Tumor Necrosis Factor and Immune Interferon Synergistically Induce Cytochrome b_{-245} Heavy-Chain Gene Expression and Nicotinamide-Adenine Dinucleotide Phosphate Hydrogenase Oxidase in Human Leukemic Myeloid Cells

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Abstract

Recombinant tumor necrosis factor (rTNF) and rIFN-γ induce in the human myeloid leukemia cell lines HL-60, ML3, and U937 the accumulation of transcripts of the X chromosome-linked chronic granulomatous disease (X-CGD) gene, encoding the 91-kD heavy chain of cytochrome b_{-245}, a component of the NADPH oxidase of phagocytic cells. The gene is induced within 6 h by either cytokine, and its accumulation is observed upon induction with rIFN-γ up to 5 d. The combined effect of the two cytokines is more than additive. rIFN-γ also induces accumulation of X-CGD mRNA in immature myeloid cells from peripheral blood of chronic myeloid leukemia (CML) patients, whereas rTNF has almost no effect. The cells from CML patients constitutively express TNF mRNA, suggesting that endogenously produced TNF may play a role in the effect of rIFN-γ on these cells. rTNF induces X-CGD gene expression in the myeloid cell lines acting, at least in part, at the transcriptional level, as shown in nuclear run-on experiments. The gene encoding the 22-kD light chain of cytochrome b_{-245} is constitutively expressed in the human myeloid cell lines and the accumulation of its transcripts is affected by neither rTNF nor rIFN-γ. rTNF and rIFN-γ act synergistically to induce the cell lines to express the cytochrome b_{-245} heterodimer (as evaluated by its visible spectrum), and to produce NADPH oxidase activity and H_2O_2 upon stimulation with phorbol diesters.

Introduction

Immune IFN-γ induces myeloid cells from normal bone marrow, from chronic myeloid leukemia (CML)[1] patients and from some acute myeloid leukemia (AML) patients to differentiate into cells with characteristics of mature monocyte/macrophages (1). Human myeloid leukemia cell lines are also induced to differentiate to monocyte/macrophages by IFN-γ (2); in this case, however, other factor(s) are required to induce complete differentiation. Among these are tumor necrosis factor (TNF) and lymphotoxin (LT) (3, 4), produced by stimulated leukocytes and acting in synergy with IFN-γ (2). Treatment of the cell lines with leukocyte-conditioned medium (or with TNF/LT and IFN-γ) induces a coordinate expression of surface antigens and functions typical of differentiated cells, closely mimicking the physiological pathway of monocytic differentiation (3, 5, 6). Both IFN-γ and TNF/LT also enhance expression and functions of surface antigens and receptors on terminally differentiated myelomonocytic cells (7–13). It is thus possible that the cytokine-induced expression of genes encoding differentiation markers relevant to maturation or to terminal differentiation of the myeloid cell lines occurs independently from a direct effect of the cytokines on commitment of the cells to a specific lineage.

HL-60 and other myeloid cell lines develop a functional oxidase system and the capability to produce reactive oxygen intermediates (respiratory burst) when treated with various differentiation inducers, including TNF/LT and IFN-γ (3, 14, 15). The precursor of these toxic oxidants, superoxide anion, is formed by a membrane-associated enzyme system, the NADPH oxidase, present only in phagocytes and dormant unless activated by a variety of stimuli such as microbial products, chemotactic peptides or phorbol diesters. NADPH, the physiologic electron source, donates a single electron to molecular oxygen (O_2) to form the superoxide anion (O_2^-). Two components are postulated to operate in this short electron transport chain: a flavoprotein and a cytochrome *b* with an unusual low midpoint redox potential of −245 mV (16, 17) and present only in phagocytic cells. Cytochrome b_{-245} is a heterodimer composed of a glycosylated 91-kD heavy chain and a nonglycosylated 22-kD light chain (18). Cytochrome b_{-245} can be identified on the basis of the typical absorbance spectrum of the heme-carrying heterodimer (19). Heme is probably carried by the 22-kD chain, but both chains are necessary for stable expression of the cytochrome (20). During differentiation of human myeloid cell lines, increased levels of cytochrome b_{-245} were detected in HL-60 cells induced with DMSO (14, 15) and in U937 macrophagic cells induced with phorbol diesters (21) or IFN-γ (22). The 91-kD heavy chain of cytochrome b_{-245} is encoded by a gene, termed X-CGD, residing at chromosomal position Xp21 (23). The gene was originally identified and cloned on the basis of genetic linkage in the majority of patients with X chromosome-linked chronic granulomatous disease (X-CGD), whose phagocytes express no or minimal NADPH oxidase activity (24, 25). The X-CGD gene was shown to be expressed only on phagocytic cells, and on the HL-60 and THP-1 cell lines after induction of maturation with DMSO and IFN-γ, respectively (24, 26, 27). IFN-γ enhances the expression of the X-CGD gene also in neutrophils and monocyte/macrophages from healthy individuals and from

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1. *Abbreviations used in this paper:* AML, acute myeloid leukemia; CML, chronic myeloid leukemia; FBS, fetal bovine serum; HRP, horseradish peroxidase; HVA, homovanillic acid; LT, lymphotoxin; MPO, myeloperoxidase; PGK, phosphoglycerate kinase; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; X-CGD, X chromosome-linked chronic granulomatous disease.

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some X-CGD patients (27–29). The gene encoding the 22 kDa subunit of cytochrome b_{-245} is, instead, constitutively expressed in a variety of cell types. The 22-kD polypeptide appears, however, to be present in stable form only in phagocytic cells (20).

In this paper, we present evidence that not only IFN-γ, in agreement with data previously reported by others (27) using the THP-1 cell line and normal myeloid cells, but also TNF induce transcription and mRNA accumulation of the X-CGD gene encoding the 91-kD chain of cytochrome b_{-245} in three human myeloid leukemia cell lines and rIFN-γ induces accumulation of X-CGD mRNA in cells from CML patients. The combined effect of the two cytokines is more than additive. X-CGD gene accumulation in the cell lines is paralleled by the appearance of cytochrome b_{-245} absorbance spectrum, of inducible NADPH oxidase activity, and of respiratory burst capability.

Methods

Cytokine preparations. Purified human recombinant IFN-γ (rIFN-γ, antiviral titer 7×10 U/mg on HeLa cells) and TNF (rTNF, cytotoxic activity titer 5×10^7 U/mg on actinomycin D-treated L-929, α subline) were kindly supplied by Dr. H. M. Shepard (Genentech Inc., South San Francisco, CA).

