Retinoic Acid and Granulocyte Colony-Stimulating Factor Synergistically Induce Leukocyte Alkaline Phosphatase in Acute Promyelocytic Leukemia Cells

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In this report we show a strong synergistic interaction between granulocyte colony-stimulating factor (G-CSF) and all-*trans* retinoic acid (ATRA) on the expression of leukocyte alkaline phosphatase (LAP) in freshly isolated acute promyelocytic leukemia (APL) blasts as well as in NB40 and HL-60 cell lines. The strong synergism observed in these cell types was not evident in two acute leukemia cell lines (K562 and GF-D8), in normal granulocytes, and in monocytes. In freshly isolated leukocytes derived from chronic myelogenous leukemia (CML), in the stable phase of the disease, a weaker interaction between ATRA and G-CSF was documented. The cross-talk between the cytokine and the retinoid was studied in detail in NB4, an immortalized APL leukemia cell line, retaining the 15-17 chromosomal translocation involving the retinoic acid receptor type α. The treatment of NB4 cells with G-CSF alone or ATRA alone leads to no increase and to minor induction in LAP activity, respectively. If the cells are treated with the two compounds simultaneously, a dramatic elevation of LAP is observed after 4 days. The synergism between G-CSF and ATRA is evident at concentrations of the retinoid between 10^{-7} and 10^{-5} mol/L and at concentrations of the cytokine between 1 and 10 ng/mL. The simultaneous presence of the two compounds is necessary to obtain maximal increase of LAP activity and the effect is cell density-dependent. Synergism is specific for G-CSF, and it is not observed with other cytokines and functional inducers of the granulocyte. The augmentation of LAP activity is the consequence of an increased transcriptional rate of the liver/bone/kidney-type (L/B/K-type) alkaline phosphatase gene, as determined by Northern blotting and nuclear run-on analysis using specific cDNA probes. Only one of the two possible alternatively spliced forms of L/B/K-type alkaline phosphatase transcript is detected in NB4 cells after stimulation with G-CSF and ATRA. This mRNA form, which is the one observed in normal polymorphonuclear leukocytes, contains the most upstream leader exon. In NB4 cells, ATRA induces G-CSF, α, and β retinoic acid receptor transcripts, whereas G-CSF has minor effects on the expression of these mRNAs.

THE ACUTE promyelocytic leukemia (APL) (M3 in the French-American-British [FAB] classification)[1,2] represents at least 10% of all the myelogenous leukemias in the adult.[3] In the bone marrow (BM), the disease is characterized by the proliferation of precursors of the myelomonocytic lineage that present most of the features of the promyelocytic cell. In its classical form, this leukemia is characterized by leukopenia and by a tendency to produce disseminated intravascular coagulation.[3] APL patients are generally responsive to various chemotherapeutic regimens and complete remission is observed in 80% of the cases with the help of supportive therapy.[4] Recently, all-*trans* retinoic acid (ATRA) has been proposed for the treatment of this type of leukemia.[5,6] Both in vivo and in vitro, this compound has been shown to induce the differentiation of APL cells toward the granulocyte.[7-9] In fact, unlike other types of leukemic blasts, the APL cell is exquisitely sensitive to the cyto-differentiating action of ATRA. This peculiar sensitivity is somehow paradoxical because the molecular hallmark of the disease is represented by a reciprocal rearrangement of chromosomes 15 and 17[10] involving the first intron of the gene coding for retinoic acid receptor type α (RARα) and the PML gene.[11-22]

The leukocyte alkaline phosphatase (LAP) is one of the isoforms[23-25] of the alkaline phosphatase family[23-31] and it is also known as the liver/bone/kidney-type (L/B/K-type) or tissue nonspecific alkaline phosphatase.* Although the primary function of this family of isoenzymes in vitro is to hydrolyze phosphomonoesters, the physiologic role of alkaline phosphatases is still unknown. Besides the neutrophilic granulocyte, the protein is expressed at high levels in the osteoblast, in the proxymal tubule of the kidney, and in various other cells and tissues at lower levels.[32] In the hematopoietic system, LAP represents a marker for the terminal differentiation of the granulocyte.[33-35] In fact, the gene is neither expressed in the lymphocytic nor in the monocytic lineage. Experiments performed in normal and leukemic cells representing various stages of the differentiation along

* Throughout this report, the alkaline phosphatase transcript expressed in polymorphonuclear leukocytes, acute promyelocytic blasts, NB4, and other hematopoietic cells is referred to as the leukocyte alkaline phosphatase (LAP) mRNA, whereas the same transcript expressed in HOS-3 osteosarcoma, liver, and other nonhematopoietic tissues and cells is referred to as the L/B/K-type alkaline phosphatase mRNA.

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the myelogenous pathway show that LAP is expressed only in the postmitotic granulocyte.[35] In this cell type, approximately 30% of the enzyme is present in its mature form as an ectoenzyme, which is anchored to the outer surface of the cell membrane through a posttranslational modification of its carboxy terminus resulting in the covalent attachment of a hydrophobic phosphatidyl inositol glycan tail. By contrast, approximately 70% of the LAP protein is present in a latent form associated with tetranectin-positive intracellular granules that can be very rapidly mobilized to the cell membrane as a consequence of chemotactic stimuli.[36]

The expression of the LAP gene is under the specific control of granulocyte colony-stimulating factor (G-CSF) in normal neutrophils and in leukemic cells obtained from the stable phase of chronic myelogenous leukemia (CML) and chronic myelomonocytic leukemia (CMML).[35,37] Furthermore, in various cell types of human and murine origin, the expression of the L/B/K-type alkaline phosphatase gene is modulated by ATRA.[38-41] These observations prompted us to study the expression of LAP in NB4, a leukemic cell line that was recently established from a case of APL. NB4 cells retain the chromosome 15-17 translocation involving RARα and they can be forced to differentiate toward mature granulocytes after treatment with ATRA in vitro.[42] Thus, they represent a unique tool for the study of the cyto-differentiating properties of retinoids in hematopoietic cells.

In this report, we show that ATRA alone has very weak effects and that G-CSF alone is not at all affecting the expression of the LAP gene in NB4 cells. When this promyelocytic cell line is simultaneously treated with the two compounds a dramatic induction of LAP activity is observed. The synergism is evident also in fresh APL blasts, in HL-60 cells, and to a lower extent in CML leukocytes. The molecular mechanisms underlying the induction is studied in NB4 cells and the possible implications of our findings are discussed.

MATERIALS AND METHODS

Cell culture conditions and reagents. The NB4 APL cell line was a kind gift of Dr Michel Lanotte (Unité INSERM 301, "Genetique cellulaire et moleculaire des leucemies," Hopital St Louis, Paris, France). This cell line retains the translocation involving chromosomes 15 and 17, which is typical of the classical form of APL.[42] HL-60 and K562 leukemic cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). GF-D8 is a granulocyte-macrophage CSF (GM-CSF)– and interleukin-3 (IL-3)–dependent cell line developed from an acute myelogenous leukemia (AML) patient.[43] Cells were routinely seeded at 3×10^5/mL in RPMI 1640 containing 10% fetal calf serum (GIBCO-BRL, Gaithersburg, MD). In the case of GF-D8, cells were routinely passaged in growth medium containing 30 ng/mL of GM-CSF. In this cell line, experiments with ATRA, G-CSF, and the combination of the two compounds were performed in the absence of GM-CSF. Cultures were free from mycoplasma as assessed using the Hoechst 33258 fluorescent dye system (Farbwerke Hoechst AG, Frankfurt, Germany). ATRA and bacterial lipopolysaccharide (LPS) were from Sigma (St Louis, MO). Stock solutions of ATRA (10^{-2} mol/L) were prepared in dimethylsulfoxide under dimmed light and stored at −80°C, protected from light until use. Recombinant human G-CSF (specific activity 10^8 U/mg protein) was from Amgen Inc, Thousand Oaks, CA. Formyl-methionyl-leucyl-phenylalanine (fMLP) was from Sigma. Recombinant human GM-CSF (specific activity 10^7 U/mg protein) was obtained from Behringwerke-Aktiengesellschaft (Marburg, Germany). Recombinant human macrophage CSF (M-CSF, specific activity 7×10^7 U/mg protein) was from Cetus (Norwalk, CT). Recombinant human interferon-α A/D (IFN-α, specific activity 4×10^7 U/mg protein) is a kind gift of Dr M. Brunda (Hoffman-La Roche, Nutley, NJ). Recombinant human interferon-γ (IFN-γ, specific activity 2×10^7 U/mg protein) was obtained from Institut Roussel Uclaf (Paris, France).

