML) myelogenous leukemia (9, 10). Cell lines and leukemic cells treated with PHA-CM stop proliferating, undergo morphological changes, and acquire phenotypic and functional characteristics of differentiated cells (9, 11, 14). Partial monocytic differentiation of human myeloid cell lines (9, 15–19) is induced by IFN- γ , a potent inducer of Fc receptors (FcR) for monomeric IgG (15), and of class II MHC antigens (9). We have shown (9) that immune interferon (IFN- γ) is as active as PHA-CM to induce monocytic differentiation of CML cells, and that treatment of PHA-CM with antibodies to IFN- γ abolishes almost completely its differentiation-inducing activity. Also, PHA-CM in which IFN- γ is neutralized with specific antibodies or 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 (18). However, IFN- γ is present in PHA-CM in concentrations insufficient to account for its differentiation-inducing activity (18), and other factors have been detected in IFN- γ -depleted PHA-CM that synergize with IFN- γ to induce differentiation (18). Synergism between PHA-CM and RA to induce differentiation of HL-60 cells has also been demonstrated (20).

With the exception of IFN- γ , the differentiation-inducing factors in PHA-CM

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¹ *Abbreviations used in this paper:* ANAE, α -naphthyl-acetate esterase; CM, conditioned medium; CSF, colony-stimulating factor; FPLC, fast-protein liquid chromatography; G-CSF, granulocyte CSF; LT, lymphotoxin; NBT, nitroblue tetrazolium; PdBU, phorbol-12,13-dibutyrate; PI, propidium iodide; RA, retinoic acid; TNF, tumor necrosis factor; TPA, 12-*o*-tetradecanoyl phorbol-13-acetate.

are poorly characterized. Olsson et al. (21) reported that Con A-induced CM obtained from human lymphocytes contained two distinct molecular species able to induce differentiation: a species of M_r 25,000 that coelutes with the colony-stimulating activity and a species of M_r 40,000 not associated with it. A differentiation-inducing factor of M_r 36,000–58,000 was partially purified from PHA-CM by Leung and Chiao (22), and a factor of M_r 46,000, acting on HL-60 cells, and in synergy with RA, on U937 cells, was purified from supernatant of the T lymphocyte line HUT-102 by Olsson et al. (23). In mice, granulocyte colony-stimulating factor (G-CSF) induces differentiation of the myelomonocytic leukemic line WEHI-3B (24). Human CSF β , a 30 kD molecule with properties similar to mouse G-CSF, was recently purified from the CM of the human bladder carcinoma cell line 5637 (5637-CM) and shown to induce differentiation of both mouse WEHI-3B and human HL-60 cells (25).

Tumor necrosis factor (TNF) and lymphotoxin (LT) are two partially homologous factors originally described on the basis of their cytotoxic or cytostatic effects on tumor cell lines: TNF as a tumor cytotoxic factor present in the serum of animals injected with endotoxin (26), and LT as a class of cytotoxic-cytostatic substances released by lymphocytes upon antigenic or mitogenic stimulation (27, 28). The cytotoxic/cytostatic effects of both cytokines are potentiated by IFN- γ (29, 30). Recently (31–35), purification and cloning of the genes for TNF and LT have allowed more detailed studies of the biology of these factors. The two cytotoxins also appear to mediate regulatory effects on various cell types, e.g., they act as growth factors on fibroblasts (36, 37) and regulate proliferation, differentiation, and functions of myelomonocytic cells at all stages of differentiation (38). In particular, we have demonstrated that a soluble factor that inhibits myeloid and erythroid colony formation in vitro is produced by NK cells (39) and has characteristics of TNF, and that LT and IFN- γ , produced by mitogen-activated T cells, act synergistically to block colony formation (40). We² and others (41) observed that TNF, LT, and INF- γ act, alone or synergistically, to activate human polymorphonuclear cell functions.

The presence of high concentrations of LT (40), and in some cases, of TNF (our unpublished observation) in PHA-CM; the synergistic effect of TNF and LT with IFN- γ , reminiscent of that observed with the differentiation-inducing factor in PHA-CM; and the original observation of Metcalf (42) that mouse postendotoxin serum induces differentiation of WEHI-3B cells prompted us to investigate the possibility that TNF and LT induce differentiation of myeloid cells. In this paper we demonstrate that TNF and LT induce monocytic differentiation of human myeloid leukemic cell lines and that their effect is potentiated synergistically by IFN- γ .

Materials and Methods

Cell Lines. All cell lines were grown in RPMI 1640 medium (Flow Laboratories, Inc., Rockville, MD). The human promyelocytic cell line HL-60 was maintained in medium supplemented with 15% FCS (Gibco, Grand Island, NY); the human promyelocytic ML3,

² B. Perussia, M. Kobayashi, M. E. Rossi, I. Anegon, and G. Trinchieri. Immune interferon enhances functional properties of human granulocytes. Role of Fc receptors and effect of lymphotoxin, tumor necrosis factor and granulocyte-macrophage colony-stimulating factor. Submitted for publication.

the histiocytic U937, the B lymphoblastoid RPMI 8866, the bladder carcinoma 5637, and the mouse mastocytoma line P815y were maintained in medium supplemented with 10% FCS. All cell lines used in this study were mycoplasma-free.

Monoclonal Antibodies. mAbs B9.8, B52.1, B33.1, and B137.17 were produced and characterized in our laboratory; OKM1, 5E9, 4F2, W6.32, and BBM1 were produced from cell lines obtained from the American Type Culture Collection (Rockville, MD); 3G8 was kindly donated by Dr. J. Unkeless (Mount Sinai Hospital, New York), and KuFc79 was a gift of Dr. P. Gambel (Medical College of Virginia, Richmond, VA). Antibodies B9.8 (IgM) (43) and OKM1 (IgG2b; anti-C3bi receptor) (44) 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) (14, 18) reacts with all peripheral blood monocytes and all α -naphthyl acetate esterase (ANAE)-positive cells in the bone marrow, and crosscompetes for binding to monocytes with antibody Mo2 (11, 45). Antibody B33.1 (IgG2a) (46) reacts with class II HLA antigens. Antibody 5E9 (IgG1) (47) reacts with the transferrin receptor and antibody 4F2 (IgG2a) (48) with a dimeric structure (40 and 80 kD) present on monocytes, activated lymphocytes, and most proliferating cell types. Antibody 3G8 (IgG1) (49) reacts with a surface molecule of M_r 50,000–72,000, 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, to the IFN- γ -inducible FcR on monocyte and myeloid cell lines.² 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 B137.17 precipitates from human monocytes and myeloid cell lines a molecule of M_r 70,000, identified as the high-affinity FcR of monomeric IgG. Antibody KuFc79 (IgG2b) (50) reacts with a 42 kD FcR species present on monocytes, granulocytes, and B cells.