Induction of myeloid cell lines. The human promyelocytic leukemia HL-60 (30) and ML3 (31) and the macrophagic U937 (32) cell lines were mycoplasma free and were grown in RPMI-1640 medium (Flow Laboratories, Rockville, MD) supplemented with 10% (ML3 and U937) or 20% (HL-60) fetal bovine serum (FBS; Flow Laboratories) (3). HL-60, ML3, and U937 cells were seeded at 1.5, 1.0, and 0.8×10^5 cells/ml, respectively. Cytokines were added at different times and left in the cultures until the cells were collected after 5 d. In some experiments, HL-60 or ML3 cells were preincubated with 20 μg/ml cycloheximide (Calbiochem-Behring Corp., La Jolla, CA) to block protein synthesis, or were exposed to 5 μg/ml actinomycin D (Calbiochem-Behring Corp.) for various intervals of time after treatment for 24 h with the cytokines to evaluate mRNA half life.

Isolation of fresh and immature myeloid cells. PMN, monocytes, and nonadherent lymphocytes were purified from peripheral blood of healthy subjects as described (9, 33). Myeloid cells were also obtained from peripheral blood of CML patients, as previously described (1). Briefly, mononuclear cells were purified from peripheral blood of four CML patients by density gradient centrifugation on Ficoll/Hypaque and were depleted of monocytes by adherence to plastic. B cells, T lymphocytes, natural killer cells, residual monocytes, metamyelocytes, and more mature myeloid cells were depleted from the nonadherent cell preparations after sensitization with a mixture of monoclonal antibodies and indirect antiglobulin rosetting and density gradient separation (34). The resulting population was composed mostly of myelocytes and more immature myeloid cells.

Northern blot analysis. Cells were collected, washed twice with cold PBS, and lysed with 4 M guanidine isothiocyanate followed by recovery of total cellular RNA by centrifugation through cesium chloride (35). Samples were fractionated on a 1% agarose-6% formaldehyde gel and blotted onto nylon membrane (Nytran; Schleicher & Schuell, Keene, NH). All gels were stained with ethidium bromide to visualize 28S and 18S ribosomal RNA bands, in order to assess the integrity of the RNA and to verify the loaded amounts. The messages for the heavy- (91 kD) and light-chain (22 kD) subunits of cytochrome b_{-245} were detected as a 5- and an 0.8-kb size mRNA, respectively, by hybridization with the 3.5-kb fragment purified from the human X-CGD cDNA (24) inserted in the Kpn/Pst I site of the Gemini 4 vector and with the 0.7-kb Hind III-purified fragment for the light chain (20), both kindly provided by Dr. S. H. Orkin (Children's Hospital Medical Center, Harvard University, Boston, MA). Constitutively expressed phosphoglycerate kinase (PGK) transcripts were detected as a 2-kb mRNA, by hybridization with the pK T218 vector containing the 1.8-kb Pst I fragment of PGK, obtained from the American Type Culture Collection (ATCC) (Rockville, MD) (36). Other cDNA probes used in this work were: human TNF detecting a 2-kb message mRNA, provided by Dr. H. M. Shepard (Genentech), pB_{AE} detecting 28S rRNA, provided by Dr. P. Curtis (Wistar Institute, Philadelphia, PA), human β actin detecting a 1.3-kb mRNA message, provided by Dr. R. Weinmann (Wistar Institute), and HLA-DRα and HLA-B7 provided by Dr. P. Wettstein (Wistar Institute). Probes were labeled using the multiprime DNA labeling system (Amersham Corp., Arlington Heights, IL) with [^{32}P]dCTP to specific activities of $\sim 10^9$ cpm/μg. Hybridization was performed (for 18 h at 42°C) in 5× SSC, 50% formamide, 5× Denhardt's solution, 0.1% SDS, 20 mM Na phosphate buffer (pH 6.5), 0.1 mg/ml denatured salmon sperm DNA, and 10% dextran sulfate, after a 5-h prehybridization incubation. Filters were then washed in 2× SSC, 0.1% SDS at room temperature (twice for 15 min each) followed by 0.1× SSC, 0.1% SDS at 65°C (twice for 30 min each), dried and exposed to Kodak X-Omat x-ray films at −70°C with intensifying screens (Lightning Plus; DuPont Co., Wilmington, DE). Rehybridization of the filters with another probe was performed after washing the membrane for 2 h at 85°C in 20 mM Tris HCl containing 0.1% SDS. Extent of hybridization was quantitated by scanning densitometry of the films with a laser densitometer (Ultraskan XL; LKB Instruments, Inc., Gaithersburg, MD).

Nuclear transcription analysis (run-on assay). This was previously described in detail (37). Briefly, nuclei ($50–70 \times 10^6$) were isolated from control and 20-h rTNF-treated HL-60 and ML3 cells; elongation of nascent RNA was performed at 26°C for 20 min in the presence of 250 μCi [^{32}P]UTP (sp act $\sim$ 3,000 Ci/mM, Amersham Corp.). Labeled RNA was purified, ethanol precipitated, resuspended at 10^7 cpm/ml, and hybridized (at 42°C for 72 h) using the same number of ^{32}P-labeled RNA counts per minute per sample with prehybridized nitrocellulose filters on which 10 μg of linearized plasmid cDNAs had been immobilized using a slot blot apparatus (Schleicher & Shuell). Plasmids used were: X-CGD and its vector Gemini 4, pB_{AE}, HLA-DRα, and HLA-B7 and their vector PBR322. Filters were then washed, dried, and exposed as described above. Quantitation was done by scanning densitometry.

Hydrogen peroxide assay. H_2O_2 production was determined by fluorimetric measurement of the conversion of homovanillic acid (HVA) (Sigma Chemical Co., St. Louis, MO) to the fluorescent 2,2'dihydroxy-3-3'-dimethyl-oxydiphenyl 5,5' diacetic acid catalyzed by horseradish peroxidase (HRP) (Sigma Chemical Co.) as described (10). For this, the cells were washed twice in PBS and suspended, 2×10^7/ml, in HBSS (Flow Laboratories) containing 1 mM $CaCl_2$ and 5.6 mM glucose (HBSSCaG). Duplicate samples of 50 μl of cell suspension were added, at 37°C, to plastic tubes containing 450 μl of reaction buffer composed of HBSSCaG, 1 mM NaN_3, 0.8 mM HVA, 20 μg/ml HRP, containing or not containing 8×10^{-7} M 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co.) as stimulus. After 30 min incubation at 37°C, the reaction was stopped by placing the tubes on ice and adding 2 ml of cold HBSS. The tubes were centrifuged at 4°C and the fluorescence of the supernatant was assessed with a spectrofluorometer (LS-5B; Perkin-Elmer Corp., Norwalk, CT); the amount of H_2O_2 in the supernatant was calculated from a standard curve constructed with known H_2O_2 concentrations.