Preparation of normal peripheral blood granulocytes, monocytes, and leukemic cells. Granulocytes and monocytes were obtained from buffy coats of normal volunteers by Ficoll-Hypaque (Seromed, Berlin, Germany) gradient centrifugation and sedimentation on dextran as described elsewhere.[34,35] Peripheral blood leukocytes from one CML patient during the stable phase of the disease were purified similarly.[35] Almost pure preparations of APL cells were obtained by dextran sedimentation of BM aspirates from two patients with a classical form of APL (M3 according to FAB classification) presenting the typical t(15-17) chromosomal translocation. This cell preparation consisted of more than 90% APL blasts as assessed by morphology. Leukemic cells were resuspended in RPMI 1640 containing 10% fetal calf serum (FCS) and incubated for 3 or 4 days in the absence and in the presence of ATRA, G-CSF, or the combination of the two compounds. At the end of the experiment, alkaline phosphatase enzymatic activity was measured in the various cell preparations.

Analysis of cell surface markers. NB4 cells were seeded at a concentration of 1×10^5/mL in RPMI 1640 containing 10% FCS and incubated for the appropriate amount of time with ATRA (10^{-5} mol/L), G-CSF (10 ng/mL), or the combination of the two compounds. The number of cells positive for the expression of CD11b, CD33, and CD16 and the mean associated fluorescence was quantitated using a FACScan analyzer (Becton Dickinson, Mountain View, CA). For the experiment presented in Fig 6, the following monoclonal antibodies (MoAbs) were used: Mo1-CD11b, My9-CD33, 1D3-CD16, and 3C11C8 (IgG1, reacting with human IFN-γ; negative control). All MoAbs were generously provided by Dr J.D. Griffin (Dana-Farber Cancer Institute, Boston, MA). The secondary reagent was an affinity-purified fluorescein isothyocyanate (FITC)-labeled goat antimouse Ig antiserum (Technogenetics, S Mauro Torinese, Italy). The same experiment was repeated by direct immunofluorescence assay using the following fluorescence-conjugated MoAbs purchased from Becton Dickinson: Phycoerythrin (PE)-conjugated Leu15 (IgG2a; anti-CD11b) and Leu M9 (IgG1; anti-CD33). Fluorescein (FITC)-conjugated Leu11a (IgG1; anti-CD16). The Simultest Control Kit (FITC and PE conjugates IgG1 and IgG2a) (Becton Dickinson) was used as negative controls.

Measurement of LAP activity. Approximately 1×10^6 NB4, APL fresh leukemic cells, or neutrophils were harvested, pelleted by centrifugation at 400*g* for 10 minutes, washed once with 0.9% NaCl, and centrifuged again. The washed cell pellet was resuspended in homogenization buffer (1 mmol/L-$MgCl_2$/1 mmol/L-$CaCl_2$/20 μmol/L-$ZnCl_2$/0.1 mol/L-NaCl/0.1% [vol/vol] Triton X-100 [Sigma]/0.05 mol/L-Tris/HCl, pH 7.4) and disrupted by pipetting.

The homogenate was used for the LAP assay, which was performed with p-nitrophenol phosphate (Sigma) as substrate according to the instruction of the manufacturer. LAP activity was normalized for the content of protein in the sample. Proteins were measured according to the Bradford method[44] using bovine serum albumin (BSA) fraction V (Sigma) as a standard. One unit of LAP activity is defined as the amount of enzyme capable of transforming 1 μmol

of substrate in 1 minute at 25°C. Enzyme assays were performed in conditions of linearity relative to the substrate and to the concentration of proteins.

Northern blotting analysis and polymerase chain reaction (PCR) amplification. Total RNA was prepared from NB4 cells, HOS-3 osteosarcoma cells, liver (obtained from a surgical specimen of normal tissue in proximity of an hepatocellular carcinoma), and granulocytes according to a modification of the guanidium isothyocyanate/CsCl method and used for Northern blotting analysis[45] and PCR amplification. The probes used for Northern blotting analysis were a full-length human (L/B/K-type) alkaline phosphatase cDNA (ATCC),[23] a human G-CSF receptor cDNA (clone D7, obtained from Dr Steven Gillis, Immunex Co, Seattle, WA),[46] and mouse RAR α1, β2, and γ1 cDNAs (from Dr Pierre Chambon, Strasbourg, France).[47] The various probes were labeled to a specific radioactivity of (1 to 2) $\times 10^9$ cpm/μg by using hexanucleotide primers and [^{32}P] dCTP.[48] PCR amplifications of the ALP transcripts were performed from total RNA after reverse transcription using the gene AMP kit (Cetus Perkin Elmer, Norwalk, CT) according to the instructions of the manufacturer. The antisense downstream oligonucleotide was common for the amplification of both exon 1A and 1B transcripts and is contained in exon 2 (5′ GCTTGGTCTCGCCAGTACTT 3′ complementary to nucleotides 255-274 of the human L/B/K-type alkaline phosphatase cDNA),[23] whereas sense upstream oligonucleotides were specific for exon 1A (5′ GCGTTGCGCTCCCGCCACTC 3′, nucleotides −196/−177 of the human L/B/K-type alkaline phosphatase gene)[28] and 1B (5′ ACTATGCCAAGCACTAGGAG 3′, nucleotides +9/+28 of the human L/B/K-type alkaline phosphatase gene),[49] respectively. For β-actin amplification, two oligonucleotides specific for the β-actin gene (5′ GCGCTCGTCGTCGACAACGG 3′, nucleotides 60-79 and 5′ GATAGCAACGTACATGGCTG 3′, complementary to nucleotides 430-449 of the human β-actin cDNA)[50] were used. The samples were subjected to 30 and 25 cycles of amplification (94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes) for alkaline phosphatase exons and β-actin, respectively. Amplifications were performed simultaneously on one third of the same reverse transcription reaction. The assays were performed in conditions of linearity in terms of RNA concentration and during the logarithmic phase of the amplification by Taq polymerase. After amplification, the samples were run on a 1.5% agarose gel. Amplified products were transferred onto nylon membranes and detected by Southern blotting analysis using ^{32}P-labeled oligonucleotides specific for exon 1A (5′ TCGCCAGTGCTCTGCGCA 3′, nucleotides −123/−106 of the human L/B/K-type alkaline phosphatase gene),[28] exon 1B (5′ GAAGCTCAGTGGTGTTAT 3′; nucleotides +151/+168 of the human L/B/K-ALP gene)[49] and β-actin (5′ GGCACCACACCTTCTACA 3′, nucleotides 298-315 of the β-actin cDNA), respectively, according to Wood et al.[51]

Table 1. Dose-Response Curve for the Induction of LAP Enzymatic Activity by ATRA in NB5 Cells

ATRA (mol/L)	LAP Enzymatic Activity (mU/mg protein)
0	4.1 ± 0.3
10^{-10}	4.5 ± 0.3
10^{-9}	4.9 ± 0.7
10^{-8}	4.7 ± 0.2
10^{-7}	6.0 ± 0.5
10^{-6}	28.4 ± 1.9*
10^{-5}	73.3 ± 6.2*
10^{-4}	27.6 ± 0.5*
PMN†	8,950.1 (1,550-15,350)

NB4 cells were seeded at an initial density of 3×10^5 cells/mL and treated for 4 days with the indicated concentrations of ATRA. Cells were harvested and LAP enzymatic activity was measured on cell homogenates. Results are expressed as mean ± SD of three separate cultures.

* Significantly higher than NB4 cells incubated in the absence of ATRA ($P < .01$, according to the Duncan's test after one-way analysis of variance).

† The results represent the mean of LAP activity measured in polymorphonuclear leukocytes obtained from the peripheral blood of 49 healthy volunteers. The intervals of confidence are indicated in parentheses.

