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# Synergistic Effect of Retinoic Acid and Calcium Ionophore A23187 on Differentiation, c-*myc* Expression, and Membrane Tyrosine Kinase Activity in Human Promyelocytic Leukemia Cell Line HL-60

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**SUMMARY**

The effect of the combination of retinoic acid (RA) and calcium ionophore A23187 on cellular differentiation was assessed in promyelocytic leukemia cell line HL-60. RA ($10^{-10}$–$2.5 \times 10^{-8}$ M) or A23187 ($4 \times 10^{-7}$ M) alone produced 15–22% differentiated cells as assessed by nitroblue tetrazolium reduction. Exposure of cells for 48 hr to the combination of $4 \times 10^{-7}$ M A23187 and $10^{-10}$–$2.5 \times 10^{-8}$ M RA resulted in 20–86% of the cells capable of reducing nitroblue tetrazolium, but with no measurable level of nonspecific esterase activity. The combination of A23187 and either dimethyl sulfoxide, 1,25-dihydroxyvitamin $D_3$, or immune interferon failed to produce a synergistic effect on differentiation. Addition of either the calmodulin antagonists, *N*-(6-aminohexyl)-5-chloronaphthalenesulfonamide and trifluoperazine, or the protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, during treatment with A23187 and RA did not block differentiation. Membrane tyrosine kinase activity was measured in cells treated with A23187 and RA in a nondenaturing gel system using the exogenous substrate poly(Glu:Tyr). Membrane-bound tyrosine kinase activity was not present in untreated or RA-treated cells but was induced by A23187 treatment alone and was markedly increased in cells 48 hr after treatment with the combination of A23187 and RA. Significantly greater reduction in c-*myc* mRNA levels was also observed 24 hr after treatment with RA and A23187 in comparison to that observed with either agent alone. These results suggest that a $Ca^{2+}$-mediated process sensitizes cells to the differentiating effect of RA and that this effect is associated with a significant reduction of c-*myc* expression and the induction of membrane tyrosine kinase activity in this cell line.

Promyelocytic leukemia cell line HL-60 can undergo morphologic changes in response to various differentiating agents which results in a phenotype with the characteristics of monocytes/macrophages or myelocytes/neutrophils (1). Although the events initiating such a process are not known, an early manifestation of differentiation is the reduction in the expression of the amplified protooncogene, c-*myc* (2, 3) when HL-60 cells are induced to differentiate along the myelocytic pathway by RA or DMSO (4) or along the monocytic pathway by either $1,25(OH)_2D_3$ (5) or TPA (6). Also associated with the differentiation process is the early induction of c-*fos* expression by TPA (7) and of c-*fms* expression by TPA or $1,25(OH)_2D_3$ (8). The induction of the latter protooncogene by TPA in the monocytic cell line ML-1 resulted in the appearance of the c-*fms* gene product p140 and its associated tyrosine kinase activity (9). Enhanced tyrosine kinase activity was also observed in the monocytic cell line U937 following treatment with TPA (10).

Recently, it has been theorized that the transduction of extracellular signals into intracellular events proceeds via two major pathways (11). One pathway is mediated by a rise in the diacylglycerol content of the plasma membrane leading to activation of protein kinase C (phospholipid- and $Ca^{2+}$-dependent protein kinase). The second pathway is mediated by a rise in the concentration of cytosolic calcium thereby leading to modulation of calmodulin-dependent reactions. In several stimulus-secretion coupling processes in various cell types, these two pathways seem to function synergistically. Activators of protein kinase C such as diacylglycerol and TPA (12, 13) and the calcium ionophore A23187 can function synergistically to elicit a physiological response in a variety of cell systems including release of serotonin from platelets (14), insulin from

**ABBREVIATIONS:** RA, retinoic acid; DMSO, dimethyl sulfoxide; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; NBT, nitroblue tetrazolium; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; W-7, *N*-(6-aminohexyl)-5-chloronaphthalenesulfonamide hydrochloride; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; IFN-$\gamma$, immune interferon; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; poly(A), polyadenylic acid; SDS; sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; EGTA; ethylene glycol bis($\beta$-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; DTT, dithiothreitol.

pancreatic islets (15), and superoxide production in neutrophils (16, 17).