Conditioned Medium. Human peripheral blood mononuclear cells (separated on a density gradient) were irradiated (20 Gy) and incubated (10^6 cells/ml) in RPMI 1640 medium supplemented with 1% PHA-M (Wellcome Research Laboratories, Beckenham, England) and 1% FBS for 72 h at 37°C. Cell-free supernatant fluid was used as crude PHA-CM preparation. Most preparations of PHA-CM contained 100–1,000 U/ml IFN- γ , and 10–40 U/ml LT. 5637-CM was obtained as cell-free supernatant fluid from 48-h culture of the bladder carcinoma cell line 5637 in RPMI 1640 medium containing 0.2% FBS. 5637-CM contained both pluripotent- and G-GSF activities (25, 51), but was devoid of IFN- γ , LT, or TNF activity.

LT and TNF Assays. Serial dilutions of samples were added to 3×10^4 mouse L-929 cells (α subline) per well in 96-well flat-bottomed microtiter plates in the presence of 1 μ g/ml actinomycin D (Calbiochem Behring Corp., La Jolla, CA). The concentrations (U/ml) of cytotoxin for each sample were defined as the reciprocal of the dilution that produced 50% cytopathic effect, as evaluated microscopically by cell rounding and detachment.

Purification of LT from RPMI 8866 Supernatant. RPMI 8866 cells (10^6 cells/ml) were cultured at 37°C, 5% CO₂ for 48 h in serum-free RPMI 1640 containing 100 ng/ml of phorbol-12,13-dibutyrate (PdBu) (Chemicals for Cancer Research, Inc., Eden Prairie, MN) (52). The cell-free supernatants were harvested and concentrated 100-fold using a Diaflow hollow-fiber cartridge (HIP 10–20; Amicon Co., Danvers, MA) and a stirred cell (PM10 membrane; Amicon Co.). The protein concentrate was applied to a metal-chelating Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column charged with CuSO₄·5H₂O (3 mg/ml) then equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. Unless otherwise cited, all the buffers used for purification contained 0.1% PEG 8000 and 0.1 mM PMSF (Sigma Chemical Co., St. Louis, MO). After washing with the same buffer, LT was eluted with 50 mM EDTA containing 1.0 M NaCl and dialyzed against 15 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl (PBS). The sample was then applied to a lentil lectin-Sepharose 4 B (Pharmacia Fine Chemicals) column, eluted, and applied to a Mono Q (Pharmacia Fine Chemicals) column connected

to a fast-protein liquid chromatography (FPLC) apparatus (Pharmacia Fine Chemicals). LT was eluted with a linear (0–0.25 M) NaCl gradient, and active fractions were concentrated and loaded on an Ultragel AcA44 (LKB Produkter, AB, Bromma, Sweden) column. After loading again on a lentil lectin–Sepharose 4 B column, and extensively washing with PEG-free PBS, LT was eluted with 200 mM α -methylmannoside in PEG-free PBS, and applied to a Pro RPC HR5/10 (Pharmacia Fine Chemicals) FPLC column. Final elution of LT was with a linear (0–50%) ethylene glycol gradient in PBS.

For iodination, proteins were mixed for 20 min at 22°C with 50 μ l of 0.1 M Tris-HCl buffer, pH 7.4, and 0.5 mCi of carrier-free Na¹²⁵I in a polypropylene tube coated with 2 μ g of 1,3,4,6-tetrachloro-3 α -6 α -diphenylglycouril. The reaction mixture was transferred to another tube and mixed with 100 μ l of NaI (2 mg/ml). Radiolabeled protein was separated on a Sephadex G25 column and eluted with 0.2% BSA in PBS. SDS-PAGE was performed on 12.5% polyacrylamide vertical slab gels. ¹⁴C-labeled mol wt standards were obtained from New England Nuclear (Boston, MA). After electrophoresis, gels were dried and labeled protein bands were detected by autoradiography.

Cytokine Preparations. Purified human rIFN- γ from *E. coli* was kindly supplied by Dr. H. M. Shepard (Genentech, Inc., South San Francisco, CA) and has an antiviral activity titer of 7×10^7 U/mg on HeLa cells. Human rTNF (90% pure, 10^7 U/mg on L-929, α subline, cells) was kindly supplied by Dr. J. S. Price (Cetus Corp., Emeryville, CA).

Induction of Myeloid Cell Lines. HL-60 cells were seeded at 1.5×10^5 cells/ml in RPMI 1640 supplemented with 15% FCS; ML3 and U937 were seeded at 10^5 and 0.75×10^5 cells/ml, respectively, in medium supplemented with 10% FCS. The various inducers of differentiation were present throughout the culture period. Cultures were performed for the indicated period in 24-well flat-bottomed plates or in plastic tissue culture flasks, depending on the volume.

Cell Morphology and Cytochemistry. Cell morphology was examined on May-Grunwald-Giemsa-stained cytocentrifuge preparations (Cytospin centrifuge; Shandon Southern Instruments, Inc., Sewickley, PA). Staining for ANAE was according to Platt (53). Ability of the cells to reduce nitroblue tetrazolium (NBT) (Sigma Chemical Co.) 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 NBT in NaCl, 0.15 M. After a 30-min incubation at 37°C in a humidified atmosphere, slides were dried, stained with Wright-Giemsa, and the percentage of cells containing blue-black formazan deposits was scored out of at least 200 cells.

Antibody-dependent Cell-mediated Cytotoxicity (ADCC). A 3-h ⁵¹Cr-release assay was carried out by incubating various numbers of effector cells in round-bottom microtiter plates with 10^4 ⁵¹Cr-labeled P815y cells sensitized with a 1:200 dilution of a rabbit anti-P815y antiserum. Supernatants were collected, and percent specific cytotoxicity was calculated as previously reported (54). Lytic units (LU) were calculated from cytotoxicity curves obtained at different E/T cell ratios using a modified van Krogh's equation as described (54). One LU₄₅ represents the number of effector cells required to mediate 45% specific ⁵¹Cr release from 10^4 target cells in the 3-h assay.

Indirect Immunofluorescence and Cell Cycle Analysis. Cells were sequentially incubated with appropriate dilutions of the different mAbs (always in the presence of 5% human serum, except for antibody B137.17) and with FITC-labeled goat F(ab')₂ anti-mouse Ig for 30 min at 0°C. The proportion of cells reactive with the different antibodies was scored on a Cytofluorograf 50H (Ortho Diagnostics, Westwood, MA) connected to a Data General MP/200 microprocessor. To determine the proportion of positive cells, the threshold fluorescence intensity was used at which 99% of the cell population treated with control supernatant from the parental myeloma cells and FITC-anti-mouse Ig antibodies were negative. Mean fluorescence intensity is reported in arbitrary units, from 1 to 200, and corresponds to the mean fluorescence channel of the positive cells. Fluorescence intensity allows comparison of results only within each experiment; in order to allow determinations on a sufficient number of cells, mean fluorescence intensity is reported only when the percentage of positive cells is >5%.