NADPH oxidase activity. This was tested as described (33). Briefly, cells were collected, washed twice in PBS, and prewarmed at 37°C in a shaking waterbath for 5–10 min (10^7 cells/ml in HBSSCaG). 8×10^{-7} M TPA or 0.002% ethanol (as solvent control) were added and, after 30 min, 0.2 ml of each cell suspension was added to 0.2 ml of reaction mixture (50 mM Hepes [pH 7.2], 2 mM diethylenetriaminopentaacetic acid [DTPA], 2 mM NaN_3, 240 μM cytochrome *c*, 0.1% Na-deoxycholate and 0.3 mM NADPH) in 24-well tissue culture trays. After an additional 10-min incubation at room temperature, NADPH oxidase activity was stopped with 0.6 ml of 50 mM Hepes (pH 7.2), 1 mM DTPA, 1 mM NaN_3 and 1 mM *p*-chloromercuribenzoate. All assays were performed in duplicate and included controls with 50 μg/ml

SOD. Cytochrome *c* reduction was calculated as the difference between absorbance values at 550 and 468 nm wavelength determined in a spectrophotometer (Ultrospec II; LKB Instruments, Gaithersburg, MD). O_2^- production was calculated as the difference between the value of cytochrome *c* reduction obtained in the absence and that obtained in the presence of SOD, using 24.5 $mM^{-1} \cdot cm^{-1}$ (38) as the extinction coefficient.

Measurement of cytochromes b. Total cytochrome *b* content was determined as described (39) by chemical reduction using a Response Tm spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Briefly, 15×10^6 cells per sample were washed twice in PBS and analyzed in acryl plastic cuvettes (Sarstedt, Inc., Princeton, NJ) in a final volume of 0.8 ml HBSSCaG with 1 mM NaN_3. Absorbance of samples from 380 to 600 nm wavelength in the oxidized state was first measured and memorized in the computer program. Samples were then reduced by adding a few grains of sodium dithionite and the difference spectrum (reduced minus oxidized) was recorded. The concentration of cytochrome b_{-245} was calculated from the difference between maximum and minimum absorbance at 428 and 410 nm, respectively, using an absorbance coefficient of 151 $mM^{-1} \cdot cm^{-1}$ (40).

To evaluate the contribution of the cytochromes *b* of mitochondria and endoplasmic reticulum to the sodium dithionite-reduced absorbance spectrum of cytochrome b_{-245}, specific quantitation of the different cytochromes *b* was performed in ML3 cells using a series of biochemical reductions, as previously described by us and others (40, 41). Briefly, 10^8 cells, incubated for 5 d with rTNF and rIFN-γ, were washed, resuspended in 3 ml of 0.34 M sucrose (containing 1 mM EDTA, 1 mM PMSF, and 10 mM Tris [pH 7.4]) and sonicated with three 5-s pulses of 50 W using a sonifier (Branson Cleaning Equipment, Co., Shelton, CT). Intact cells, cell debris, and nuclei were removed by centrifugation at 400 *g* for 10 min; the postnuclear supernatant was spun for 1 h at 100,000 *g*, and the pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) at a concentration of ~ 3 mg/ml, measured using a protein assay (Bio-Rad Laboratories, Richmond, CA) (42). The pellet suspension was subjected to the following sequential treatments: (*a*) no addition (oxidized state); (*b*) 10 μg/ml antimycin A (Sigma Chemical Co.) addition and 10 mM sodium succinate-induced reduction; (*c*) 0.1 mM NADH-induced reduction; and (*d*) sodium dithionite-induced reduction. After each treatment, absorbance spectral scanning (380–600 nm wavelength) was recorded and memorized. The difference spectra were plotted each time after electronic subtraction of the memorized spectrum recorded after the previous treatment. The content in the pellet of mitochondrial cytochromes *b*, which are specifically reduced by succinate in the presence of antimycin A (43), was determined from difference spectrum (succinate reduced)-(oxidized). The content of endoplasmic reticulum cytochrome b_5, which is specifically reduced by NADH (44), was determined by subtracting the spectrum of mitochondrial cytochromes *b* from the spectrum of the NADH-reduced sample. Finally, the difference spectrum for cytochrome b_{-245} was obtained by subtracting the mitochondrial and endoplasmic reticulum cytochrome *b* difference spectra from the sodium dithionite-reduced sample and cytochrome b_{-245} was quantitated from the 428-nm peak, as described above.

Results

Induction of X-CGD gene expression by TNF and IFN-γ in human myeloid cell lines. To study the accumulation of mRNA for the heavy chain of cytochrome b_{-245}, Northern blot analysis was performed using the X-CGD cDNA probe with total RNA from human myeloid cell lines treated for various times with rTNF, rIFN-γ, or a combination of the two cytokines. Fig. 1 shows the results of one representative experiment out of at least four performed with identical results on each cell line. Uninduced cultured ML3 and U937 cells did not express X-CGD transcripts, whereas HL-60 cells expressed them constitutively at low levels. As previously reported (3), the majority of the cells from each of the three cell lines cultured in the presence of 100 U/ml rTNF and/or 1,000 U/ml rIFN-γ were induced to express, after 5-d culture, differentiation markers and functional properties (antibody-dependent cell-mediated cytotoxicity, phagocytosis) of monocyte-macro-