In the present study, we report the synergistic effect of RA and A23187 on the induction of differentiation in HL-60 cells. This process was further characterized by the level of expression of c-*myc* RNA, as well as the appearance of membrane-bound tyrosine kinase activity found recently to be associated with differentiation in this cell line (18).

## Experimental Procedures

**Materials.** [$\gamma$-$^{32}$P]ATP (2900 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Phenylmethylsulfonyl fluoride, NBT, $\alpha$-napthyl butyrate, RA, poly(Glu:Tyr) (4:1), W-7, and trifluoperazine were purchased from Sigma Chemical Co. (St. Louis, MO). 1,25(OH)$_2$D$_3$ was a gift from Dr. Milan R. Uskokovic, Hoffman La Roche Inc. (Nutley, NJ), H-7 was generously provided by Dr. Hiroyoshi Hidaka, Mie University School of Medicine (Tsu, Japan). IFN-$\gamma$ ($10^6$ units/mg) was purchased from Cellular Products (Buffalo, NY). Nick-translated c-*myc* DNA probe ($3 \times 10^8$ dpm/$\mu$g), a Cla-EcoR1 subclone of human c-*myc* third exon, was obtained from Oncor, Inc. (Gaithersburg, MD). The $\beta$-tubulin c-DNA probe (pT-2) was a generous gift from Dr. Don W. Cleveland, The Johns Hopkins University School of Medicine (Baltimore, MD). The probe was labeled ($5.8 \times 10^8$ dpm/$\mu$g) by nick translation by Lofstrand Laboratories (Gaithersburg, MD).

**Cell culture.** HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 40 mM Hepes (pH 7.4), and gentamicin, 50 $\mu$g/ml, at 37° under an air atmosphere containing 5% $CO_2$. Cell inocula were $5 \times 10^6$ cells/10 ml of medium in 25-cm$^2$ plastic flasks, $1.5 \times 10^7$ cells/30 ml of medium in 75-cm$^2$ flasks, or $5 \times 10^7$ cells/100 ml of medium in 175-cm$^2$ flasks. Cell number was determined with a model ZM Coulter counter and viability was determined by the ability of cells to exclude trypan blue.

**Histochemical assays.** Nonspecific esterase activity was measured in Cytospin preparations by the method of Ansley and Ornstein (19) using $\alpha$-naphthyl butyrate as substrate. The ability of cells to reduce NBT to formazan was assessed by the method described by Breitman *et al.* (20).

**Measurement of c-*myc* and $\beta$-tubulin mRNA.** Total cellular RNA was extracted in 6 M urea:3 M LiCl (21). Poly(A)-enriched RNA was fractionated by passage through an oligo(dT)-cellulose column. Non-poly(A)RNA was eluted in 10 mM Tris-HCl(pH 7.6):0.5% SDS:0.5 M NaCl buffer, and poly(A)RNA was then eluted with 10 mM Tris-HCl (pH 7.6):0.5% SDS buffer (21). RNA was precipitated with 2 volumes of ethanol at −20° and quantitated by UV absorbance. Eight $\mu$g of poly(A)RNA were denatured with 7.4% (v/v) formaldehyde and 50% (v/v) formamide and separated electrophoretically in 1% agarose gels containing 6.6% formaldehyde:40 mM Hepes (pH 7):10 mM sodium acetate:2 mM EDTA, and the latter buffer without formaldehyde was used as the running buffer. Gels were run at 25 V overnight at room temperature. Transcript size was estimated by a comparison with rRNA markers which were detected by ethidium bromide staining. The RNA in the gel was transferred electrophoretically to Zeta Probe membrane (Bio-Rad, Richmond, CA) for 1 hr at 0.3 amp and then 2 hr at 1 amp at 4° in a buffer containing 25 mM $Na_2HPO_4$ (pH 6.5). RNA blots were baked for 2 hr at 80° under vacuum and prehybridized for 2 hr at 37° in 50% formamide:0.1% SDS:0.001% diethylpyrocarbonate:5× SSC (1 × = 0.15 M NaCl:0.015 M sodium citrate):50 mM $Na_2HPO_4$ (pH 6.5):200 $\mu$g/ml of yeast RNA:200 $\mu$g/ml of sonicated denatured salmon sperm DNA:0.1% Ficoll:0.1% bovine serum albumin:0.1% polyvinylpyrrolidone. Hybridization was carried out for 16–18 hr at 37° in the prehybridization solution containing 10% dextran sulfate and approximately $2 \times 10^6$ dpm/ml of c-*myc* or $\beta$-tubulin [$^{32}$P]DNA probe. After hybridization, blots were washed three times with 1× SSC:0.1% SDS for 10 min at room temperature and three times with 0.1× SSC:0.1% SDS for 10 min at 50°. Blots were dried and autoradiographed using Kodak X-Omat XAR-5 film. The mRNA levels were quantitated by densitometry of the autoradiographs (Hoefer Scientific GS 300 scanning densitometer).