Table 2. Effect of Various Stimuli Alone or in Combination With ATRA on LAP Enzymatic Activity in NB4 Cells

Stimulus	LAP Enzymatic Activity (mU/mg protein) −ATRA	+ATRA
None	14.1 ± 0.8	93.9 ± 9.3*
LPS	43.3 ± 6.0*	160.9 ± 29.0*
fMLP	17.1 ± 0.6	13.6 ± 1.8
GM-CSF	18.5 ± 1.5	13.5 ± 1.8
M-CSF	20.4 ± 1.8	14.6 ± 2.0
G-CSF	13.8 ± 0.3	2779.1 ± 23.1*
IFN-α A/D	15.0 ± 2.1	36.0 ± 3.5*
IFN-γ	17.5 ± 3.4	31.7 ± 1.6*

NB4 cells were seeded in RPMI 1640 containing 10% FCS at an initial density of 3×10^5 cells/mL. Cells were incubated with the indicated compounds in the absence or in the presence of ATRA (10^{-5} mol/L) for 4 days before harvesting. LAP enzymatic activity was measured in cell homogenates. Each experimental value is the mean ± SD of three separate cultures. LPS, bacterial lipopolysaccharide (10 μg/mL); fMLP, formyl-methionyl-leucyl peptide (10^{-8} mol/L); GM-CSF, human recombinant granulocyte-macrophage colony-stimulating factor (100 ng/mL); M-CSF, human recombinant macrophage colony-stimulating factor (13 ng/mL); G-CSF, human recombinant granulocyte colony-stimulating factor (10 ng/mL); IFN-α A/D, interferon α A/D (25 ng/mL); IFN-γ, interferon γ (50 ng/mL).

* Significantly higher relative to NB4 cells incubated with the appropriate stimulus in the absence of ATRA ($P < .01$, according to the Duncan's test after one-way analysis of variance).

Nuclear transcription run-on assay. Nuclear transcription run-on assay was performed as described by Eick and Bornkamm.[52] Plasmids used for the experiments were pBluescript (Stratagene, La Jolla, CA), pBR322 (GIBCO-BRL, Bethesda, MD), glucose 6-phosphate dehydrogenase,[53] and L/B/K-type alkaline phosphatase cDNAs.[23] Autoradiograms of both Northern blotting analysis and nuclear run-on assays were quantitated by laser-scanning densitometry with a laser beam densitometer (300 A computing densitometer Fast Scan; Molecular Dynamics, Sunnyvale, CA).

RESULTS

G-CSF cooperates with ATRA in inducing LAP enzymatic activity in NB4 cells. The effect of ATRA on the expression of LAP enzymatic activity was studied in NB4 acute promyelocytic cells. As shown in Table 1, unstimulated NB4 cells express LAP enzymatic activity at levels near the threshold for determination (7 ± 3 mU/mg protein). After 4 days, ATRA induces LAP activity in a dose-dependent manner starting at a concentration of 10^{-6} mol/L, reaching its

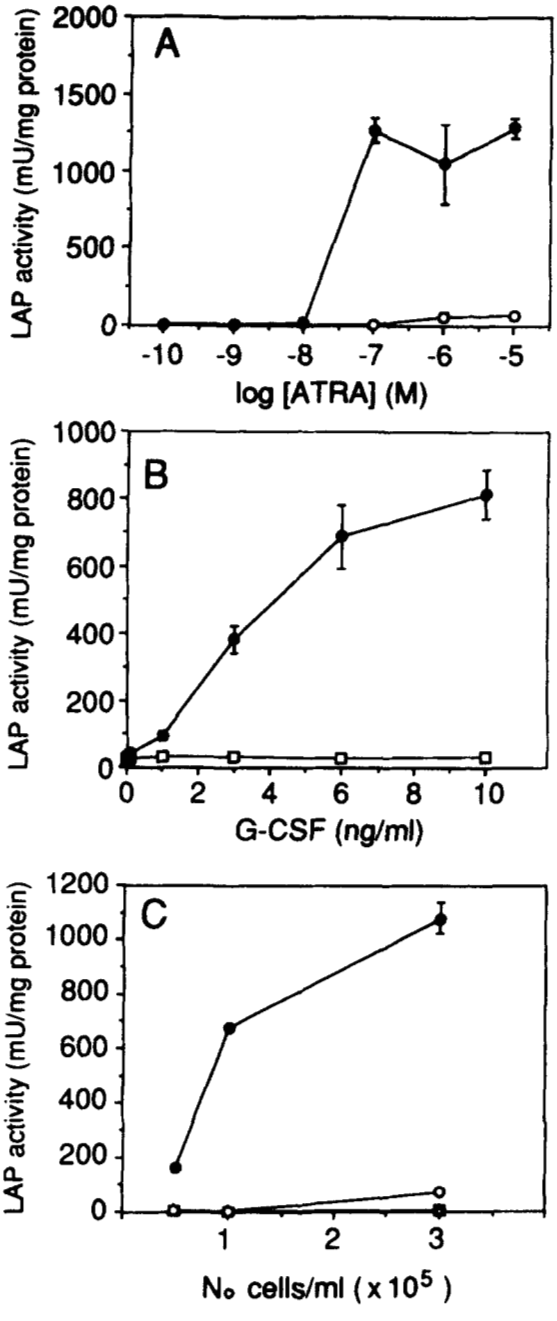

Fig 1. Cooperation between ATRA and G-CSF on the induction of LAP enzymatic activity in NB4 cells, and effect of cell density on LAP specific activity. (A) LAP activity was measured in cell homogenates obtained from NB4 cells cultured with the indicated concentration of ATRA either in the presence (●) or in the absence (○) of 10 ng/mL of G-CSF for 4 days. Results obtained from cultures grown in medium alone and in medium containing G-CSF (10 ng/mL) alone are not indicated because they are below the limit of detection of the enzymatic assay. Each experimental value is the mean ± SD from three separate cell cultures. (B) LAP activity was measured in cell homogenates obtained from NB4 cells grown with the indicated amount of G-CSF either in the presence (●) or in the absence (□) of 10^{-8} mol/L ATRA for 4 days. Results obtained from cultures grown in medium alone are not indicated because they are below the limit of detection of the enzymatic assay. The specific activity of LAP after treatment with ATRA alone at a concentration of 10^{-8} mol/L was 45 ± 5 mU/mg protein. (C) NB4 cells were seeded at the indicated density in medium alone (△), medium containing 10 ng/mL G-CSF (□), medium containing 10^{-8} mol/L ATRA (○) and medium containing the combination of ATRA and G-CSF (●). LAP enzymatic activity was measured in cell homogenates after 4 days of culture. Each experimental value is the mean ± SD of three cultures.

maximum at 10^{-5} mol/L and declining at 10^{-4} mol/L. The decrease in the level of induction observed at 10^{-4} mol/L is caused by toxic effects produced on NB4 cells by the retinoid at this high concentration (data not shown). Despite the fact that an approximately 18-fold induction in LAP levels is attained after incubation with ATRA at 10^{-5} mol/L, the specific activity is much lower than that of normal granulocytes. Several functional or differentiation inducers of the monocyte and the granulocyte, and of their precursors were thus tested alone or in combination with ATRA at 10^{-5} mol/L to study their effects on the expression of LAP activity in NB4 cells. Table 2 shows that f-MLP, GM-CSF, M-CSF, IFN-α A/D, and IFN-γ each alone or in combination with ATRA do not induce LAP significantly. LPS induces LAP activity three times and it shows an additive effect with ATRA. G-CSF (10 ng/mL) in combination with ATRA dramatically induces the expression of LAP (approximately 200-fold) in NB4 cells after 4 days of incubation. In NB4 cells, after treatment with G-CSF and ATRA, the levels of LAP are in the same range as that observed in peripheral neutrophils obtained from normal volunteers (see Table 1). The inducing effect is observed only in the presence of the retinoid, because G-CSF alone does not significantly increase the expression of LAP enzymatic activity.