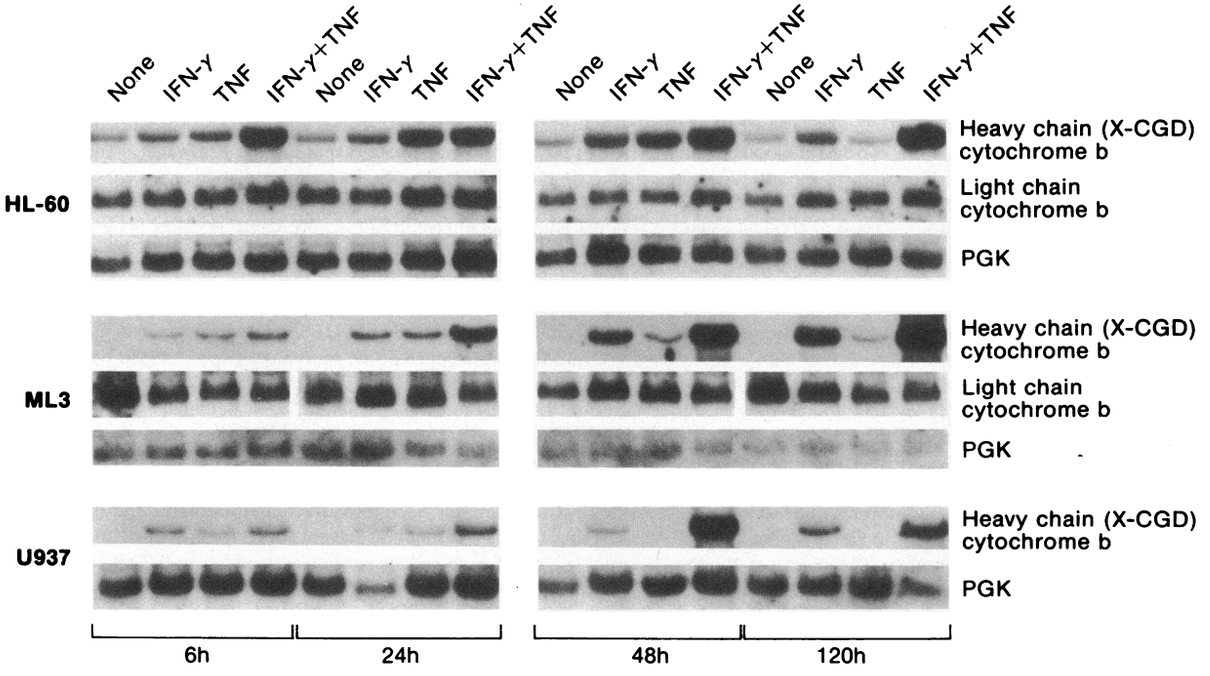

Figure 1. Kinetics of accumulation of mRNA for the heavy chain (X-CGD) and light chain of cytochrome b_{-245} in human myeloid cell lines cultured for different times in medium, 1,000 U/ml rIFN-γ, 100 U/ml rTNF, or a combination of the two cytokines. Amount of RNA per lane was: HL-60, 10 μg; ML3, 12 μg; U937, 15 μg. The same filters were subsequently hybridized with the PGK cDNA probe.

phages. Decreased proliferation and expression of proliferation-associated surface markers were concomitantly detected. All effects were synergistically induced by the two cytokines in combination (data not shown).

Both rIFN-γ and rTNF enhanced or induced accumulation of X-CGD mRNA in all three cell lines (Fig. 1). The effect was evident as early as 6 h after induction with either cytokine. Peak levels of X-CGD mRNA accumulation were observed 24–48 h after induction with either cytokine. They declined at later times with rTNF but further increased up to 5 d during induction with rIFN-γ. Cells from all cultures, up to 5 d, were > 90% viable. The mRNA accumulation was quantitated by densitometric scanning of the films. In ML3 cells treated with rIFN-γ and rTNF, an increase of X-CGD mRNA accumulation at 6, 24, 48, and 120 h, was respectively 1.33-, 2.25-, 2.40-, and 9.09-fold higher than that expected for an additive effect (i.e. the sum of the accumulation induced by rTNF and rIFN-γ, separately, at the same concentrations) and in U937 cells these values were 0.72-, 2.18-, 2.24- and 2.58-fold, respectively. In HL-60 cells accumulation of X-CGD mRNA was observed in uninduced cells and it was increased by induction with rIFN-γ and rTNF. In the experiments in Fig. 1 the densitometric values for the accumulation induced at 6, 24, 48, and 120 h by the two cytokines in combination were 1.42-, 1.00-, 2.25-, and 2.50-fold higher than those expected for an additive effect. Dose-response experiments (not shown) confirmed the synergism between the two cytokines: doses of rTNF or rIFN-γ up to 10 times higher than those used in Fig. 1 did not induce, alone, X-CGD mRNA accumulation at levels higher than those obtained using the two cytokines in combination at the same concentrations as in Fig. 1.

In all experimental conditions, the transcripts for the light chain of cytochrome b_{-245} were expressed at similar levels in HL-60, ML-3 (Fig. 1), and U937 cells (not shown).

Rehybridization of the same filters with a probe for PGK, a gene constitutively expressed in the three cell lines, demonstrated that approximately equivalent amounts of mRNA were loaded in each lane (Fig. 1). HL-60 and ML3 cells treated for 5 d with a combination of rTNF and rIFN-γ expressed levels of X-CGD mRNA in the range of those present in peripheral blood PMN and monocytes (Fig. 2), whereas U937 cells expressed lower levels (Fig. 1 and results not shown).

Nuclear run-on transcription assays were performed to analyze whether or not rTNF enhances the transcription rate of the X-CGD gene. Fig. 3 shows the results of one representative experiment out of three performed with the HL-60 cell line and of two with the ML3 cell line. Increases of 6.0- and 5.3-fold in transcription rate of the X-CGD gene and of MHC class I (HLA-B7) genes, respectively, were observed in HL-60 cells treated for 20 h with rTNF. Transcription of MHC class II (HLA-DRα) genes was minimal in both samples (Fig. 3). The results on transcription of MHC class I and class II genes reflect those obtained on mRNA accumulation of the same genes in Northern blot analysis (not shown). Identical transcription rate of 28S rRNA was detected in both samples (not shown).

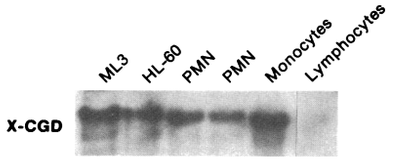

Figure 2. Northern blot analysis of the accumulation of X-CGD mRNA in differentiated ML3 and HL-60 cells (1,000 U/ml rIFN-γ and 100 U/ml rTNF, 5-d cultures), peripheral blood PMN, monocytes, and lymphocytes. 10 μg RNA was loaded per lane.

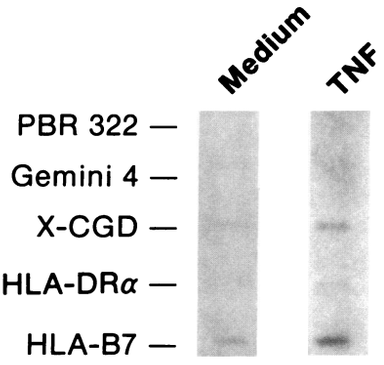

Figure 3. Induction of transcription of the X-CGD gene (nuclear run-on analysis) in HL-60 cells in response to a 20-h treatment with 100 U/ml rTNF. Equal amounts of ^{32}P-labeled mRNA from uninduced and rTNF-induced HL-60 cells were hybridized with X-CGD cDNA (Gemini 4 vector), HLA-DRα cDNA (PBR322 vector), HLA-B7 cDNA (PBR322 vector), and with the two vectors immobilized on nitrocellulose filters.

Half life of X-CGD mRNA in ML3 and HL-60 cells was also measured and found to be ~ 6 h in control and induced cells (not shown).

Induction of X-CGD gene expression in myeloid cells from CML patients. We analyzed the effect of rIFN-γ and rTNF on X-CGD gene expression in the cells from four CML patients. Northern blot analysis was performed using total RNA obtained from enriched preparations of peripheral blood immature myeloid cells (mostly myelocytes or earlier myeloid cells) cultured for different times in the presence of rTNF and/or rIFN-γ. X-CGD mRNA accumulation was expressed at variable levels in freshly isolated cells from four donors and increased when the cells were cultured in medium. rIFN-γ enhanced the expression of the X-CGD gene at 24 and 120 h, and this effect was more pronounced with cells from donors expressing low initial levels of X-CGD mRNA, as in the representative experiment shown in Fig. 4; rTNF induced no enhancement by itself and did not synergize with rIFN-γ. TNF mRNA was constitutively expressed in the cells from all four CML patients. Its accumulation was enhanced in rIFN-γ-treated cells from the patient shown in Fig. 4. TNF mRNA transcripts were detected in the 3 myeloid cell lines only after 5 days of culture with both rTNF and rIFN-γ (not shown).