**Preparation of cell-free extracts.** Cells were washed twice with Hanks' balanced salts solution deficient in $Ca^{2+}$ and $Mg^{2+}$ and containing 20 mM EDTA and twice with this buffer lacking EDTA. The cell pellet was suspended in 50–100 $\mu$l of buffer containing 50 mM Tris-HCl (pH 7.5):1 mM phenylmethylsulfonyl fluoride:2 mM EGTA:10 mM DTT:5 $\mu$g/ml of aprotinin:0.1% Triton X-100, sonicated for 5 sec at 4°, and centrifuged for 6 min at 15,000× *g* at 4° in an Eppendorf microcentrifuge. The pellet was then extracted in the same manner with buffer containing 1% Triton X-100. Protein concentrations in the 0.1 and 1% Triton X-100 extracts were determined by the method of Bradford (22) with bovine serum albumin as a standard.

**Tyrosine kinase assay.** One-tenth and 1% Triton X-100 extracts containing 25 $\mu$g of protein were dissolved in sample buffer containing 63 mM Tris-HCl (pH 6.8):5 mM DTT:10% glycerol:0.001% bromophenol blue and separated electrophoretically at 4° in 13.5 × 14 cm gels of 0.75 mm thickness [4.5% acrylamide:0.24% bisacrylamide gel and containing 0.375 M Tris-HCl (pH 8.8):10% glycerol] using 25 mM Tris-HCl (pH 8.3):0.192 M glycine as the running buffer. Electrophoresis was carried out at 60 V for 1 hr and then 200 V for 4 hr. After electrophoresis, the gel was soaked twice with cold 20 mM Hepes (pH 7.4) for 15 min and soaked in 20 mM Hepes containing 5 mg/ml poly(Glu:Tyr) (4:1) (6 ml/gel) at room temperature for 30 min. Gels were then incubated at 37° for 30 min with [$\gamma$-$^{32}$P]ATP (33 $\mu$Ci/ml):20 mM Hepes (pH 7.4):1 mM CTP:10 mM $MgCl_2$:0.2 M EGTA:1 mM DTT:50 $\mu$M ATP:3 mM $MnCl_2$:5 mg/ml poly(Glu:Tyr) (4:1) (6ml/gel). After incubation, gels were rinsed once with water and then washed at room temperature on a shaker with 5% trichloroacetic acid:0.01 M sodium pyrophosphate until less than 500 counts were detected in the washes (usually five 30-min washes). Gels were dried and autoradiographed using Kodak X-Omat XK-1 film.