For cell cycle analysis, cells previously stained or not with mAbs by indirect immunofluorescence were fixed in ice-cold 70% ethanol for 10 min, spun, and resuspended at 22°C

in PBS containing RNase (62 $\mu\text{g}/\text{ml}$) (Sigma Chemical Co.) and propidium iodide (PI) (Sigma Chemical Co.) (0.2 $\mu\text{g}/\text{ml}$ for simultaneous analysis of antigenic phenotype and cell cycle, 10 $\mu\text{g}/\text{ml}$ for analysis of cell cycle only). Cells were analyzed with the Cytofluorograf 50H, and the proportion of cells in G_0/G_1 , S, and G_2/M was determined using an integration program on the MP/200 microprocessor.

[^3H]TdR and [^3H]Uridine Incorporation and Autoradiography. Cells were cultured in 96-well flat-bottomed microtiter plates (10^5 cells/ml, 200 $\mu\text{l}/\text{well}$) for the indicated periods of time, and pulsed for the last 6 h of culture with 1 $\mu\text{Ci}/\text{well}$ of [^3H]TdR or [^3H]uridine (New England Nuclear); cells were collected on glass-fiber with an automated cell harvester (Skatron, Sterling, VA), and the cell-associated radioactivity was assayed by liquid scintillation. For autoradiography, cells were pulsed for 1 h with 5 $\mu\text{Ci}/\text{ml}$ of [^3H]TdR, smears were prepared on microscope slides, covered with NBT-2 emulsion (Eastman Kodak Company, Rochester, NY), and developed after a 2-d exposure.

Chemiluminescence. HL-60 cells in Krebs buffer (10^6 cells/ml) were added (0.5 ml) to scintillation vials, followed by addition of 0.5 ml of Krebs buffer containing 4×10^{-6} M 3-aminophthalhydrazide (Luminol; Sigma Chemical Co.), and dark-adapted for 1 h at 22°C. TPA (10^{-7} M, Chemicals for Cancer Research Inc.) was then added, and chemiluminescence was measured at 22°C in a liquid scintillation counter in the out-of-coincidence mode (55); cpm of each sample were recorded at 2-min intervals. Luminol alone gave a background of $2.5 \pm 1.4 \times 10^3$ cpm.

Phagocytosis-induced Protein Iodination. This assay was carried out according to the method of Clark and Szot (56) modified for microtiter plates. 50 μl of cell suspension (5×10^6 cells/ml in Krebs buffer containing 400 nCi/ml ^{125}I (Amersham Corporation, Arlington Heights, IL) were added, in triplicate, to 50 μl of a suspension of serum-activated zymosan (2 mg/ml) in Krebs buffer containing 50 mg/ml BSA (Sigma Chemical Co.) as a source of protein. After 1-h incubation at 37°C, the reaction was stopped by adding 30 μl of a solution of 1 mM sodium metabisulfite and 10 mM KI, and proteins were precipitated upon addition of 30 μl of 10% TCA. The suspension was layered on silicone oil in microsediment tubes (Sarstedt, Princeton, NJ) and spun at 13,000 g for 2 min; the tips of the tubes containing the TCA-precipitable proteins were cut and pellet-associated cpm were counted in a gamma counter. The number of ^{125}I nmoles incorporated in the TCA-precipitable proteins was calculated from the amount of ^{125}I and from the absolute number of cpm added per well. Background precipitable cpm in control wells containing the reaction mixture but no cells were subtracted, and were always <0.1% of input cpm.

Results

Purification of LT. LT was purified from serum-free conditioned medium of PdBU-stimulated RPMI 8866 cells (52). LT produced by RPMI 8866 cells bound tightly to metal-chelating Sepharose charged with Cu^{2+} ions and to lentil lectin-Sepharose. This protein had M_r of ~40,000–60,000, by gel filtration. The final preparation was purified ~340-fold with a 3% recovery of activity and a sp act of 6.5×10^6 U/mg of protein. From 11 liters of cell supernatant, 2 μg of purified LT were obtained. SDS-PAGE of LT with or without DTT revealed a major protein band of M_r 20,000 consistent with the M_r of LT purified from RPMI 1788 as reported by Aggarwal et al. (31), and two minor protein bands of undetermined origin at 60,000 and 18,000 M_r positions (Fig. 1, lane 2). The LT purified from RPMI 8866 cells also bound to mAb produced against RPMI 1788 LT (not shown). The 20,000 M_r form of LT is probably a biologically active degradation product of a native 25,000 form, as shown by Aggarwal et al. (32) for RPMI 1788 LT.

Induction of Differentiation of HL-60 Cells by LT and TNF and Synergism with IFN- γ . A proportion of HL-60 cells cultured for 5 d in the presence of LT and

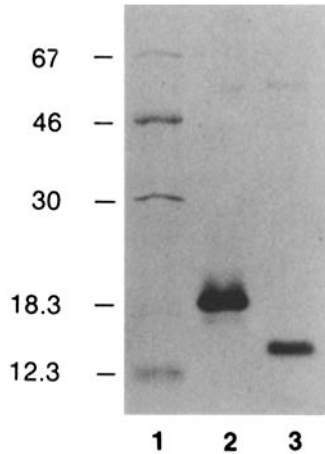


FIGURE 1. Electrophoresis of purified LT and rTNF. LT, purified from supernatant of RPMI 8866 cells, and rTNF were labeled with ^{125}I and run on an SDS-12.5% polyacrylamide slab gel under reducing conditions. Bands were detected by autoradiography. Lane 1, mol wt markers; lane 2, LT; lane 3, rTNF.

TNF expressed the monocyte-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). The expression of the myelomonocytic antigen recognized by antibody B9.8 was also increased (not shown), whereas the proportion of cells bearing transferrin receptor, as detected with antibody 5E9, was slightly decreased (Fig. 2D). HL-60 cells cultured in the presence of LT and TNF also mediated low levels of ADCC (Fig. 2A). rIFN- γ alone (100 U/ml) induced ADCC activity in HL-60 cells, but had only a minimal effect on the expression of monocytic or myelomonocytic antigens and on ANAE or NBT reduction activities (Fig. 2). Addition of rIFN- γ to LT and TNF potentiated their ability to induce differentiation markers in HL-60 cells (Fig. 2). The proportion of cells induced to express surface differentiation antigens was additive when rIFN- γ and LT or TNF were simultaneously present; however, in the range of all concentrations tested, simultaneous addition of rIFN- γ and cytotoxins induced a higher proportion of cells than that induced by each substance used separately. 4 U/ml of rIFN- γ were sufficient to significantly potentiate the ability of TNF to induce ADCC (Fig. 3A) or ANAE activity (Fig. 3B) in HL-60 cells, and the effects of IFN- γ and TNF were synergistic, as shown in the isobologram analysis in the insets in Fig. 3. A significant proportion of the cells expressed differentiation markers after 24-h culture in the presence of rIFN- γ and TNF, and this proportion increased up to day 4-6, when maximal differentiation was usually observed under the culture conditions used (not shown).