Effect of protein synthesis inhibition on X-CGD gene expression. The myeloid cell lines were treated with 20 μg/ml cycloheximide 1 h before and during an 8-h incubation of the cells in the presence of rIFN-γ and/or rTNF. Cell viability was always > 90%, as assessed by vital dye exclusion. Cycloheximide did not prevent the induction of X-CGD mRNA by rIFN-γ in ML3 cells (Fig. 5, *top,* representative of four experiments). In HL-60 cells, cycloheximide alone superinduced (4.5-fold) the constitutive level of expression of X-CGD mRNA and rIFN-γ treatment in the presence of cycloheximide induced a further 1.5-fold enhancement (Fig. 5, *bottom,*

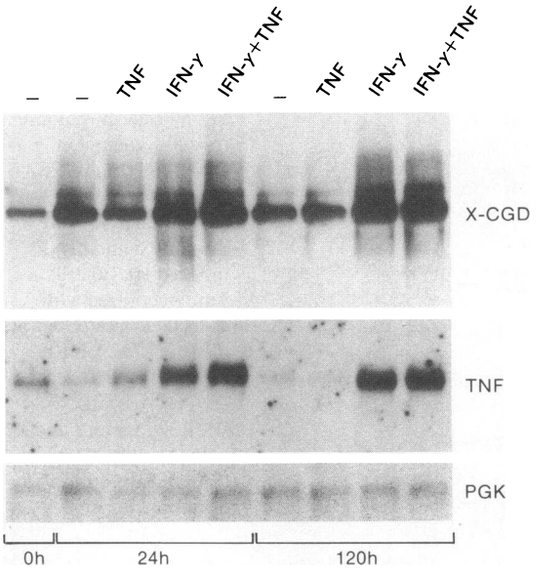

Figure 4. Northern blot analysis of total RNA from immature myeloid cells from a patient with CML, cultured for 1 or 5 d in medium or in the presence of 1,000 U/ml rIFN-γ and/or 100 U/ml rTNF. 5 μg RNA were loaded per lane, and the filter was sequentially hybridized with X-CGD, TNF, and PGK cDNA probes.

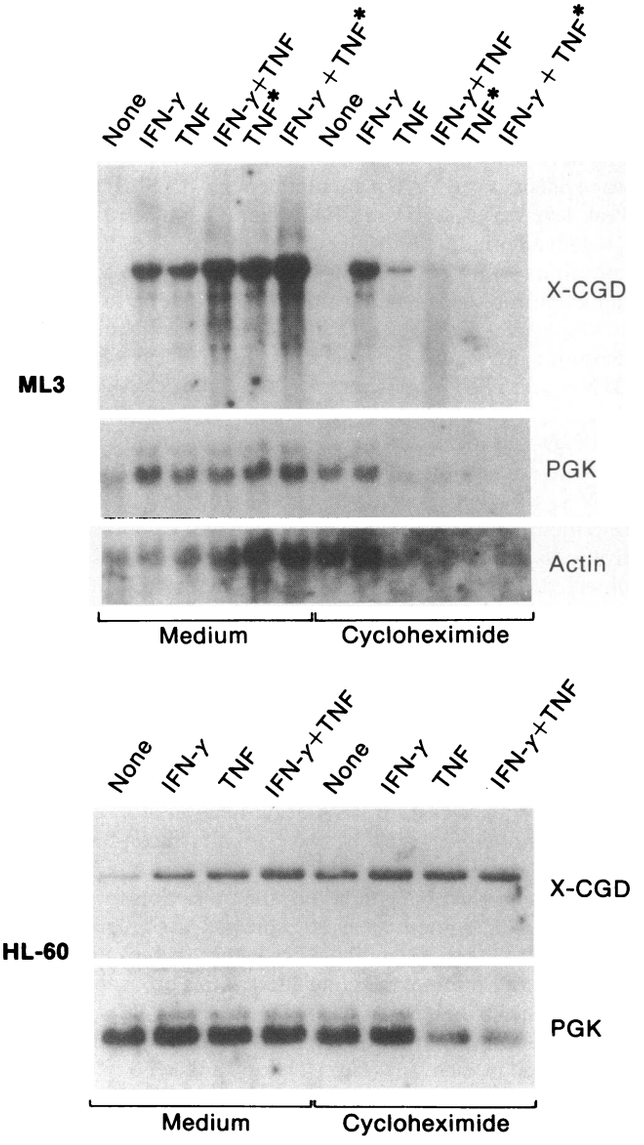

Figure 5. Effect of cycloheximide on the induction of accumulation of X-CGD mRNA in ML3 and HL-60 cells treated with rIFN-γ and/or rTNF. Cells were preincubated (for 1 h at 37°C) in the absence or presence of 20 μg/ml cycloheximide before rIFN-γ and/or rTNF were added and then incubated for a further 8 h. 10 μg total RNA were loaded per lane. Filters were sequentially hybridized with X-CGD, PGK, and β actin cDNA probes. The concentrations of cytokines were: 100 U/ml rTNF; 1,000 U/ml rTNF*; 1,000 U/ml rIFN-γ.

representative of three experiments). Addition of cycloheximide to ML3 and to HL-60 cells treated with rTNF resulted in an altered pattern of ethidium bromide staining of the RNA: additional discrete bands of migration faster than those of 28S and 18S rRNA were detected (not shown). Northern blot analysis of RNA obtained from ML3 cells treated with rTNF in the presence of cycloheximide showed an almost complete disappearance of the constitutively expressed mRNA for PGK and β actin (Fig. 5). In ML3 cells cultured in the presence of 100 U/ml rTNF and cycloheximide, X-CGD mRNA accumulation was increased 1.85-fold compared with that observed in control cells in the presence of cycloheximide alone. Accumulation of X-CGD mRNA in HL-60 cells treated with rTNF in the presence of cycloheximide was also increased (1.41-fold), although the accumulation of PGK mRNA was significantly reduced under the same conditions (Fig. 5). In both cell lines, induction of X-CGD mRNA, as measured by densitometry scanning, was, however, completely suppressed by cycloheximide when higher doses of rTNF or a combination of rTNF and rIFN-γ were used (Fig. 5).

Cytochrome b_{-245} content of myeloid cell lines induced with rTNF and/or rIFN-γ. The presence of cytochrome b_{-245} was evaluated by measuring its spectrum of absorbance in the 100,000-*g* pellet of the three cell lines. Uninduced cultured cells did not show the typical spectrum of cytochrome b_{-245}. As illustrated in Fig. 6, showing the results of a representative experiment with ML3 cells, both rTNF and rIFN-γ induced the cytochrome b_{-245} spectrum in the three cell lines: the effect of the combination of the two cytokines was at least additive. We then evaluated whether the spectrum obtained with induced ML3 cells reflects the participation of the different types of cytochrome *b*. As calculated from the different spectra shown in Fig. 7, the cytochrome *b* spectrum in 100,000-*g* pellet from rTNF- and rIFN-γ-induced cells depended on cytochrome b_{-245} by > 85% and on endoplasmic cytochrome b_5 for < 15%, whereas mitochondrial cytochromes *b* were almost nondetectable.