## Results

**Induction of differentiation of HL-60 cells by RA and A23187.** The differentiation of HL-60 cells was assessed by measuring their capacity to reduce NBT, as well as their nonspecific esterase activity. Fig. 1 illustrates the morphology of the cells treated with RA and A23187 after the NBT reaction. Treatment of cells for 48 hr with either $10^{-10}$–$2.5 \times 10^{-8}$ M RA or $4 \times 10^{-7}$ M A23187 produced 17% and 22% NBT-positive cells, respectively. The combination of $4 \times 10^{-7}$ M A23187 with $10^{-10}$–$2.5 \times 10^{-8}$ M RA produced a dose-dependent increase in NBT-positive cells (Fig. 2) with no discernable elevation in nonspecific esterase activity (results not shown). The extent of differentiation was dose-dependent for A23187 to a concentration of $4 \times 10^{-7}$ M (Fig. 3), and was maximal after a 48-hr exposure interval (Fig. 4). A23187 ($10^{-6}$ M) alone or in combination with RA reduced cell viability by 50%, whereas lower concentrations were not cytotoxic. The synergistic effect exhibited by the combination of A23187 and RA did not occur with other differentiating agents such as IFN-$\gamma$, DMSO, or 1,25(OH)$_2$D$_3$ (results not shown).

**The effect of antagonists of protein kinase C and calmodulin.** In order to discern whether protein kinase C- or calmodulin-dependent processes were directly involved in the induction of differentiation by A23187 and RA, cells were treated simultaneously with either the protein kinase C inhibitor, H-7, or the calmodulin antagonists, W-7 and trifluoperazine, at maximally permissive concentrations as determined by dose-response cytotoxicity assays (Table 1). Neither H-7 ($10^{-5}$ M), W-7 ($2 \times 10^{-4}$ M), nor trifluoperazine ($10^{-5}$ M) blocked differentiation induced after exposure to RA and A23187. Tri-