HL-60 cells treated with TNF and rIFN- γ were smaller and more irregular and vacuolated than control cells, and nucleoli and primary granules, present in undifferentiated cells, were usually not detectable. A small proportion of the cells became plastic-adherent and acquired macrophage-like morphology; giant multinucleated cells were often observed. Cell viability in the cultures was not

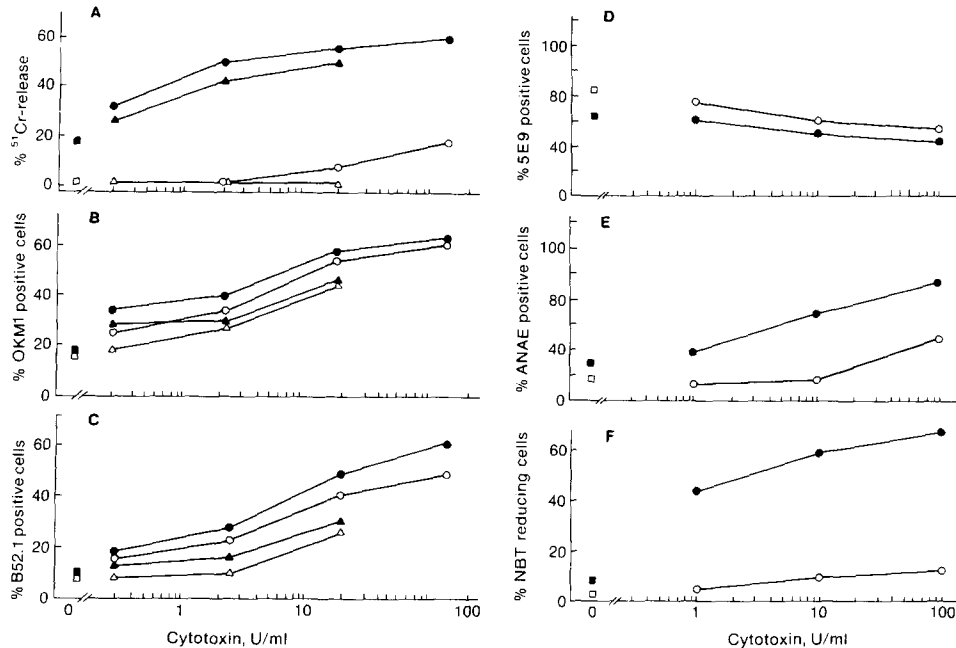


FIGURE 2. Induction of differentiation of HL-60 cells by rTNF, LT, and rIFN- γ . HL-60 cells were incubated in the presence of the indicated concentrations of cytotoxins with or without 100 U/ml rIFN- γ , and assayed for: A, ADCC activity (E/T ratio, 50:1); B, reactivity with antibody OKM1; C, reactivity with antibody B52.1; D, reactivity with antibody 5E9; E, ANAE activity; F, ability to reduce NBT. Cells were cultured in medium containing: \square , no cytokine; \blacksquare , 100 U/ml rIFN- γ ; \circ , rTNF; \bullet , rTNF and 100 U/ml rIFN- γ ; Δ , LT; \blacktriangle , LT and 100 U/ml rIFN- γ .

affected by the treatment and cell recovery from the cytokine-treated cultures was 70–90% of that from the untreated control cultures.

Effect of TNF and IFN- γ on Differentiation of HL-60, ML3, and U937 Cell Lines. The effect of 5 U/ml rTNF, in combination or not with 100 U/ml rIFN- γ , in inducing various differentiation markers in two other human myeloid cell lines was tested and compared with the effect of PHA-CM and 5637-CM, used at 25% final concentration. PHA-CM contains IFN- γ , LT, CSF activity, and possibly other differentiation-inducing factors. 5637-CM lacks IFN- γ , LT, and TNF, and its ability to induce differentiation of cell lines has been attributed to its CSF content. PHA-CM and 5637-CM induced ADCC activity in all three cell lines. rTNF and rIFN- γ , acting synergistically, induced higher levels of ADCC activity in HL-60 cells than did PHA-CM or 5637-CM at the concentrations used. rTNF and rIFN- γ induced lower ADCC activity than PHA-CM in both ML3 and U937 cells (not shown). Class II HLA antigens, detected by antibody B33.1, were induced in ML3 cells only by rIFN- γ and by IFN- γ -containing PHA-CM (Table I). rTNF (5 U/ml) and 5637-CM did not induce class II antigens; however, rTNF potentiated the induction by rIFN- γ . rTNF, used at 5 U/ml, induced a significant proportion of HL-60, but not of the other cell lines, to express the monocyte-specific antigen recognized by antibody B52.1 (Table I). rIFN- γ (100 U/ml) was a poor inducer of this antigen, but it potentiated the