The kinetics of induction of cytochrome b_{-245} spectrum in intact ML3 and HL-60 cells is shown in Fig. 8. rIFN-γ and rTNF induced detectable cytochrome b_{-245} levels, as judged by its absorbance spectrum, starting between day 3 and 4 and reaching maximal values at day 5 of culture.

NADPH oxidase activity and respiratory burst in myeloid cell lines induced by rTNF and/or rIFN-γ. In parallel with the kinetics of cytochrome *b* spectrum expression in the three myeloid cell lines, we tested NADPH oxidase activity and respira-

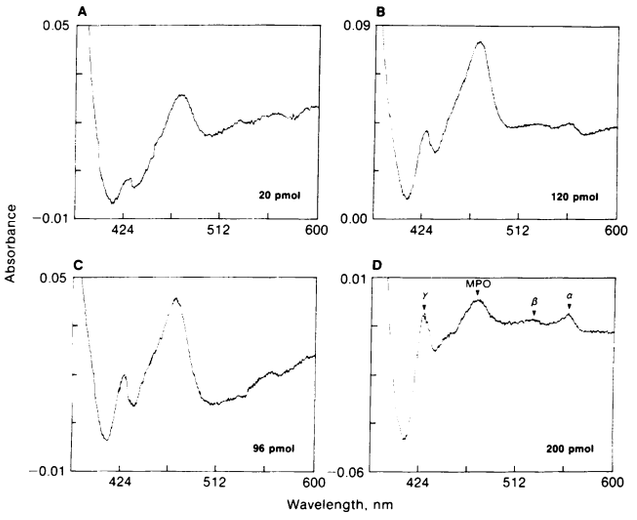

Figure 6. Reduced minus oxidized difference spectra of 100,000 *g* pellet of ML3 cells cultured for 5 d in: (*A*) medium; (*B*) 1,000 U/ml rIFN-γ; (*C*) 100 U/ml rTNF; (*D*) 1,000 U/ml rIFN-γ and 100 U/ml rTNF. Different absorbance spans were used for the four spectra, as indicated on abscissa. The relative concentrations of cytochrome b_{-245}, as measured by the 428-nm peak, is indicated in each panel. In *D* the position of γ (428 nm), β (530 nm), and α (558 nm) absorbance peaks of cytochrome b_{-245} and the myeloperoxidase (MPO) (475 nm) peak are indicated.

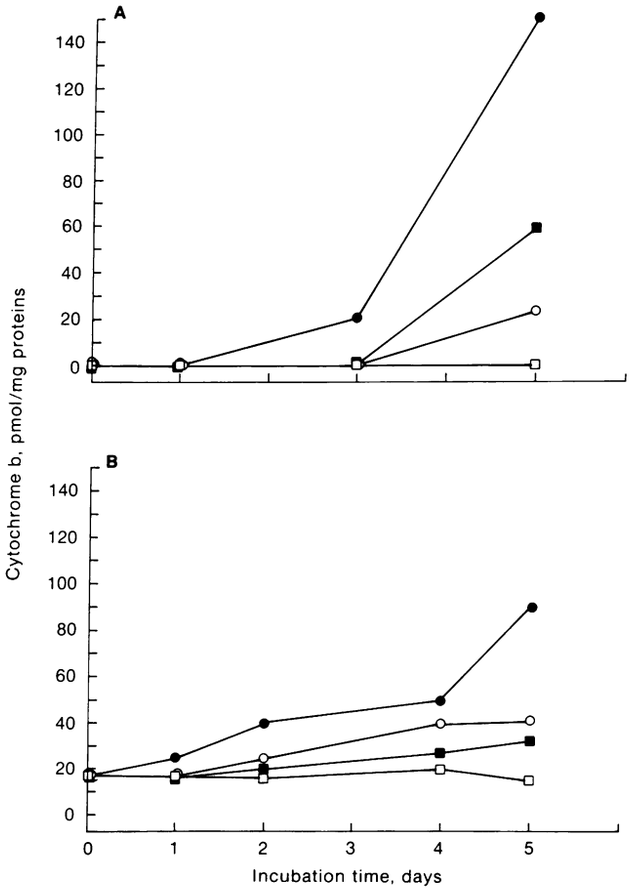

Figure 8. Relative concentrations of cytochrome b_{-245} (evaluated on the basis of the 428-nm absorbance peak) in (*A*) ML3 and (*B*) HL-60 intact cells cultured for the indicated times in (□) medium, (■) 1,000 U/ml rIFN-γ, (○) 100 U/ml rTNF, and (●) 1,000 U/ml rIFN-γ and 100 U/ml rTNF. The experiment in each panel is representative of two performed with each cell line.

tory burst function measured by H_2O_2 production in response to TPA. The results of a representative experiment with the ML3 cell line are shown in Fig. 9. H_2O_2 production in response to TPA (Fig. 9 *A*) was detected by day 2–3 in the cells treated with both rTNF and rIFN-γ but was only minimal at day 5 in the cells treated with either cytokine alone. TPA-induced NADPH oxidase activity paralleled the induction of respiratory burst ability in the cells (Fig. 9 *B*). Table I summarizes the results on H_2O_2 production and NADPH oxidase activity induced in response to TPA by the cells of the three myeloid cell lines cultured for 5 days with rTNF and/or rIFN-γ. In all cases the two cytokines induced both activities and their combined effect was more than additive. At 5 d, rIFN-γ and rTNF together induced, in ML3 and HL-60 cells, levels of H_2O_2 production, after TPA stimulation, 13- and 2-fold greater, respectively, than that expected for an additive effect. Dose-response curves and isobologram analysis of H_2O_2 production (not shown) confirmed that the two cytokines are strongly synergistic in their inducing effect. The K_m of NADPH oxidase in ML3 cells induced by rTNF and rIFN-γ was ~ 25 μM, similar to that of monocyte/macrophages (45) (not shown).

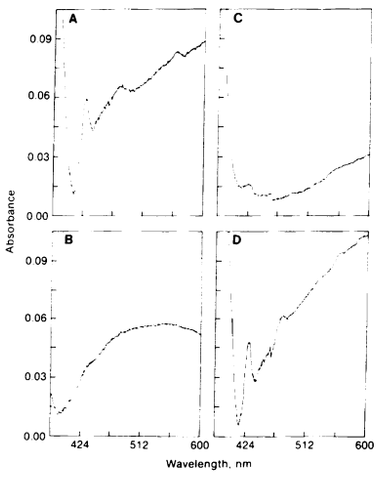

Figure 7. Difference spectra of 100,000 *g* pellet of ML3 cells cultured for 5 d in the presence of 1,000 U/ml rIFN-γ and 100 U/ml rTNF. (*A*) (sodium dithionite reduced)-(oxidized): total cytochromes *b* spectrum; (*B*) (succinate reduced)-(oxidized): mitochondrial cytochromes *b* spectrum; (*C*) (NADH reduced)-(succinate reduced): endoplasmic cytochrome b_5 spectrum; (*D*) (sodium dithionite reduced)-(succinate and NADH reduced): cytochrome b_{-245} spectrum.