As shown in Fig 1A, in the presence of ATRA at 10^{-7} mol/L, G-CSF at 10 ng/mL produces a maximal induction of LAP activity after 4 days of incubation. A remarkable elevation in the expression of LAP is observed if the concentration of ATRA is increased from 10^{-8} mol/L to 10^{-7} mol/L. In the presence of 10^{-8} mol/L ATRA, the dose-response curve for G-CSF between 0.1 and 10 ng/mL was studied and it is shown in Fig 1B. A significant increase in the expression of LAP is evident with G-CSF at a concentration of 1 ng/mL, but the cytokine at 10 ng/mL gives an almost 10-fold higher induction. Concentrations of G-CSF higher than 10 ng/mL were not tested because, in the presence of ATRA, the cytokine becomes cytotoxic for NB4 cells at concentrations above 30 ng/mL (data not shown). Figure 1C shows that the number of cells initially present in the culture flask is also an important parameter for the effect of the combination between ATRA and G-CSF on LAP induction. After 4 days, LAP enzymatic activity is augmented by the cytokine and the retinoid, regardless of the number of cells originally present, whereas the specific activity of the induced enzyme is progressively higher as the inoculum of NB4 cells is increased.

The time course for the induction of LAP activity by G-CSF and ATRA in NB4 cells is presented in Fig 2A. Whereas G-CSF does not affect LAP at any time point and ATRA

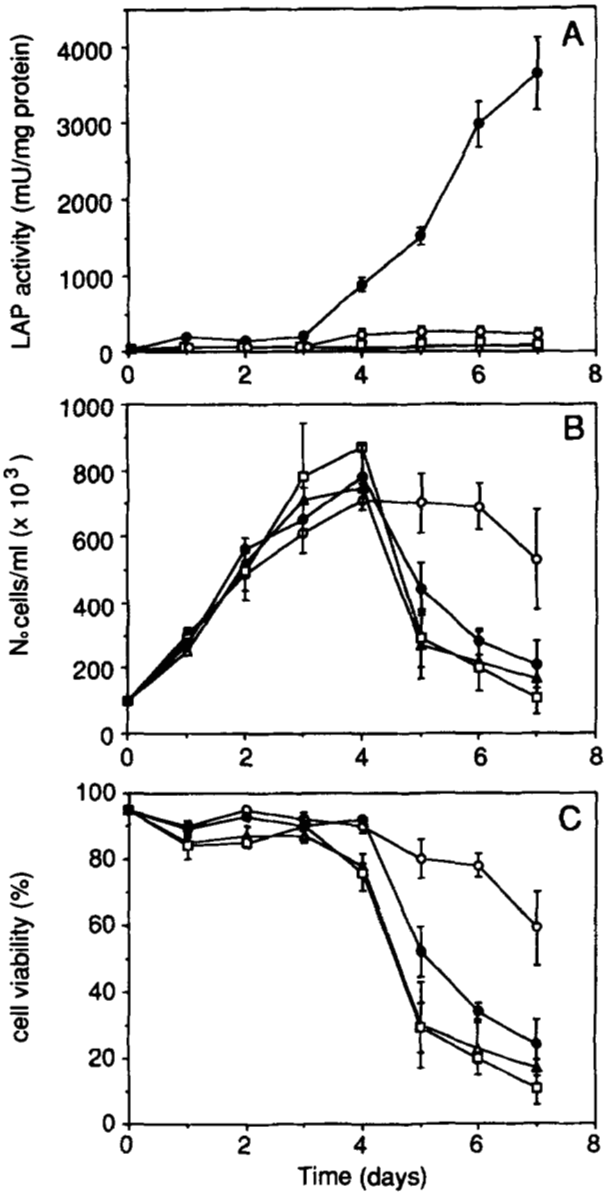

Fig 2. Time course of the cooperative interaction between G-CSF and ATRA in the induction of LAP enzymatic activity. Effects of the combination on cell proliferation and viability. NB4 cells (1 × 10^5/mL) were treated with medium alone (△), medium containing 10^{-5} mol/L ATRA (○), 10 ng/mL G-CSF (□), and 10^{-5} mol/L ATRA + 10 ng/mL G-CSF (●), respectively, for the indicated amount of time. At the end of each treatment, cells were collected and processed for the measurement of LAP enzymatic activity (A), or an aliquot of the cultures was counted in a Bürker chamber after staining with trypan blue so as to assess the total number of cells (B) and their viability (C). Each experimental value is the mean ± SD of three separate cultures.

causes only marginal changes, the combination of the cytokine and the retinoid induces the enzyme specific activity dramatically. After challenge with G-CSF and ATRA, the increase in LAP activity is almost linear from the third to the seventh day of culture, although, as shown in Figs 2B and 2C, a progressive decrease in the total number and the viability of NB4 cells is evident from the fourth day on. As far as this last observation, it is important to mention that the high level of cell mortality at 5 and especially at 6 and 7 days does not affect the interpretation of the results observed, even at these time points, because separation of viable from dead or damaged cells on Percoll gradients shows that more than 90% of the LAP enzymatic activity measured after ATRA or ATRA + G-CSF treatment is associated with live cells regardless of their proportion in culture (data not shown). As illustrated by Figs 2B and 2C, ATRA has a positive effect on cell viability that is counteracted by G-CSF starting from the fourth day. Therefore, the remarkable induction of LAP after treatment of NB4 cells with ATRA and G-CSF is not caused by a cumulative increase in the enzymatic activity consequent to a longer survival rate of cells differentiated by the retinoid alone.

The order of addition of G-CSF and ATRA to the culture medium is important for the synergistic interaction between the two compounds. Figure 3 shows that the synergism is evident if ATRA and G-CSF are simultaneously added to the medium and left in contact with NB4 cells for 5 days.

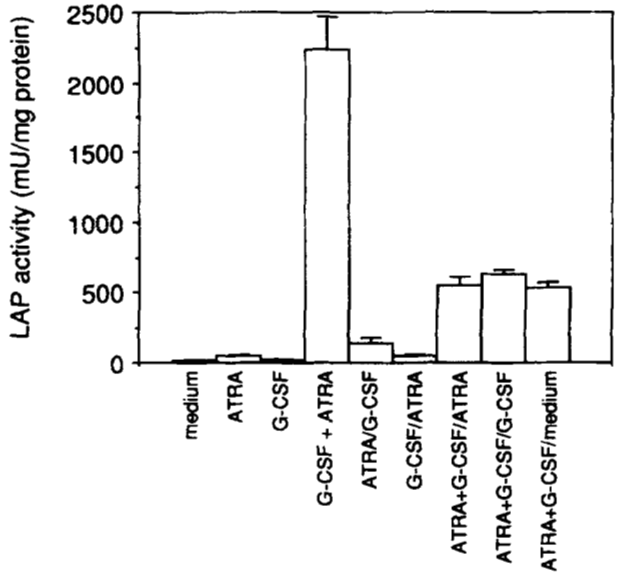

Fig 3. Influence of the order of addition of the compounds on the synergism between G-CSF and ATRA. NB4 cells were grown in the absence or in the presence of the indicated compounds (10^{-5} mol/L ATRA; 10 ng/mL G-CSF; or the combination of the two) for 3 days. Cells were washed with fresh medium and further incubated for 2 days as indicated. The slash in between the indication of treatments represents the removal of the medium after the first 3 days of incubation. For example, G-CSF/ATRA means that the cells were grown in the presence of 10 ng/mL G-CSF for the first 3 days, washed, and incubated for another 2 days in the presence of 10^{-5} mol/L ATRA. Control cultures were grown for 5 days, in medium alone (medium), medium containing 10^{-5} mol/L ATRA (ATRA), medium containing 10 ng/mL G-CSF (G-CSF), and medium containing 10^{-5} mol/L ATRA + 10 ng/mL G-CSF (ATRA + G-CSF). At the end of each treatment, cells were collected, homogenized, and LAP enzymatic activity was measured. Each experimental value is the mean ± SD of three separate cultures.

Table 3. Effect of ATRA, G-CSF, and the Combination of the Two Compounds on LAP Enzymatic Activity in PMNs and Various Types of Leukemic Cells

	LAP Enzymatic Activity (mU/mg protein)			
Cell Type	Medium	ATRA	G-CSF	ATRA + G-CSF
APL pt. 1	51 ± 4	58 ± 7	220 ± 14*	1,197 ± 104†
APL pt. 2	90 ± 14	128 ± 10	71 ± 15	1,656 ± 80†
CML	27 ± 4	86 ± 9*	56 ± 6*	196 ± 29†
HL-60	13 ± 1	18 ± 1	14 ± 1	259 ± 47†
GF-D8	4 ± 1	6 ± 1	5 ± 1	9 ± 3

3×10^5/mL fresh leukemic blasts obtained from the BM of APL patients, and the same number of HL-60 or GF-D8 cells were treated with medium alone (medium), medium containing 10^{-5} mol/L ATRA (ATRA), 10 ng/mL G-CSF (G-CSF), and 10^{-5} mol/L ATRA + 10 ng/mL G-CSF (ATRA + G-CSF), respectively, for 4 days. Peripheral blood CML leukocytes were treated with the same compounds for 1 day. The results are the mean ± SD of three separate cultures.