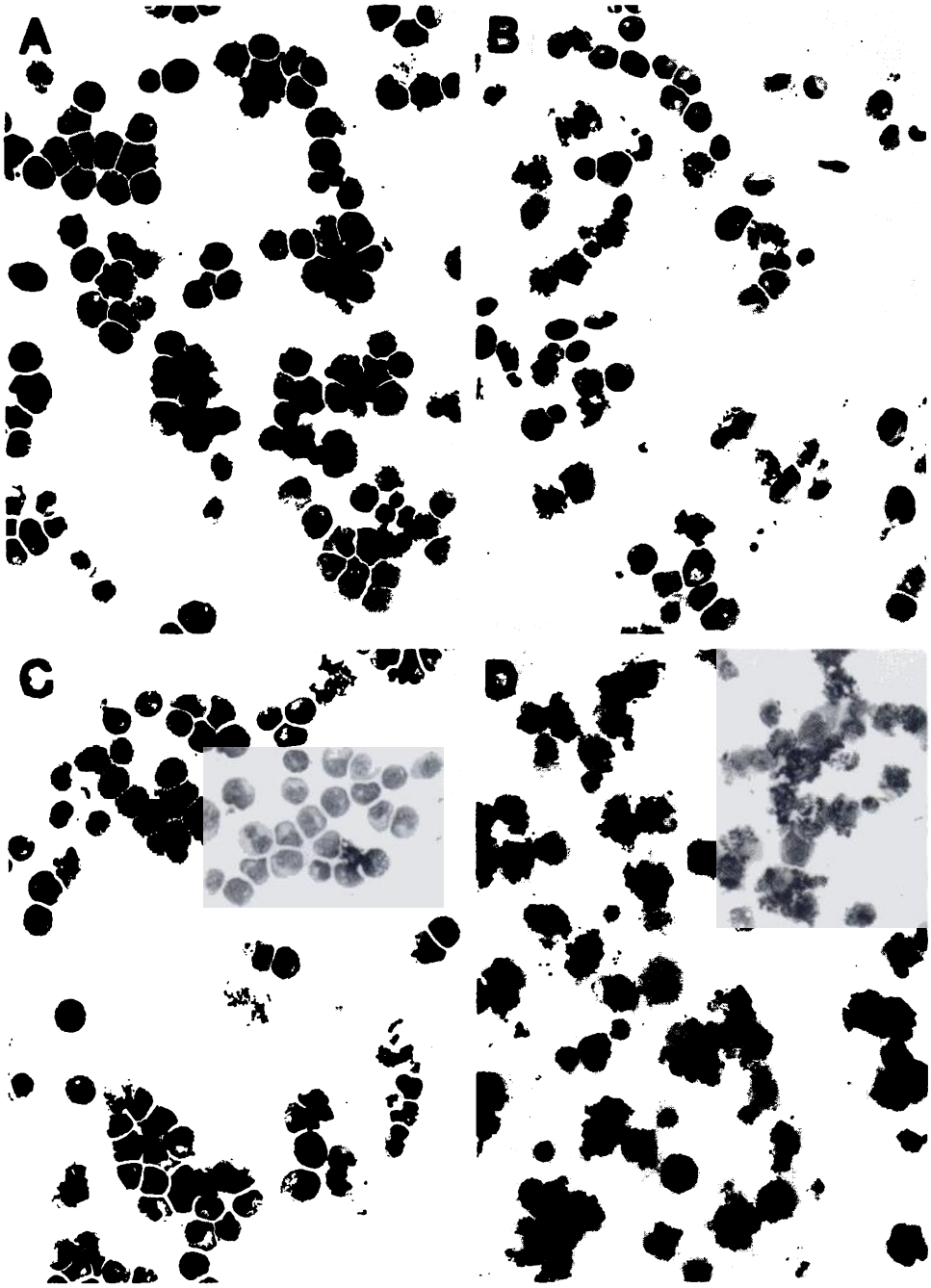


**Fig. 1.** Morphology of HL-60 cells treated with A23187 and RA after NBT reduction. Cytospin slide preparations of cells following the NBT reaction were stained with Wright-Giemsa. A, Uninduced cells; B, cells treated for 48 hr with $4 \times 10^{-7}$ M A23187; C, cells treated for 48 hr with $2.5 \times 10^{-8}$ M RA; and D, cells treated for 48 hr with the combination of $2.5 \times 10^{-8}$ M RA and $4 \times 10^{-7}$ M A23187.

fluoperazine alone resulted in a small increase in NBT-positive cells, whereas W-7 and H-7 were without effect on differentiation.

**c-*myc* mRNA levels.** HL-60 cells are known to possess the amplified cellular oncogene c-*myc* (2, 3). To assess whether the expression of c-*myc* was altered after treatment for 24 hr with RA and A23187, Northern blot analysis of poly(A)RNA was carried out (Fig. 5). In order to compare the selective changes in c-*myc* mRNA content, the RNA blot was hybridized with a $^{32}$P-labeled c-*myc* DNA probe and autoradiographed, followed by removal of the c-*myc* signal and rehybridization with a $\beta$-tubulin DNA probe (Fig. 5). After normalizing the changes in c-*myc* mRNA expression relative to the changes in $\beta$-tubulin mRNA, it was found that c-*myc* mRNA was preferentially reduced by either $2.5 \times 10^{-8}$ M RA or $4 \times 10^{-7}$ M A23187; however, a greater than additive reduction in c-*myc* mRNA was observed after treatment with the combination of RA and A23187 (Fig. 5). The reduction in c-*myc* mRNA after RA and/or A23187 treatment correlated with their respective effects on differentiation and preceded the appearance of NBT-positive cells (Fig. 4).

**Tyrosine kinase activity.** We have observed recently the appearance of membrane-bound tyrosine kinase activity in association with the mature monocyte phenotype in HL-60 cells produced by immune interferon and tumor necrosis factor (18). Thus, tyrosine kinase activity was measured in 1% Triton X-100-solubilized extracts of cells by a nondenaturing electrophoretic gel assay using the synthetic substrate, poly(Glu:Tyr)