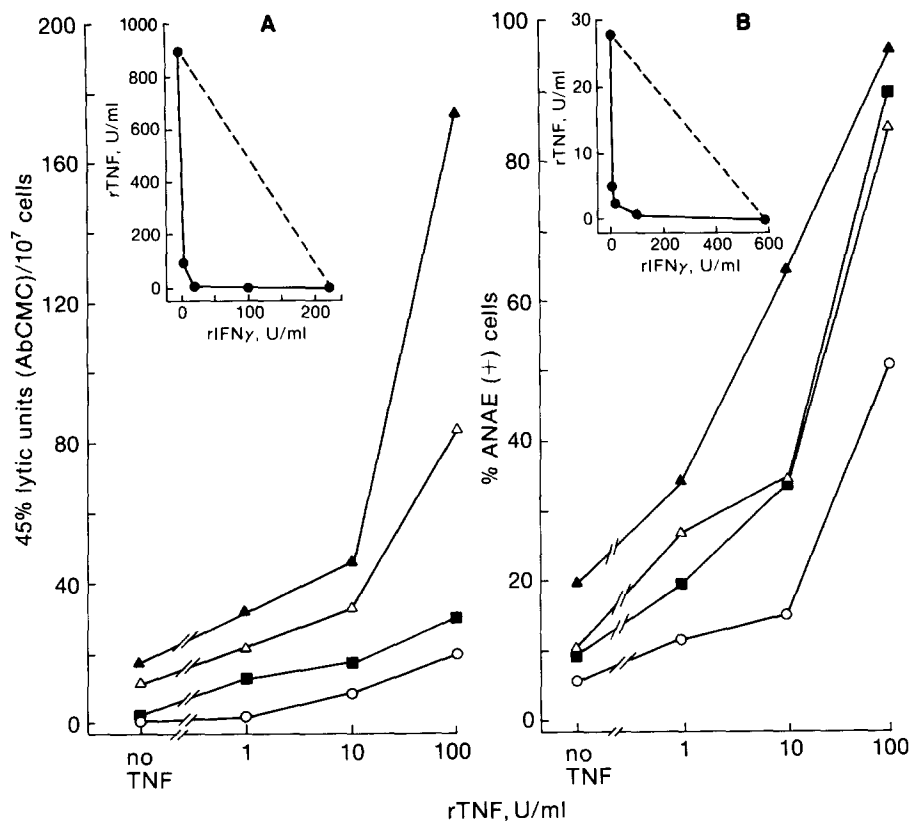


FIGURE 3. Synergism between rTNF and rIFN- γ in inducing ADCC activity (A) and ANAE activity (B) in HL-60 cells. HL-60 cells were cultured for 5 d in the presence of the indicated concentrations of rTNF and: ○, no rIFN- γ ; ■, 4 U/ml rIFN- γ ; △, 20 U/ml rIFN- γ ; or ▲, 100 U/ml rIFN- γ . At the end of the incubation, the cells were washed and tested for ADCC against antibody-sensitized P815y cells, and for ANAE. The insets in the figures are isobolograms depicting synergism between rTNF and rIFN- γ . The filled circles represent doses of cytokines that induce 30 LU per 10^7 HL-60 cells (A) or that render 30% of HL-60 cells positive for ANAE (B). The broken lines represent the hypothetical amounts of rTNF and rIFN- γ required to cause the same effects assuming the interaction was additive.

induction by rTNF. Expression of the receptor for C3bi, recognized by antibody OKM1, was increased in all three cell lines upon incubation with any of the inducers. Expression of OKM1 antigen was induced on HL-60 cells by rTNF and rIFN- γ more efficiently than by either PHA-CM or 5637-CM, which were more effective inducers on U937 cells (Table I). Expression of OKM1 antigen was induced on ML3 cells by a combination of rTNF and rIFN- γ more effectively than by 5637-CM, but almost as effectively as by PHA-CM.

Proliferation and Cell Cycle Analysis of Differentiation Cells. ~1–2 U/ml rTNF were sufficient to inhibit [³H]TdR incorporation by 50% on day 5, and rIFN- γ potentiated the inhibition in all three cell lines. [³H]TdR incorporation by HL-60 cells was increased several-fold upon 24-h culture in the presence of rTNF, either alone or with rIFN- γ ; this effect was not detectable on day 2, and inhibition of [³H]TdR incorporation occurred at later times (Table II). By contrast, inhi-

TABLE I

Effect of rTNF, rIFN- γ and Other Differentiation Inducers on Surface Antigen Expression by Human Myeloid Cell Lines

Inducer	Percentage cells positive								
	HL-60			ML3			U937		
	B33.1	B52.1	OKM1	B33.1	B52.1	OKM1	B33.1	B52.1	OKM1
None	0.0	0.7	13.6 (85)*	0.0	0.1	6.2 (39)	1.3	0.0	7.0 (46)
rIFN- γ , 100 U/ml	0.0	1.9	15.5 (94)	32.2 (52)	0.9	28.9 (42)	0.8	0.0	16.0 (58)
rTNF, 5 U/ml	0.0	8.4 (106)	29.9 (101)	1.5	0.6	28.9 (49)	0.2	0.0	11.6 (46)
rIFN- γ , 100 U/ml + rTNF, 5 U/ml	2.0	13.2 (130)	36.6 (101)	48.9 (64)	1.9	57.6 (57)	0.8	0.2	32.2 (60)
PHA-CM, 25%	0.0	12.4 (123)	28.9 (97)	10.3 (31)	2.9	70.6 (68)	1.0	1.8	65.7 (111)
5637-CM, 25%	0.0	12.0 (152)	24.2 (113)	1.2	2.1	20.7 (43)	1.2	2.0	45.8 (103)

Cell lines were incubated for 5 d with the indicated inducers and assayed by indirect immunofluorescence for the expression of the antigens recognized by the three mAbs.

* Numbers in parentheses represent the average intensity of fluorescence on the positive cells (arbitrary units from 1 to 200).

TABLE II

Effect of rTNF on Proliferation, Thymidine Uptake and Uridine Uptake in HL-60 Cells

Day	Cells in S/G ₂ /M*		Cells incorporating [³ H]TdR [‡]		[³ H]TdR uptake [§]		[³ H]Uridine uptake [§]	
	No TNF	20 U/ml rTNF	No TNF	20 U/ml rTNF	No TNF	20 U/ml rTNF	No TNF	20 U/ml rTNF
	%				cpm			
1	49	51	27.0	33.0	2,614	7,658	4,658	6,831
2	56	63	33.5	49.5	6,782	7,054	6,804	6,134
3	56	44	41.0	34.5	ND	ND	ND	ND
4	51	43	41.1	21.5	18,258	6,126	18,247	5,929

HL-60 cells were incubated for the indicated periods of time in culture medium with or without 20 U/ml rTNF.

* The proportion of cells in the S/G₂/M phases of the cell cycle was determined by flow-cytometry analysis of cells stained with PI.

[‡] Proportion of [³H]TdR-incorporating cells was evaluated by autoradiography after a 1-h pulse with [³H]TdR (5 μ Ci/ml).

[§] Incorporation of [³H]TdR and [³H]uridine was evaluated on cells cultured in flat-bottomed microtiter plates (200 μ l/well, initial concentration 2×10^4 cells/well).

bition of [³H]TdR incorporation by rTNF in ML3 and in U937 cells was significant at 24 h, and became more dramatic at later times (not shown).

The effect of rTNF on proliferation of HL-60 cells was also analyzed by cell cycle analysis, autoradiography, and incorporation of [³H]uridine (Table II). The number of HL-60 cycling cells (S/G₁/M phases) was slightly increased at 24 and 48 h, and decreased on days 3 and 4 of culture. Analogous results were obtained when the number of cells incorporating [³H]TdR was evaluated by autoradiography: the number of positive cells was increased compared to control cells on days 1 and 2, whereas a decrease of almost 50% was observed on days 3 and 4. The results of [³H]uridine incorporation paralleled those of [³H]TdR, with stimulation of incorporation at 24 h and a decrease on day 4.

The expression of differentiation markers on cells in different phases of the cell cycle was analyzed by flow cytometry, with simultaneous analysis of surface antigens (indirect immunofluorescence) and of cell cycle (PI staining). The results