*Significantly higher relative to cells incubated with medium alone ($P < .01$, according to the Duncan's test after one-way analysis of variance).

† Significantly higher relative to the sum of the values of G-CSF and ATRA separately ($P < .01$, according to the Tukey's test after two-way analysis of variance and evaluation of the F of interaction).

On the other hand, if cells are treated with ATRA for 3 days before washing, addition of fresh medium containing G-CSF and further incubation for 2 days, the synergism is abolished. Similarly, no induction of LAP is observed if cells are incubated with G-CSF for 3 days and ATRA for the subsequent 2 days. Interestingly, treatment of NB4 cells with the combination of G-CSF + ATRA for 3 days followed by incubation for the remaining 2 days with medium alone and medium containing ATRA or G-CSF leads to submaximal induction of LAP activity. Taken together, these data indicate that there is no priming of ATRA or G-CSF on NB4 cells, and the contemporaneous presence of the cytokine and the retinoid is necessary to obtain maximal increase in the amounts of LAP.

ATRA and G-CSF induce the expression of LAP enzymatic activity in fresh leukemic cells obtained from APL patients, in HL-60 cells, and to a lower extent in CML leukocytes. To evaluate whether the synergism between ATRA and G-CSF is peculiar to NB4 or if it is a more general phenomenon, the effect of the two compounds on the expression of LAP enzymatic activity was studied in BM blasts obtained from three APL cases, peripheral blood leukocytes of one CML patient, as well as in HL-60, K-562, and GF-D8 cell lines. The results are presented in Table 3. Freshly isolated APL blasts show low but detectable levels of LAP activity (50 ± 2 U/mg protein for patient 1 and 80 ± 7 for patient 2, respectively; mean ± SD of three determinations) and the amount of the enzyme is not influenced by 4 days of culture in medium alone. If APL blasts are treated for the same amount of time with ATRA and G-CSF, LAP is dramatically induced. When cells are treated with ATRA alone, no increase in the enzyme is observed. In one APL case, a small but significant augmentation of LAP activity is evident after treatment with G-CSF. At 4 days, the viability of APL cells is approximately 90% and very similar in all the experimental groups. Furthermore, ATRA either alone or in the presence of G-CSF produces a cytostatic effect. Indeed, if the number of cells in the culture incubated with medium alone is taken as 100%, percentage values (mean ± SD of three separate cultures of APL blasts from patient 1) of 70% ± 19%, 41% ± 13%, and 58% ± 10% were obtained after incubation with G-CSF, ATRA, and the combination of the two compounds, respectively. HL-60 cells behave essentially as NB4 cells after treatment with the various compounds. In fact, G-CSF and ATRA have no effect on LAP whereas the cytokine in combination with ATRA causes a remarkable induction. Freshly isolated leukemic cells obtained from one CML patient express low levels of LAP in basal conditions. As expected,[54,55] after 1 day of treatment, LAP activity is maximally induced by treatment with G-CSF and ATRA alone. The simultaneous incubation with the two compounds leads to a weak synergistic increase in the levels of the enzyme. After 1 day, the viability of cells in all the experimental groups is similar and approximately 80%. No further augmentation in LAP levels is observed if cells are cultured with the various stimuli up to 2 days. Starting from the third day, the viability of cells is very low (<10%) in all the experimental groups and it prevents further studies. GF-D8 cells express levels of LAP that are at the limit of detection of the assay in basal conditions, similarly to K-562 (data not shown) and freshly isolated monocytes. The three cell types do not upregulate the expression of LAP after treatment for 1, 2, 3, or 4 days with ATRA, G-CSF, or the combination of the two compounds (only data at 4 days for GF-D8 cells are shown). Normal granulocytes express high levels of LAP activity constitutively and no further increase is observed upon stimulation for 1 day with the various compounds alone or in combination (data not shown). With this last cell type, experiments cannot be protracted beyond 1 day because granulocytes have a very limited life span in culture.

G-CSF and ATRA induce the expression of the same LAP transcript present in normal neutrophils. To investigate the molecular mechanism underlying the induction of LAP activity after treatment with the combination of G-CSF and ATRA, Northern blotting analysis on RNA extracted from NB4 cells was performed using a full-length cDNA probe specific for human L/B/K-type alkaline phosphatase.[23] This cDNA hybridizes to an mRNA of approximately 2.5 kb in human peripheral neutrophils.[34,35] Preliminary experiments showed that NB4 cells do not express detectable levels of the LAP transcript in basal conditions or after treatment with medium alone, medium containing ATRA (10^{-5} mol/L) and medium containing G-CSF (10 ng/mL) for various amounts of time up to 96 hours (data not shown). As shown in Fig 4A, after simultaneous treatment with the cytokine and the retinoid, high levels of a 2.5-kb transcript are observed only after 72 hours and they remain constant at least until 96 hours of incubation. Comparing the data reported in Figs 4A and 2A, it is clear that the upregulation in LAP mRNA precedes by 1 day the increase in LAP enzymatic activity. The gene coding for the L/B/K-type alkaline phosphatase in

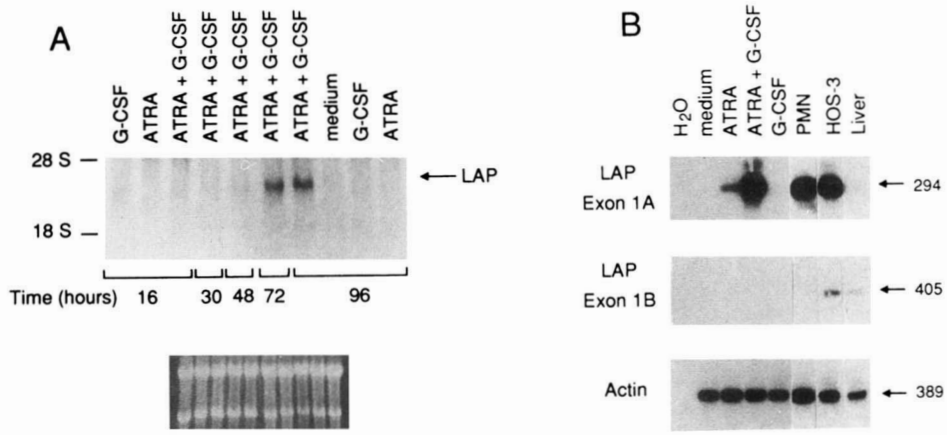

Fig 4. LAP mRNA induction and usage of exon 1A and 1B of the L/B/K-type alkaline phosphatase gene in NB4 cells after treatment with ATRA, G-CSF, or the combination of the two compounds. (A) Total RNA (10 μg for each lane) was extracted for Northern blotting analysis from NB4 cells incubated in the absence (medium) or in the presence of G-CSF (10 ng/mL), ATRA (10^{-5} mol/L), or the combination of ATRA + G-CSF for the indicated amount of time. The position of the size markers (28 S and 18 S rRNAs) is indicated on the left. The position of the LAP transcript is indicated by an arrow. A picture of the ethidium bromide staining of the RNA is shown at the bottom. (B) Total RNA (1 μg) from human liver, PMNs, HOS-3 osteosarcoma, and from NB4 cells treated with medium alone (medium), 10 ng/mL G-CSF (G-CSF), 10^{-5} mol/L ATRA (ATRA), and the combination of the two compounds (ATRA + G-CSF) for 4 days was reverse transcribed and the resulting cDNA mixture was split in three identical aliquots and PCR amplified using oligonucleotide couples specific for L/B/K-type alkaline phosphatase exons 1A and 1B, and for β-actin, respectively. As a negative control, a blank sample (H_2O) was reverse transcribed and PCR amplified exactly as above. The products of the amplification were run on a 1.5% agarose gel and blotted on nylon membranes and hybridized to ^{32}P-labeled synthetic oligonucleotides specific for alkaline phosphatase exons 1A and 1B, and β-actin, respectively. The oligonucleotides used for the hybridization were distinct and internal to the couple of oligonucleotides used for the amplification step. The expected size of the amplified products is 294, 405, and 389 base pairs for L/B/K-alkaline phosphatase exon 1A, exon 1B, and β-actin, respectively, and it is indicated on the right side with arrows.