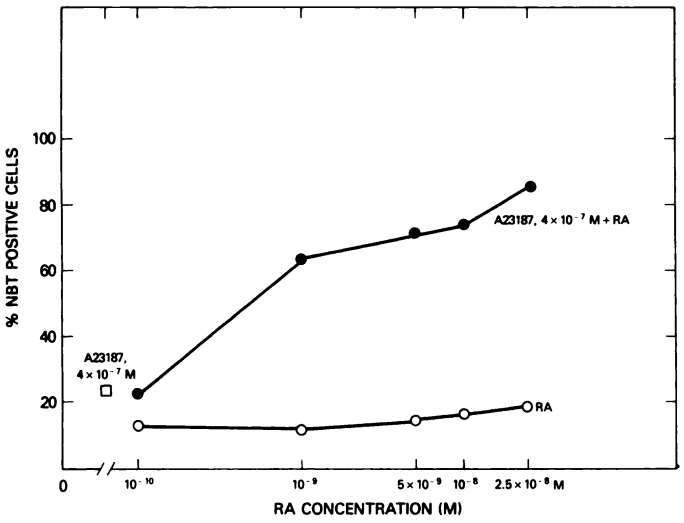


**Fig. 2.** Differentiation of HL-60 cells following treatment with A23187 and varying concentrations of RA. Cells were exposed for 48 hr to 4 × $10^{-7}$ M A23187 and the indicated concentrations of RA, and NBT-positive cells were determined as described in Experimental Procedures. Each value is the mean of three to four experiments where the standard error did not exceed 5%.

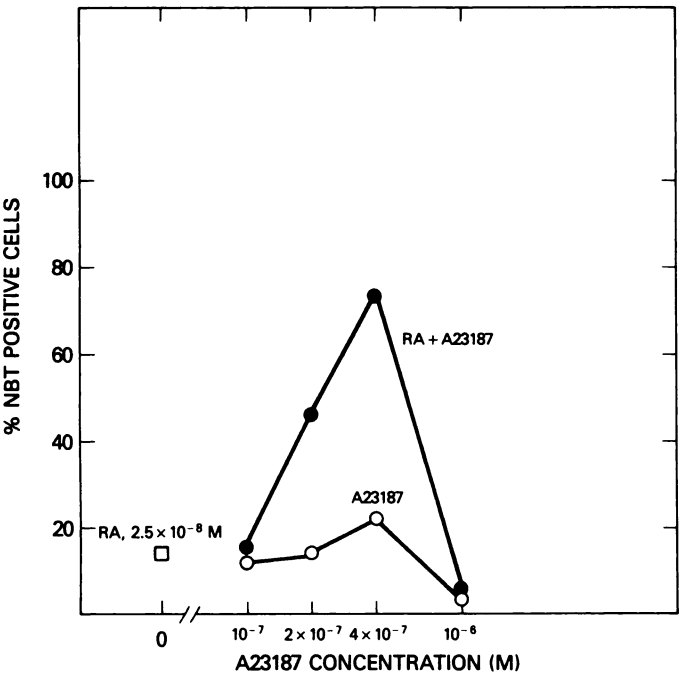


**Fig. 3.** Differentiation of HL-60 cells after treatment with RA and varying concentrations of A23187. Cells were exposed for 48 hr to 2.5 × $10^{-8}$ M RA and the indicated concentrations of A23187, and NBT-positive cells were determined as described in Experimental Procedures. Each value is the mean of three experiments where the standard error did not exceed 5%.

(Fig. 6, *lanes 5–8*). A marked elevation in membrane-bound tyrosine kinase activity was observed after 48 hr of exposure to a combination of 2.5 × $10^{-8}$ M RA and 4 × $10^{-7}$ M A23187. RA at 2.5 × $10^{-8}$ M did not increase tyrosine kinase activity, but 4 × $10^{-7}$ M A23187 resulted in a moderate induction of activity. In order to assess the contribution of autophosphorylation to the tyrosine kinase activity detected in the gel, assays were conducted in the absence of poly(Glu:Tyr) (Fig. 6, *lanes 1–4*).