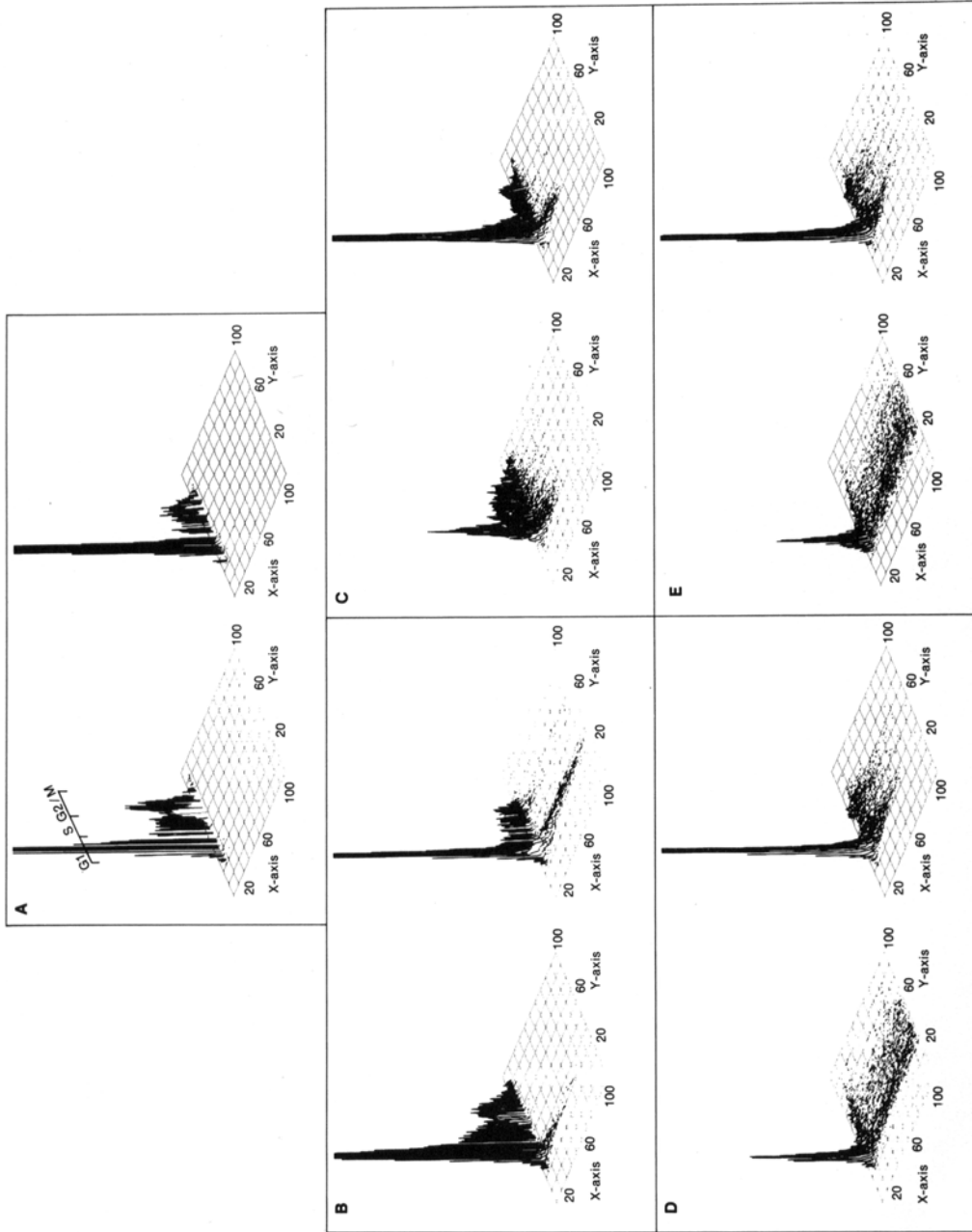


FIGURE 4. Simultaneous analysis of surface antigen expression (indirect immunofluorescence) and cell cycle (PI staining) in HL-60 cells cultured in the presence or absence of rTNF and rIFN- γ . HL-60 cells were cultured for 5 d in the absence (*Left*) or in the presence (*Right*) of 20 U/ml rTNF and 100 U/ml rIFN- γ . A, no first antibody; B, antibody B52.1; C, antibody OKM1; D, antibody 5E9; E, antibody 4F2. x axis green fluorescence (indirect immunofluorescence); y axis red fluorescence (DNA content, PI staining); z axis, cell number.

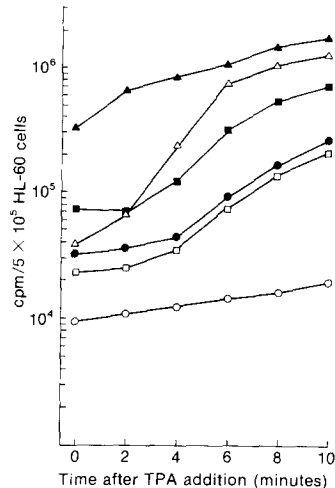


FIGURE 5. Effect of rTNF, LT, and rIFN- γ on the luminol-enhanced chemiluminescence of HL-60 cells. HL-60 cells were cultured for 5 d in the presence of the inducers, washed, and tested for chemiluminescence in the presence of 1.6×10^{-7} M TPA. HL-60 cells were cultured in the presence of: O, culture medium; ●, 100 U/ml rIFN- γ ; Δ , 20 U/ml rTNF; ▲, 20 U/ml rTNF and 100 U/ml rIFN- γ ; □, 20 U/ml LT; ■, 20 U/ml LT and 100 U/ml rIFN- γ .

are illustrated in Fig. 4. Treatment of HL-60 cells with rTNF and rIFN- γ induced a decrease of cycling cells (S/G₂/M) from 40 to 25% on day 5 (Fig. 4A). The monocyte-specific antigen recognized by antibody B52.1 (Fig. 4B) was expressed on a small proportion of cells in G₁ among the noninduced HL-60 cells, but was expressed at high density on a significant proportion of induced cells in G₁ phase. The myelomonocytic antigen recognized by antibody OKM1 (C3bi receptor, Fig. 4C) was expressed in a proportion of uninduced HL-60 cells independent of the phase of the cell cycle, and after induction, became more highly expressed, particularly on cells in the G₁ phase. The transferrin receptor recognized by antibody 5E9 (Fig. 4D), and the antigen recognized by antibody 4F2 (Fig. 4E) were expressed at high density on all uninduced HL-60 cells; after culture with rTNF and rIFN- γ , the intensity of expression of the two antigens decreased on all cells, and a proportion of cells in the G₁ phase did not express the antigens, whereas all cycling cells remained positive.

Effect of TNF, LT, and IFN- γ on Chemiluminescence and Phagocytosis by HL-60 Cells. HL-60 cells did not respond to TPA with luminol-enhanced chemiluminescence (Fig. 5). After 5-d culture in the presence of rIFN- γ , spontaneous and TPA-induced chemiluminescence were increased by 3- and 20-fold, respectively. HL-60 cells cultured in the presence of rTNF and LT alone had high levels of spontaneous and TPA-induced chemiluminescence, which were enhanced when rIFN- γ was present during the culture with either cytokine. HL-60 cells cultured for 5 d with 20 U/ml rTNF and 100 U/ml rIFN- γ showed a 30- and 100-fold increase in spontaneous and TPA-induced chemiluminescence, respectively.