the human,[28] † as well as in the mouse[56,57] and in the rat,[58] contains two leader exons, 1A (about 30 kb upstream from exon 2) and 1B (about 4 kb upstream from exon 2), and it has the potential to produce two alternatively spliced mRNAs differing in their 5′ untranslated regions.[23,49,57] Because the L/B/K-probe used for the Northern blotting analysis does not distinguish the two transcripts, RNA extracted from NB4 promyelocytes grown in medium alone and in medium containing G-CSF, ATRA, or the combination of the two compounds was used to amplify specifically the LAP mRNAs containing exon 1A and exon 1B by RT/PCR. Figure 4B shows that the transcript containing exon 1A is the only one present in NB4 cells treated with ATRA and G-CSF contemporaneously. This is similar to what is observed in polymorphonuclear leukocytes, where the transcript containing exon 1A is the only one expressed in basal conditions. HOS-3 and human liver RNA were used as positive controls for the expression of exon 1A and 1B, respectively. HOS-3 osteosarcoma cells synthesize predominantly the transcript containing exon 1A, although very low levels of the mRNA containing exon 1B are also detectable. On the other hand, the major L/B/K-type alkaline phosphatase mRNA expressed in human liver is the one containing exon 1B, as expected on the basis of the data published by Matsuura et al.[49] Notice that, in contrast to what is observed after Northern blotting analysis, the high sensitivity of the RT/PCR assay allows the detection of the signal for the LAP mRNA containing exon 1A even after treatment with ATRA alone. This confirms that the relatively minor induction of LAP enzymatic activity after treatment with the retinoid (see Tables 1 and 2) is correlated with an upregulation of the corresponding transcript.

The accumulation of high levels of LAP mRNA is the result of an increase in the transcriptional rate of the LAP gene triggered by the treatment of NB4 cells with G-CSF and ATRA. Figure 5A illustrates a representative nuclear run-on analysis for the LAP and the glucose-6-phosphate dehydrogenase (G6PDH) genes after treatment of NB4 cells, for 72 and 96 hours, with medium, ATRA, G-CSF, or the combination of the retinoid and the cytokine. In these studies, G6PDH was chosen as a reference to assess the specificity of the changes in the transcription of the LAP gene, because it is a housekeeping gene and its transcriptional activity as well as steady-state mRNA levels (not shown) are relatively constant in NB4 cells in all the experimental conditions tested. The average results of the densitometric analysis of the LAP nuclear run-on signals (after normalization for the level of expression of the G6PDH gene) obtained from two independent experiments performed at 4, 72, and 96 hours are shown in Fig 5B. Very low but detectable levels of transcription of the LAP gene are observed when cells are incubated in medium alone for various lengths of time.

† The most upstream leader exon of the L/B/K-type alkaline phosphatase gene is referred to as exon 1A whereas the other is referred to as exon 1B throughout this manuscript, although the two exons were first described as 1B and 1L, respectively, by Matsuura et al.[49]

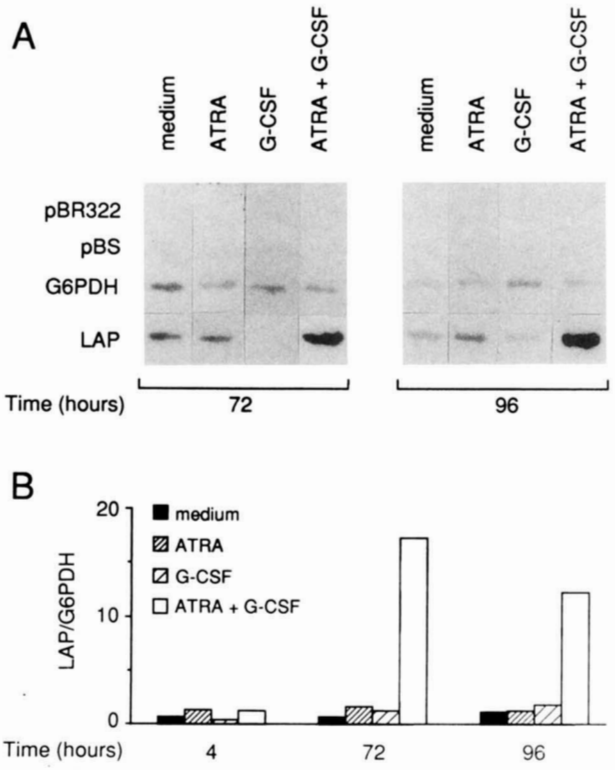

Fig 5. Effect of G-CSF, ATRA, and the combination of the two compounds on the transcriptional activity of L/BK/-type alkaline phosphatase gene. (A) Nuclear run-on assays were performed using nuclei isolated from NB4 cells treated for the indicated amount of time with medium alone or with medium containing ATRA (10^{-5} mol/L), G-CSF (10 ng/mL), and the combination of the two compounds. NB4 cells (3.6×10^7) were seeded in 120 mL of growth medium, the culture was divided into 4 equal portions, and treated as explained above. At the indicated time, an appropriate aliquot of cells was harvested and nuclei were isolated. The probes used in this experiment were glucose 6-phosphate dehydrogenase cDNA (G6PDH) and L/B/K-type alkaline phosphatase (LAP). Plasmid pBluescript (pBS) and pBR322 were used as controls for nonspecific hybridization. (B) The average results of the densitometric analysis of the LAP nuclear run-on signals, obtained in two independent experiments performed after treatment of NB4 cells with the indicated stimuli for 4, 72, and 96 hours are shown. Results are normalized for the transcriptional activity of the G6PDH gene and are expressed as the ratio of the optical density of the run-on signal measured for LAP and G6PDH. The optical density obtained from the scanning of the pBR322 and pBS signals was always negligible (<1% to 2% of the corresponding LAP and G6PDH signals).

Treatment of NB4 cells with ATRA alone has no significant and reproducible effects on the transcriptional activity of the gene at any time point. The contemporaneous treatment of NB4 cells with ATRA and G-CSF for 4 hours does not affect the levels of transcription of the LAP gene. By contrast, the combination of the retinoid and the cytokine dramatically augments the level of transcription of the LAP gene at 72 and 96 hours. The kinetics of the increase in the transcriptional activity of the LAP gene after treatment with G-CSF and ATRA are correlated with those for the accumulation of LAP mRNA (compare Figs 4A and 5).

G-CSF along with ATRA regulates the expression of CD33 and CD11b in NB4 cells. To study the effect of G-CSF on the differentiation of NB4 by ATRA, the cells were analyzed for the expression of CD11b and CD16, two markers of the late myeloid differentiation, and CD33, a marker of the early myeloid differentiation. The experiment shown in Fig 6 illustrates data obtained from one of two highly reproducible experiments performed by either indirect or direct (data not shown) immunofluorescence assays. The expression of CD11b is very low in NB4 cells after culturing in medium alone for 3 days (7.5% positive cells with a mean fluorescence of 36.0) or 4 days (4.0% positive cells with a mean fluorescence of 41.7). No significant differences are observed after culturing with G-CSF. If NB4 cells are treated in the presence of ATRA for 3 days, a clear induction in the number of $CD11b^+$ cells (46.2% with a mean fluorescence of 46.1) is observed. The levels of this myeloid marker are not further increased after 4 days in culture with the retinoid (50.7% positive cells with a mean fluorescence of 45.6). After 3 days, the combination of ATRA and G-CSF, leads to a small but reproducible increase in the number of CD11b positive cells (67.2% with a mean fluorescence intensity of 59.2). After 4 days, an additional slight elevation in the number of $CD11b^+$ cells is observed (76.4% with a mean associated fluorescence of 67.2). CD33 is normally decreased during the maturation of myeloid precursors toward the granulocyte. The basal expression of this antigen on NB4 and the mean associated fluorescence (68.0% and 42.8, respectively) is not affected after 3 days in culture with G-CSF or ATRA alone. The combination of ATRA and G-CSF decreases the number of $CD33^+$ cells (47.5% with a mean fluorescence of 40.7). After 4 days in culture, NB4 exposed to ATRA alone showed a very minor decrease in the expression of CD33 (56.3% positive cells with a mean fluorescence of 42.3) whereas the simultaneous addition of G-CSF leads to a more marked reduction in the percent of positive cells (37.5% with a mean fluorescence of 42.5). CD16 is never expressed on the plasma membrane of NB4 cells either in the absence or in the presence of ATRA, G-CSF, and the combination of the two compounds.