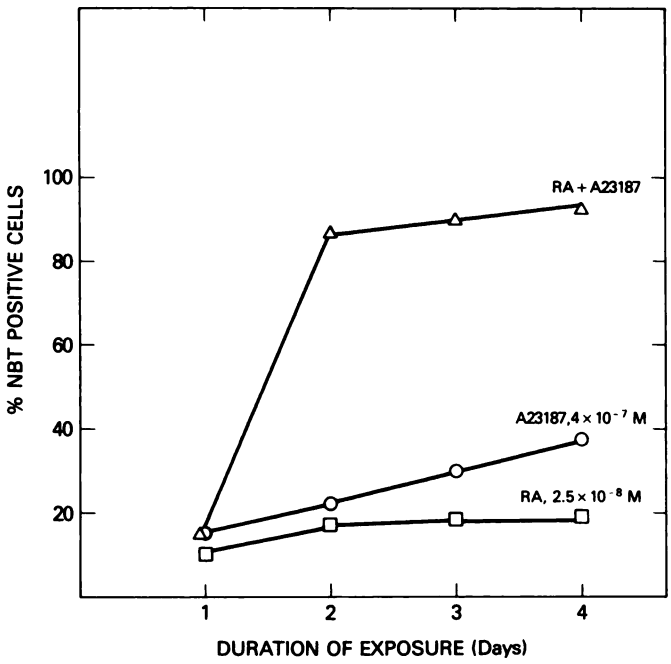


**Fig. 4.** Time course of the induction of differentiation by RA and A23187. Cells were exposed to 2.5 × $10^{-8}$ M RA and 4 × $10^{-7}$ M A23187, and NBT-positive cells were determined at the indicated time period as described in Experimental Procedures. Each value is the mean of three to four experiments where the standard error did not exceed 5%.

TABLE 1

**Effect of trifluoperazine, W-7, or H-7 on proliferation and differentiation in retinoic acid- and A23187-treated cells**

Cells were cultured with RA and A23187 in the presence and absence of either W-7, H-7, or trifluoperazine for 48 hr, and cell number, cell viability, and NBT-positive cells were determined as described under Experimental Procedures.

| Treatment | Cell growth (% of control) | Cell viability (% of cells excluding trypan blue) | Percentage of NBT-positive cells |
|---|---|---|---|
| Control | 100 | 100 | 8 |
| A23187, 4 × $10^{-7}$ M | 75 | 92 | 30 |
| RA, 2.5 × $10^{-8}$ M | 101 | 94 | 14 |
| Trifluoperazine, 1 × $10^{-5}$ M | 76 | 95 | 24 |
| W-7, 2 × $10^{-4}$ M | 104 | 86 | 10 |
| H-7, 1 × $10^{-5}$ M | 66 | 97 | 11 |
| RA + A23187 | 67 | 87 | 84 |
| RA + A23187 + Trifluoperazine | 67 | 67 | 90 |
| RA + A23187 + W-7 | 68 | 74 | 72 |
| RA + A23187 + H-7 | 65 | 75 | 91 |

Minimal autophosphorylation was observed in the extracts from A23187- and RA-treated cells, indicating that the assay detected mainly tyrosine phosphorylation.

## Discussion

Several studies have explored the synergism between calcium ionophore A23187 and activators of protein kinase C such as TPA. This combination of agents increases synergistically amylase secretion by pancreatic acini (23), activation of glycogen phosphorylase in hepatocytes (24), and activation of the respiratory burst in neutrophils (16, 17, 25). Similar investigations with RA and A23187 have not been reported, but RA is known to potentiate the differentiation-inducing ability of interferon-$\alpha$ and -$\beta$ (26), as well as cholera toxin, prostaglandin $E_2$, dibutyryl cAMP, and a T lymphocyte differentiation-inducing