Discussion

Myeloid cell lines, treated with IFN-γ and/or TNF or with other differentiation inducers, become able to respond to external stimuli (such as zymosan, immune complexes, phorbol diesters, Con A) with a respiratory burst (3, 14, 15, 21). In this study, we analyzed the expression of the X-CGD gene, encoding the 91-kD heavy chain of cytochrome b_{-245}, a component of the NADPH oxidase system, and the expression of the gene encoding its 22-kD light chain, to obtain information on the sequence of differentiation events leading to respiratory burst capability in myeloid cells and to start analyzing the molecular mechanisms involved in their induction by IFN-γ and TNF.

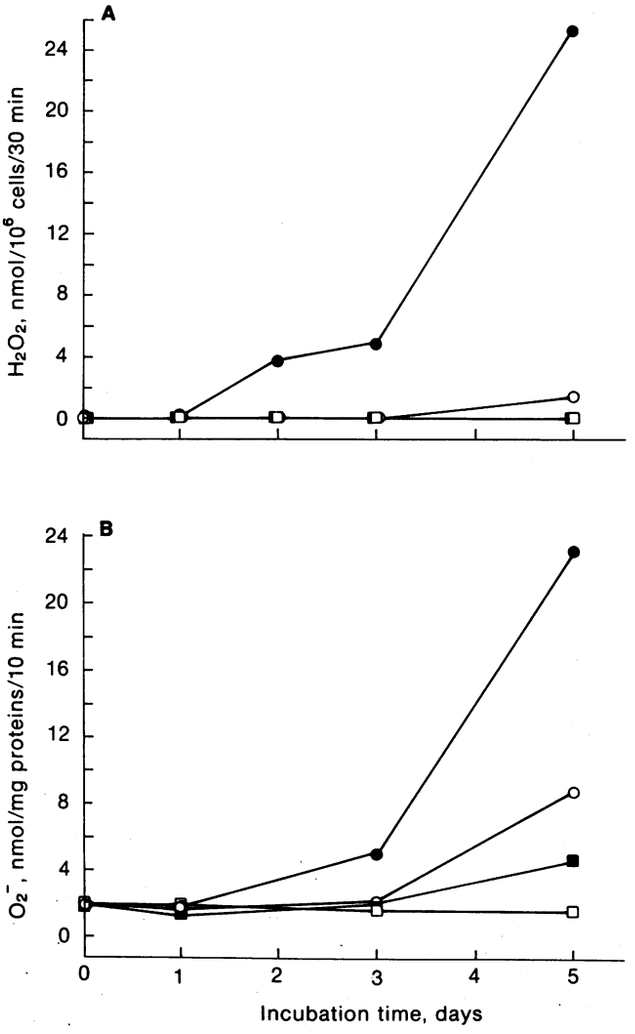

Figure 9. (*A*) H_2O_2 production by ML3 cells and (*B*) NADPH oxidase activity in ML3 cell lysates in response to 8×10^{-7} M TPA. ML3 cells were cultured for the indicated times in: (□) medium, (■) 1,000 U/ml rIFN-γ, (○) 100 U/ml rTNF, and (●) 1,000 U/ml rIFN-γ and 100 U/ml rTNF.

Table I. H_2O_2 Production by Intact Cells and NADPH Oxidase Activity in Lysates from Human Myeloid Cell Lines

Cell line*	Inducer‡	H_2O_2 production	NADPH oxidase	
		TPA-treated	Resting	TPA-treated
		nmol H_2O_2/ 10^6 cells/30 min	*nmol O_2^-/ mg protein/10 min*	
HL-60	Medium	3.8±1.2§	2.7	16.2
	rIFN-γ	13.0±3.0	3.1	31.5
	rTNF	17.0±3.0	4.5	38.0
	rIFN-γ + rTNF	48.8±8.0	5.0	71.0
ML3	Medium	<0.1	1.1	1.1
	rIFN-γ	2.2±0.3	1.8	13.7
	rTNF	1.8±0.5	1.8	10.1
	rIFN-γ + rTNF	54.2±11.2	5.0	59.0
U937	Medium	<0.1	2.7	2.8
	rIFN-γ	4.4±1.0	2.2	7.3
	rTNF	2.4±0.5	0.8	3.3
	rIFN-γ + rTNF	15.5±5.0	1.9	21.6

* Cells were cultured for 5 d in the presence of the indicated inducers. H_2O_2 production and NADPH oxidase activity were measured on washed resting cells or on cells stimulated with 8×10^{-7} M TPA, as described in Methods.
‡ rIFN-γ was used at 1,000 U/ml, rTNF at 100 U/ml.
§ Mean±SD, $n = 3$. H_2O_2 production in resting cells from each cell line was always < 0.1 nmol in any of the conditions tested.

X-CGD transcripts are not present constitutively in ML3 and U937 cells, but can be detected at low levels in HL-60 cells. The observed constitutive expression of the X-CGD gene in HL-60 cells could be due to a low-level expression in all cells or, more likely, to the expression in a small proportion of spontaneously differentiating cells present in the cultures. Induction or enhancement of X-CGD mRNA accumulation can be detected in the three cell lines as early as 6 h after induction with either rTNF or rIFN-γ. The effect of rTNF reaches a maximum at 24–48 h, but it declines soon after 24 h induction in the U937 cells. The combined effect of the two cytokines is more than additive at all times of induction tested.

When immature myeloid cells from four CML patients were studied, one clear difference with the cell lines was observed. The myeloid cells (myelocytes or earlier cells) expressed constitutively transcripts of X-CGD gene and their levels increased in culture at days 1 and 5, consistent with the spontaneous differentiation of these cells in culture to neutrophils (1). rIFN-γ significantly increased X-CGD expression, but rTNF failed to do so, either directly or in combination with rIFN-γ. Unlike myeloid cell lines, which are poorly induced to differentiate by rIFN-γ or rTNF separately (3), immature myeloid cells from CML patients have been previously shown (1) to be induced to differentiate by rIFN-γ alone, and we also observed (unpublished results) that rTNF, alone or in combination with rIFN-γ, has only a modest effect on cells from several CML patients. The results reported here indicate that TNF transcripts are expressed in freshly isolated immature myeloid cells from four CML patients analyzed. Their expression is increased severalfold upon rIFN-γ treatment in at least one of these patients. It is therefore possible that endogenous TNF production plays a role in the spontaneous expression of the X-CGD gene and synergizes with exogenously added rIFN-γ in enhancing its expression. Endogenously produced TNF is, however, unlikely to play a role in the differentiation of the cell lines: in these, TNF mRNA is expressed only after 5 d of induction with a combination of rTNF and rIFN-γ, suggesting that TNF production takes place only after differentiation is induced.