ATRA increases the expression of the G-CSF receptor mRNA. To understand the cross-talk between retinoids and G-CSF in NB4 cells, the levels of the transcripts coding for the various forms of RAR and for the G-CSF receptor were investigated and the results are shown in Fig 7. In basal conditions, at time 0 (data not shown) as well as after 16 and 96 hours in the presence of growth medium, NB4 cells express at least three different transcripts for the RARα, including the aberrant form derived from the fusion between the PML and the retinoid receptor. The levels of the various forms of RARα are slightly increased by treatment with ATRA or the combination of ATRA and G-CSF but not by G-CSF alone at 16 and particularly at 96 hours. NB4 cells left in basal medium for 0 (data not shown), 16, and 96 hours do not contain appreciable amounts of RARβ; however, ATRA but not G-CSF induces its expression already after 16 hours of incubation. The levels of RARβ remain constant up to 96 hours in the presence of the retinoid. G-CSF, in combination with ATRA, does not further augment the amounts of RARβ relative to the retinoid alone. The

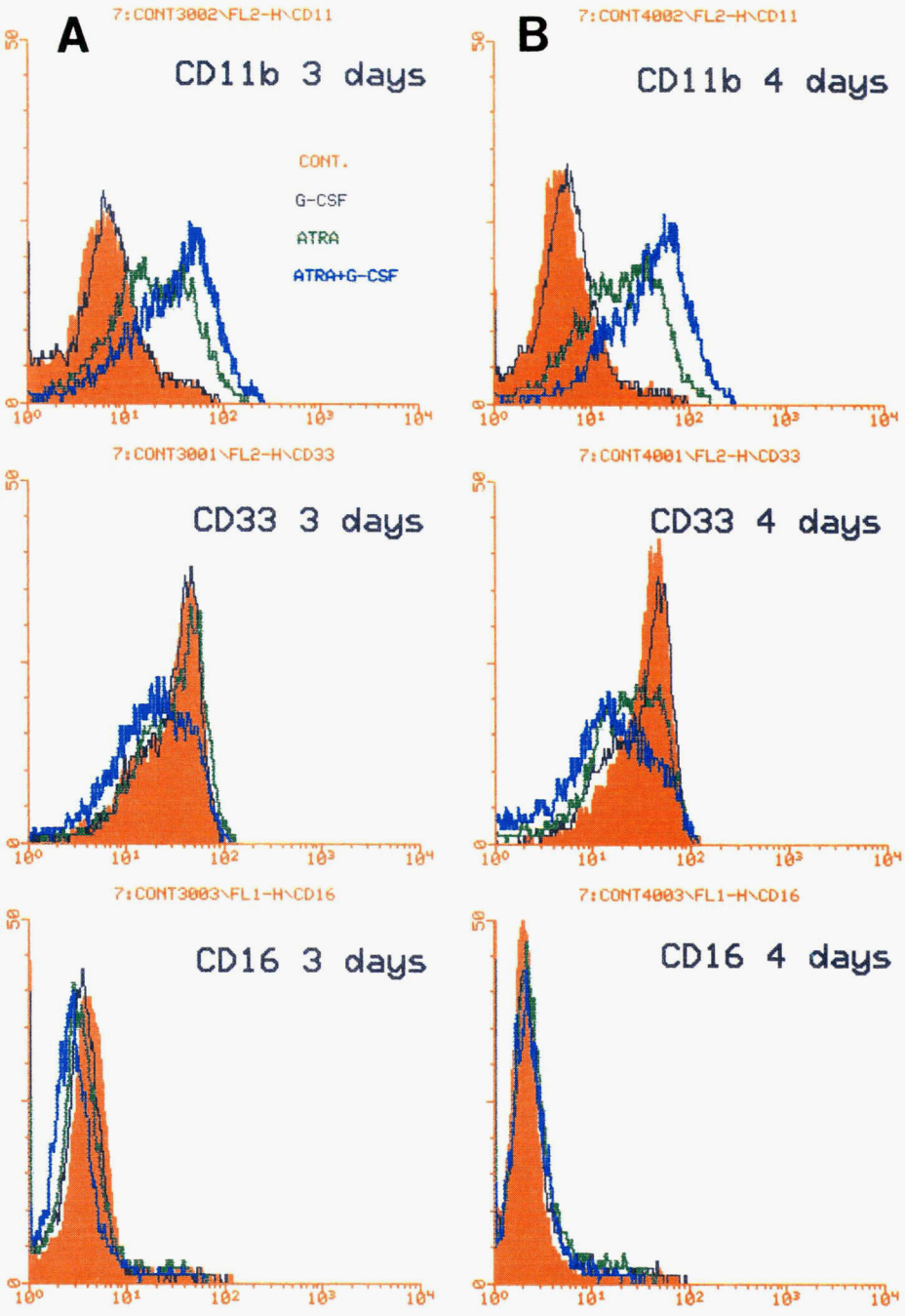

Fig 6. Effect of G-CSF, ATRA, and the combination of the two compounds on the expression of the myeloid surface markers CD11b, CD33, and CD16. NB4 cells (1×10^5/mL) were incubated with medium alone (red tracing), G-CSF (10 ng/mL, black tracing), ATRA (10^{-5} mol/L, green tracing), or the combination of the two compounds (blue tracing) for the indicated amount of time. The number of cells positive for the expression of CD11b, CD33, and CD16 and the mean associated fluorescence were quantitated by flow cytometry. The fluorescence-activated cell sorter tracings for cells decorated with the irrelevant MoAb 3C11C8 are not shown in the figure because they were superimposable to those obtained for CD16. The values for the percentage of positive cells (A) and the mean-associated fluorescence (B) were as follows: (A) (Control [3.0, 2.7]; G-CSF [2.3, 2.4]; ATRA [3.8, 1.5]; ATRA + G-CSF [1.9, 0.4]). (B) (Control [38.3, 34.2]; G-CSF [41.7, 33.0]; ATRA [35.3, 28.0]; ATRA + G-CSF [32.6, 23.6]).

transcript for RARγ, as determined by Northern blotting analysis, is not expressed in this cell line either in basal conditions or after treatment with G-CSF, ATRA, or the combination of the two compounds (data not shown). As shown at the bottom of Fig 7, remarkable changes in the amounts of G-CSF receptor mRNA are observed as a consequence of ATRA treatment. After 0 (data not shown), 16, or 96 hours of incubation in the presence of medium alone, similar levels of a G-CSF receptor message with an apparent size of 2.5 kb is expressed in NB4 cells. Whereas G-CSF does not affect the expression of this mRNA, the transcript is induced approximately 10-fold after treatment with ATRA for 16 hours and its levels remain constant up to 96 hours. Cotreatment of cells with ATRA and G-CSF does not further increase the expression of the G-CSF receptor transcript.