The ability of HL-60 cells to ingest C3-activated zymosan particles was tested using the method of phagocytosis-induced protein iodination (Table III), which evaluates the function of the myeloperoxidase-H₂O₂-halide system in phagocytosing cells. Both rTNF and rIFN- γ increased the phagocytic ability of HL-60

TABLE III
*rTNF and rIFN- γ Induce HL-60 Cells to Mediate Iodination of
 Proteins upon Phagocytosis of Serum-activated Zymosan*

rIFN- γ	¹²⁵ I oxidized* by HL-60 cells given rTNF (U/ml)			
	0	1	10	100
	<i>nmol</i>			
0	0.32	0.78	1.30	1.60
20	1.64	2.19	2.51	2.33
100	2.41	2.94	2.99	2.47

HL-60 cells were cultured for 5 d in the presence of rIFN- γ and rTNF at the indicated concentrations, and assayed for ability to iodinate BSA when stimulated to phagocytize by serum-activated zymosan.

* TCA-precipitable ¹²⁵I oxidized by 2.5×10^5 HL-60 cells during 1-h incubation.

TABLE IV
Expression of FcR on HL-60 Cells Treated with rTNF and rIFN- γ

mAb	Percentage of cells staining positively with mAbs treatment with:			
	None	rTNF	rIFN- γ	rTNF + rIFN- γ
None	1.0	2.0	2.1	3.2
3G8	10.7 (72)*	18.5 (75)	18.7 (87)	25.1 (84)
KuFc79	83.2 (43)	78.2 (44)	72.4 (45)	77.7 (51)
B137.17	83.9 (53)	71.2 (44)	93.4 (104)	94.5 (133)

HL-60 cells were cultured for 5 d in the presence of rTNF (20 U/ml), rIFN- γ (100 U/ml), or a combination of the factors. Percent 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 the average intensity of fluorescence (on an arbitrary scale from 1 to 200) of the positive cells.

cells in a dose-dependent manner. The amount of ¹²⁵I oxidized per cell by HL-60 cells induced by a combination of the two cytokines was approximately half of that oxidized by fresh peripheral blood polymorphonuclear cells (5.3 ± 0.6 nmoles per 2.5×10^5 cells/h).

Expression of Different FcR Types on HL-60 Cells Induced with rTNF and rIFN- γ . The results of the analysis of FcR expression on HL-60 cells during differentiation are shown in Table IV. The low-affinity FcR of neutrophils, NK cells, and macrophages recognized by antibody 3G8 was poorly induced by rTNF or rIFN- γ . The expression of the 42 kD FcR of neutrophils, monocytes, and B cells recognized by antibody KuFc79 was not significantly affected by rTNF and rIFN- γ . 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. However, rTNF potentiated the effect of rIFN- γ in inducing the receptor.

Discussion

In this paper we demonstrate that TNF and LT, two cytotoxins produced by different cell types, are potent inducers of myeloid cell differentiation along the monocytic lineage. HL-60, and to a lesser extent, ML3 and U937 cells, cultured for a few days in the presence of rTNF or LT at concentrations of $\sim 10^{-11}$ M, coordinately express a series of differentiation markers, surface antigens, enzymatic activities, and cellular functions typical of monocyte/macrophages, while concomitantly showing a reduced proliferative ability. Most cells are induced to express the various differentiation markers at high density, and for most markers, the induction is synergistically potentiated by IFN- γ . This terminal differentiation of leukemic cell lines, induced by the synergistic effect of cytotoxins and IFN- γ , closely resembles that induced by lymphokines contained in PHA-CM (8, 14, 18). PHA-CM contains both LT and IFN- γ , and we have shown that synergism between the two lymphokines is required to inhibit proliferation of normal myeloid precursor cells by PHA-CM (40). LT and IFN- γ are both produced by T cells, and we proposed that secretion of the two lymphokines is responsible for hematopoietic colony inhibition *in vitro* by activated T cells, and possibly for bone marrow failure in some pathological conditions (40). Experiments in progress using a sensitive and specific RIA have detected low concentrations of TNF in some, but not all, PHA-CM. We have also observed that PHA and other cell mitogens induce production of TNF by peripheral blood mononuclear cells at early times of culture. Therefore, PHA-CM could contain different factors able to affect cellular differentiation, depending on the culture conditions and on the time of its harvest used in the different studies; this might explain some of the minor discrepancies of the results obtained in different laboratories (18, 21-23).

HL-60 cells, cultured in the presence of TNF or LT alone, are efficiently induced to express some differentiation markers, e.g., myelomonocytic and monocytic antigens. Although IFN- γ alone is not very effective in inducing these surface markers, it potentiates the effect of cytotoxins, although a clear synergistic effect is not observed. Both cytotoxins and IFN- γ induce ADCC activity, ANAE, NBT reduction ability, phagocytosis, and chemiluminescence in HL-60 cells, and the two classes of factors synergize to induce these cellular markers and functions. Cytotoxins alone, at the doses used in this study, do not induce expression of high-affinity FcR or class II MCH antigens on myeloid cells, whereas IFN- γ is very efficient in inducing both types of surface molecules; TNF, however, potentiates this effect of IFN- γ .

We have previously (18) shown that the differentiation-inducing ability of PHA-CM on HL-60 and ML3 cells is due to the synergistic effect of IFN- γ and one or more undefined differentiation-inducing activities. The effect of IFN- γ -depleted PHA-CM is very similar to that of TNF and LT, i.e., significant induction of surface myelomonocytic antigens, modest or no effect on ADCC and ANAE, and no effect on FcR and class II antigen expression (18). The synergistic effect of IFN- γ -depleted PHA-CM and rIFN- γ precisely mimics that between cytotoxins and rIFN- γ described in the present study. The concentrations of both LT and IFN- γ in PHA-CM are sufficient to account for the differentiation-inducing effect of PHA-CM on HL-60 cells. However, differen-

tiation factors other than LT and IFN are probably present in the PHA-CM, as suggested by (a) studies showing heterogeneity of differentiation-inducing factors in PHA-CM (21), (b) the inability of anti-LT antibodies to completely suppress the differentiation-inducing ability of unfractionated PHA-CM (our unpublished results), and (c) the present observation that cytotoxins and IFN- γ are efficient inducers of differentiation of HL-60 and ML3, but less so of U937 cells, whereas PHA-CM effectively induces all three cell lines. 5637-CM, which contains CSF- β but neither cytotoxins nor IFN- γ , is also a potent inducer of differentiation of the three cell lines (25, 57). IFN- γ only modestly potentiated the differentiation induced by 5637-CM (our unpublished observation). Whether CSF- β or other CSF types play a role in the induction of differentiation of cell lines by PHA-CM is still undetermined.

HL-60 cells stimulated with TPA, Ca²⁺ ionophore, and bacterial LPS produce high levels of TNF (35) with 10–20 min of stimulation, suggesting the possibility that HL-60 cells constitutively produce TNF and that differentiation in these cells upon exposure to IFN- γ occurs in response to autocrine stimulation. However, using RIA sensitive to TNF concentrations lower than 10⁻¹² M, we could not detect any TNF produced by HL-60 cells, either constitutively or upon stimulation with IFN- γ . IFN- γ has been shown (58) to induce TNF production by normal peripheral blood mononuclear cells and by fresh bone marrow samples containing these cells. This observation could perhaps explain why IFN- γ , a modest inducer of differentiation of myeloid cell lines (18), is as potent as PHA-CM in inducing monocytic differentiation in fresh preparations of bone marrow cells from both healthy donors or CML patients (9).