The kinetics of expression of cytochrome b_{-245} in the three cell lines, as measured by its visible spectrum, approximated, with some delay, the kinetics of expression of X-CGD mRNA. In HL-60 and U937 cells induced with DMSO and IFN-γ, respectively (14, 22), the cytochrome *b* spectrum was previously shown to be due almost exclusively to cytochrome b_{-245}. Our data indicate that also in the ML3 cell line, never analyzed before, the increased cytochrome *b* content after induction with rIFN-γ and rTNF depends almost entirely upon

the increase in cytochrome b_{-245}. The cytochrome b_{-245} spectrum requires the presence of both its subunits (18, 20, 46). Because the gene encoding the 22-kD chain is constitutively transcribed in the myeloid cell lines, it is not surprising that the expression of the X-CGD gene encoding the 91-kD chain is the limiting factor for the expression of the cytochrome b_{-245} spectrum. The kinetics of expression of NADPH oxidase activity and respiratory burst capability (as evaluated by measurement of H_2O_2 release in response to TPA) approximated that of cytochrome b_{-245} appearance, but were somehow delayed compared to the appearance of X-CGD transcripts. One possibility that could explain these data is the existence of other limiting cofactors, such as the flavoprotein or the cytosolic cofactor activity, whose expression has been shown to be affected by differentiation inducers (22, 47). The absence of cofactors or an inefficient membrane translocation or utilization of cytochrome b_{-245} (48, 49) could result in expression of X-CGD mRNA in the absence of NADPH oxidase activity and may explain the observation that, in ML3 cells, rTNF and rIFN-γ synergize more efficiently to induce respiratory burst capability than in inducing X-CGD gene expression or the cytochrome b_{-245} spectrum.

rIFN-γ enhances the transcription rate of X-CGD gene in monocyte/macrophages (reference 27 and our unpublished results). Accumulation of X-CGD transcripts in HL-60 and ML3 cells induced by rTNF as well as by rIFN-γ (27) depends, at least in part, on an induced increase in transcription rate of the gene. Our data can not distinguish conclusively whether the observed synergism between the two inducers operates at the transcriptional rather than at the postranscriptional level. However, the observation that X-CGD transcripts half-life is not affected by treatment of the cells with rTNF and/or rIFN-γ, is compatible with a transcriptional regulation.

To investigate the mechanism of induction of maturation/differentiation by rTNF and rIFN-γ, it was important to determine whether de novo protein synthesis in the myeloid cell lines was required for the effect of the two cytokines on X-CGD gene expression. Induction of X-CGD mRNA accumulation by rIFN-γ was still observed in cells in which protein synthesis was inhibited by cycloheximide. The use of cycloheximide to block protein synthesis did not affect cell viability during the 8-h incubation period, however, an alteration in the pattern of ethidium bromide staining of RNA was observed consistently in all the samples treated with rTNF and cycloheximide. This resulted also in disappearance, or significant reduction, of transcripts constitutively present in the cells, such as PGK or β actin. A similar effect has been reported by Tobler et al. (50) who attributed it to both a nonspecific reduction of transcription rate and enhanced RNA degradation. However, in our experiments, rTNF was still able to induce detectable levels of X-CGD mRNA in ML3 cells, even in conditions in which PGK and β actin expression were completely suppressed. In HL-60 cells, cycloheximide itself superinduced accumulation of X-CGD mRNA and greater accumulation was demonstrable in the presence of rTNF. Because PGK mRNA was also significantly decreased, the ratio between X-CGD mRNA and PGK mRNA significantly increased, indicating both an absolute and a relative induction of X-CGD gene expression. Although these results are difficult to interpret unequivocally, they suggest that induction of X-CGD gene expression by rTNF is, at least in part, independent from de novo protein synthesis. Other effects of TNF on gene expression, such as decreased c-*myc* expression in HL-60 (50, 51) and HeLa (52) cells, and induced expression of c-*fos*, c-*myc* (53), and IFN-β2 (54, 55) in fibroblasts, do not require de novo protein synthesis. Enhancement of class I MHC gene expression by TNF was reported to be prevented by cycloheximide, but those results are difficult to interpret because no control of constitutively expressed genes was reported (13).

The three different myeloid cell lines used in our studies have different origins. Their phenotypic characteristics are different, both before and after differentiation, but they all respond with similar patterns to rIFN-γ and/or rTNF (3) treatment. These observations suggest that the phenomena described here are not peculiar to a single aberrant malignant cell line, but reflect physiological regulation of myelomonocytic cell differentiation. This conclusion is also supported by the observation that the results are reproducible, at least in part, with leukemic cells freshly separated from CML patients. Also, normal bone marrow myeloid cells have been previously shown to undergo differentiation under conditions similar to those used here (1).

Our results indicate that both rIFN-γ and rTNF rapidly (6 h) induce expression of the X-CGD gene in myeloid cell lines acting, at least in part, at the transcriptional level. The X-CGD gene in the cell lines appears therefore already in a transcriptionally poised state and TNF and IFN-γ at early times of incubation neither affect commitment of the cells nor induce a cascade of events, involving subsequent synthesis of specific proteins, along a programmed pathway of differentiation. In this respect, IFN-γ, TNF, or their combination appear to induce maturation rather than genuine differentiation of the cell lines. However, at least in the case of rIFN-γ and of the combination of rIFN-γ and rTNF, the accumulation of X-CGD mRNA continues to increase until day 5, suggesting slow maturation at the single cell level, or recruitment of an increasing number of cells in the maturation process. The commitment of cells to respond with a coordinate expression of differentiation markers might be caused by the inducers themselves, thus acting as real differentiation inducers, or might be a stochastic event, with TNF and IFN-γ simply recruiting the responsive cells to terminal maturation. The protein encoded by the X-CGD gene is required for a specific function expressed only by mature phagocytic cells, and its expression should be expected to occur only at late stages of myelomonocytic differentiation. However, the three leukemic cell lines used in this study are arrested at a relatively late stage of maturation and already express, at least on a proportion of the cells, some markers of terminally differentiated cells. It is not surprising that other differentiation markers might be easily inducible by cytokines without requiring prior progression along the differentiation pathway. Further information on the mechanism of maturation/differentiation induced by these cytokines will be obtained by comparing results on cell lines with those obtained using normal myeloid cells and by comparing the mechanisms of the induction of the X-CGD gene with those of the induction of genes expressed only late during the culture of the cell lines with TNF/IFN-γ such as complement receptor 1 or TNF.

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