DISCUSSION

LAP is a membrane protein that is expressed at high levels in neutrophilic granulocytes[34,35] and it is a marker for the terminal differentiation of this cell type.[33,35] The specific ac-

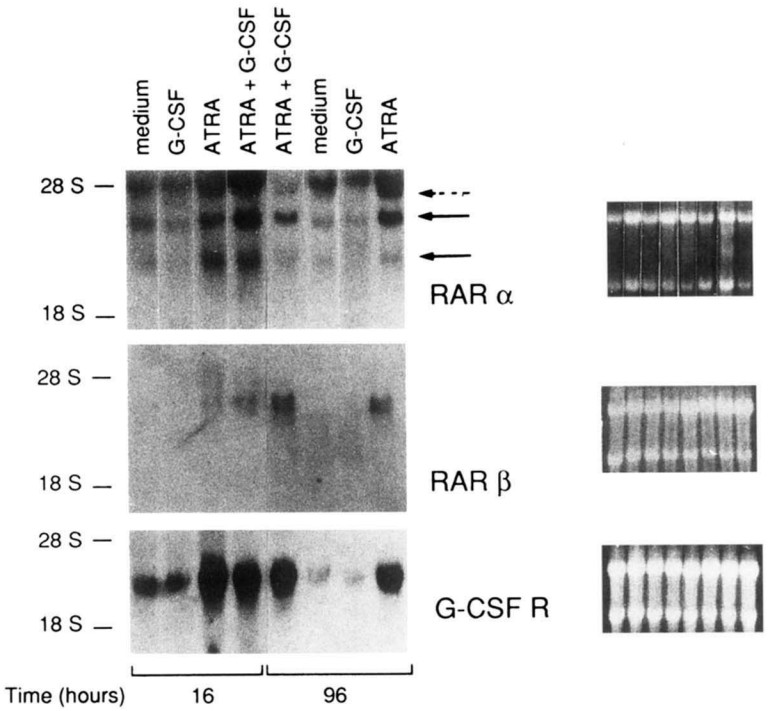

Fig 7. Effect of G-CSF, ATRA, and the combination of the two compounds on the steady-state levels of RARα and β, and G-CSF receptor mRNAs. Total RNA was extracted from NB4 cells treated with G-CSF (10 ng/mL), ATRA (10^{-5} mol/L), or the combination of the two compounds for the indicated amount of time. Three equivalent aliquots (20 μg each) of the extracted RNA were loaded on separate gels and subjected to Northern blotting analysis. The three membranes were hybridized with probes specific for RARα and β, and G-CSF receptor, respectively. The experiment was repeated a second time with similar results. On the right, the position of the PML-RARα fusion mRNA is indicated by a dotted arrow, whereas the position of the other RARα transcripts is indicated by solid arrows. The ethidium bromide staining of each gel is shown on the right side and it shows that an approximately equal amount of RNA was added in each lane.

tivity of the enzyme increases with cell aging and it is particularly high in the fraction of the circulating cells marginalized on the endothelium of blood vessels.[59-61] In neutrophils, the expression of LAP protein is under a short- and a long-term regulation. A rapid translocation and activation of the protein from specific intracellular granules to the outer cell membrane is caused by chemotactic agents.[36] Long-term regulation of the steady-state levels of the protein is achieved through the control of the expression of the gene coding for LAP by physiologic and pharmacologic regulators of the myeloid cells such as ATRA and G-CSF.[35,62] In CMML and in CML cells, during the stable phase of the disease, LAP mRNA can be specifically upregulated by treatment with G-CSF both in vitro[35] and in vivo.[37]

In this report, we show a dramatic induction of LAP activity in freshly isolated APL blasts, as well as in their derivative cell line NB4 after in vitro treatment with the combination of ATRA and G-CSF. This effect is not observed in normal granulocytes, monocytes, and in two AML cell lines. Furthermore, although a weak synergistic interaction between the retinoid and the cytokine has been observed in CML by Sato et al[54,55] and confirmed in this study, induction of LAP by simultaneous treatment with ATRA and G-CSF is much less efficient in this cell type than in APL blasts. Interestingly, upregulation of the expression of LAP by the combination of ATRA and G-CSF is also evident in HL-60, a cell line of APL origin, frequently used as a paradygm of normal in vitro cell differentiation toward the granulocyte. Increased levels of LAP activity in HL-60 show that the cross-talk between ATRA and G-CSF is independent of the PML-RARα fusion protein that is not present in this cell line whereas it is characteristically expressed in APL blasts and in NB4 cells. Because freshly isolated APL blasts as well as NB4 and HL-60 cells are morphologically and biochemically very similar to the promyelocyte, it would be tempting to speculate that strong synergistic interaction between the vitamin A derivative and the cytokine requires not only a cell type that responds to ATRA and G-CSF but also a peculiar cellular differentiation stage. In this respect, it would be interesting to know whether G-CSF and ATRA are acting in concert to induce LAP not only in leukemic promyelocytes at pharmacologic doses but also in the normal conterparts at physiologic concentrations.

The molecular mechanism underlying the induction of LAP was studied in detail in NB4 cells. In this cell line, the induction of LAP is probably the result of both direct and indirect effects of G-CSF and ATRA on the accumulation of the same transcript expressed in normal neutrophilic granulocytes, ie, the mRNA containing exon 1A. The regulation of LAP mRNA by G-CSF in NB4 cells is clearly different from the direct control on the transcript exerted by the cytokine in CML and CMML leukocytes.[35] In the former cell line, the increase in LAP mRNA is triggered by the combination of G-CSF and ATRA, but not by G-CSF alone as in the latter cell types. Furthermore, in NB4, induction of LAP is slow and mainly the result of a transcriptional activation, whereas it is relatively fast and early posttranscriptional in CML and CMML.[35]

In NB4 cells, LAP induction by the cytokine in combination with ATRA is correlated with differentiation toward mature granulocytes. In fact, the expression of LAP is augmented by ATRA and G-CSF when the majority of cells have accumulated granules inside the cytoplasm and present a U-shaped or lobated nucleus. Morphologically, G-CSF

does not have appreciable effects on NB4 cells. When treated with the cytokine alone, NB4 promyelocytes maintain their relative undifferentiated appearance whereas they show the same kind of morphologic differentiation as after incubation with the retinoid alone, when treated with the combination of G-CSF and ATRA. Biochemically, relative to ATRA alone, the combination of G-CSF and the retinoid enhances the expression of CD11b, a molecule present on the cell membrane of mature granulocytes, whereas it slightly decreases the levels of CD33, a marker of early myeloid precursor cells. However, the biochemical differentiation of NB4 is still incomplete and may require the involvement of other cytokines besides G-CSF because it does not lead to the expression of CD16, which is normally present in mature granulocytes.

As to the mechanism of the interaction between the cytokine and the retinoid, theoretically, it is possible that ATRA is sensitizing NB4 promyelocytes to respond to G-CSF or, by converse, G-CSF could render the cells more susceptible to the action of ATRA. The cross-talk between the two compounds on the induction of the expression of the gene encoding LAP might be at multiple points along the cascade of events triggered by the two agents separately. The first elements in the two pathways are represented by the receptors for G-CSF and ATRA, respectively. G-CSF has minor effects on the amounts of the various forms of RAR (α and β) mRNAs expressed in NB4 cells in basal conditions or after treatment with ATRA, whereas ATRA has a strong inducing effect on G-CSF receptor mRNA(s). If the increase in G-CSF receptor transcripts caused by ATRA is accompanied by a concomitant augmentation of the cell surface protein, the synergistic interaction between the retinoid and the growth factor could be easily explained and it would be the result of the sensitization of NB4 cells to the effects of G-CSF by ATRA. However, the scenario is probably more complicated than that and we believe that the upregulation of G-CSF receptors is necessary but not sufficient for the induction of LAP gene expression in NB4 cells. Firstly, increase in G-CSF receptor mRNAs is a relatively fast process, being already maximal within 16 hours after the treatment with ATRA, whereas the induction of the LAP transcript requires at least 3 days. Secondly, treatment of NB4 cells with ATRA for 2 or 3 days followed by washing and addition of G-CSF should lead to an increase in LAP mRNA similar to that observed after simultaneous treatment with the retinoid and the cytokine, which is not observed in our experimental conditions (see Fig 3). Thirdly, the specific activity of LAP after induction with G-CSF and ATRA is linearly dependent on the number of cells incubated with the two compounds. Taken together these data suggest that the induction of LAP is a complex event that requires the simultaneous presence of ATRA and G-CSF and cooperation among NB4 cells, perhaps to maintain critical levels of a secreted factor or to allow cell to cell contact.

Further studies are required to verify whether the cross-talk between G-CSF and ATRA is active only in the regulation of LAP in APL blasts or it is a more general phenomenon involving other cytokine or retinoid regulated genes. Nevertheless, we believe that induction of LAP in NB4 cells represents a good experimental model to elucidate the molecular basis of the cross-talk between ATRA and G-CSF at the single gene level.

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