The synergism between IFN- γ and cytotoxins is similar to that previously observed for the cytotoxic/cytostatic effects of the cytotoxins (29, 30, 39, 40). IFN- γ induces a twofold increase of surface expression of the TNF receptors in most cell lines (59–61). Such an increase probably accounts, in part, for the synergistic effect between TNF and IFN- γ , but other mechanisms are likely to play a role, as shown by significant synergistic effects on cell lines in which increased expression of TNF receptors is not detected (61). The differential efficiency of IFN- γ in synergizing for the various effects of TNF on HL-60 cells suggests that the synergy between IFN- γ and TNF cannot be explained solely on the basis of an increased TNF receptor expression. The unique ability of IFN- γ to increase the expression of high-affinity FcR on myeloid cells (15) can in part explain the synergism with TNF in inducing ADCC activity in these cells, but this effect does not account for the strong synergism in the induction of FcR-independent markers such as ANAE, chemiluminescence, or NBT reduction. Of the three types of FcR analyzed in this study, the expression of the high-affinity FcR for monomeric IgG is strongly enhanced on HL-60 cells induced to differentiate by rIFN- γ and rTNF, whereas only a minor proportion of cells is induced to express the low-affinity receptor recognized by antibody 3G8 (49). The expression of a third type of FcR, recognized by antibody KuFc79 (50), is not changed during differentiation of HL-60 cells. Although partial correlation is observed between the expression of high-affinity FcR and ADCC mediated by the three cell lines, the exact role played by the three types of FcR in endowing the cells with ADCC activity remains to be determined.

As in HL-60 cells induced to differentiate by PHA-CM (14, 62), the proliferative capacity of myeloid cell lines in the presence of TNF for several days was inhibited, as indicated by a decrease in [^3H]TdR incorporation and in the number of cells in the S/G₂/M phases of the cell cycle. The monocytic surface antigen recognized by antibody B52.1, expressed in a very small proportion of HL-60 cells in G₁ phase, was expressed in a much larger proportion of G₁ cells after induction with TNF and IFN- γ . These results could be interpreted on the basis of a cell cycle-dependent expression of B52.1 antigens. However, we previously (14) observed that, upon induction with PHA-CM, a subset of HL-60 cells stops proliferating and coordinately expresses B52.1 antigen, ANAE activity, C3b receptor, and ability to mediate ADCC. It is more likely that the differentiated cells accumulate in G₁ and express B52.1 antigen; the small proportion of B52.1⁺ cells in G₁ phase in control HL-60 cultures probably represents the small number of cells spontaneously differentiating along the monocytic pathway.

Expression of the transferrin receptor was previously shown (63) to decrease on HL-60 cells induced to differentiate, correlating with the decreased proliferation in the culture. In this paper, we show that the expression of transferrin receptor and of the proliferation-associated antigen recognized by antibody 4F2 decreases on HL-60 cells induced to differentiate by TNF and IFN- γ . A population of cells in G₁ phase, which do not express transferrin receptor or 4F2 antigen, can be identified in induced HL-60 cells, whereas all cells still proliferating in the presence of TNF and IFN- γ express the two antigens.

When [^3H]TdR incorporation and cell cycle analysis were performed on HL-60 cells at early times of culture, a stimulatory effect of TNF was observed. This effect was observed using both high TNF concentrations and doses lower than those inducing differentiation. Anti-TNF antibodies blocked the stimulation effect (not shown), indicating that this was not due to contaminants present in the TNF preparations. These results, showing increased [^3H]TdR incorporation by TNF-treated cells, constitute further evidence that the observed effects of TNF on differentiation of HL-60 cells are not secondary to a generalized toxic effect of the cytotoxin. The results obtained with TNF on HL-60 cells closely resemble those reported by Elias et al. (64), who demonstrated a very similar effect of PHA-CM on HL-60 but not on U937 cells. The strong increase in [^3H]TdR and [^3H]uridine incorporation was paralleled by a modest increase, at 24 and 48 h, in the proportion of cycling cells (PI analysis) and of cells incorporating [^3H]TdR (as analyzed by autoradiography). Thus, as demonstrated by Elias et al. (64) with PHA-CM, the effect of TNF on [^3H]TdR uptake might rest, in large part, on an increased TdR accumulation in the intracellular nucleotide triphosphate pool, and also in an expanded population of cells in active DNA synthesis.

Increased production of cytotoxins and IFN- γ is observed *in vivo* in various pathological situations such as infections, tumor growth, autoimmune diseases, and during immune response. Indeed, these cytokines act directly on the proliferative ability of infectious agents or tumor cells, and indirectly, on the cellular types involved in both adaptive and natural immune resistance to these pathogens. We² (38–40, 65) and others (41) have shown that cytotoxins and IFN mediate complex effects on cellular proliferation, differentiation, and functional

activities of myelomonocytic cells at all stages of maturation, from the committed precursor cells to the terminally differentiated polymorphonuclear cells and macrophages. Other cytotoxin-induced effects are targeted on cell types such as fibroblasts, adipocytes and hematopoietic cells (36, 37, 66). Thus, these soluble mediators might be active at low concentrations in physiological conditions and represent a hormone-like class of substances, that, through suppressive and stimulating synergistic and antagonistic effects, contribute to the homeostasis of the whole organism, and in particular, the hematopoietic system.

Summary

We show that the cytotoxins tumor necrosis factor (TNF) or lymphotoxin (LT), at concentrations of $\sim 10^{-11}$ M induce monocytic differentiation of human myeloid cell lines. After 5 d of culture in the presence of rTNF and LT, a significant proportion of the myeloid cell lines express monocyte differentiation antigens and ANAE activity, and become able to reduce nitroblue tetrazolium (NBT) and mediate low levels of ADCC against tumor target cells. These markers of differentiation, however, are maximally induced when rIFN- γ , at concentrations as low as 4 U/ml, is present simultaneously with the cytotoxins, and the two classes of cytokines act synergistically to induce terminal differentiation. The appearance of monocytic antigens is accompanied by acquisition of morphology and other functional properties of mature monocytic cells, such as chemiluminescence and phagocytosis, and by expression of FcR for monomeric IgG. A decrease in cell proliferation accompanies induced differentiation, and is not due to the cytotoxic properties of TNF or LT, as indicated in simultaneous analysis of surface phenotype and cell cycle. The lack of cytotoxicity of TNF on the HL-60 cell line is also demonstrated by the enhancing effect of TNF on HL-60 cell growth and nucleoside uptake in the first 2 d of culture. These data show that the cytotoxins TNF and LT mediate complex effects on cells of the myelomonocytic lineage and, in synergy with IFN- γ , can fully induce immature myeloid cells to differentiate into cells with phenotypic, functional, and proliferative characteristics of terminally differentiated myelomonocytic cells.

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