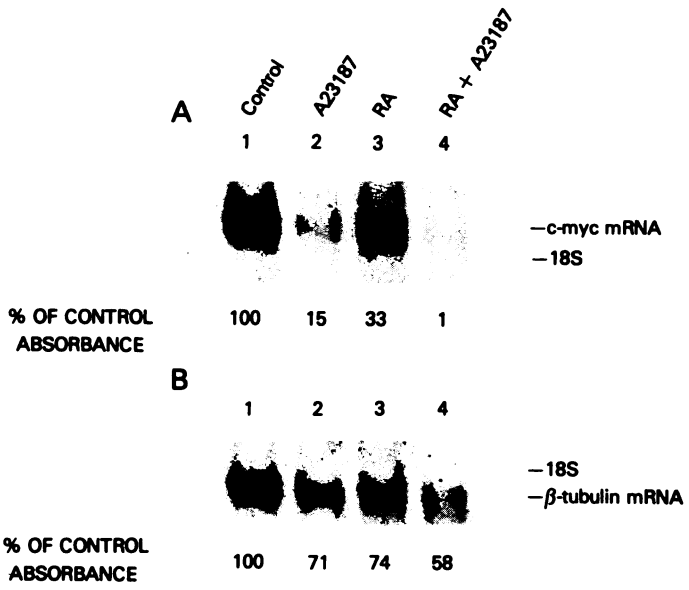


**Fig. 5.** c-*myc* RNA expression in HL-60 cells following 24 hr treatment with A23187 and RA. Eight μg of poly(A)RNA were size fractionated by agarose gel electrophoresis and transferred by Northern blotting as described in Experimental Procedures. A, Autoradiograph of the blot hybridized with [$^{32}$P]c-*myc* DNA; B, autoradiograph of the same blot after dehybridization of the c-*myc* DNA and rehybridization to a [$^{32}$P]β-tubulin DNA probe. *Lane 1*, control; *lane 2*, A23187 ($4 \times 10^{-7}$ M)-treated cells; *lane 3*, RA ($2.5 \times 10^{-8}$ M)-treated cells; *lane 4*, A23187 + RA-treated cells. Absorbance was measured by densitometric scanning of the autoradiograph as described under Experimental Procedures.

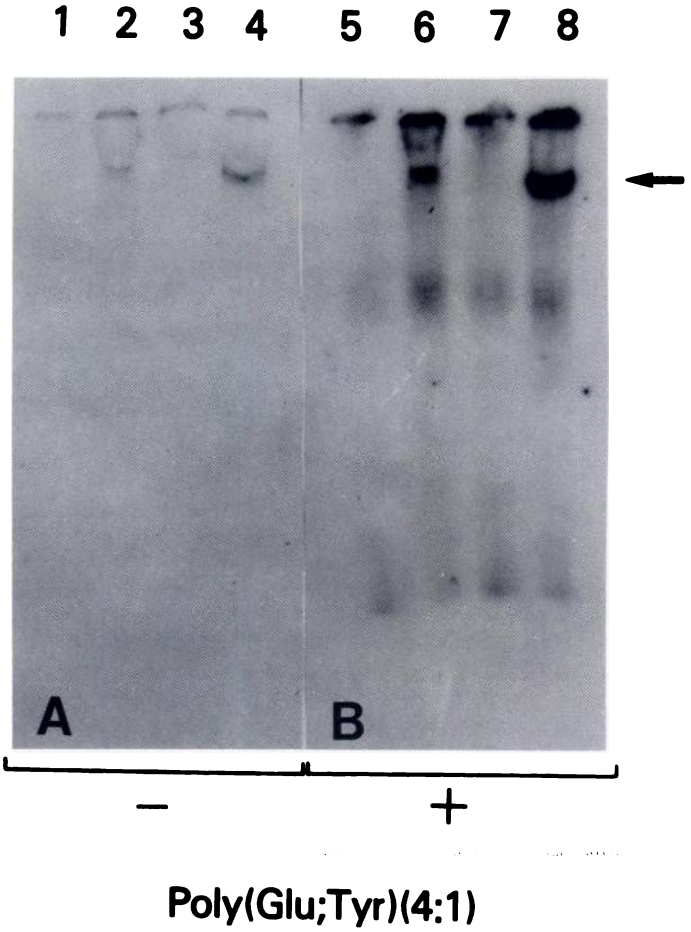


**Fig. 6.** Tyrosine kinase activity in extracts from HL-60 cells. Tyrosine kinase in 1% Triton extracts was measured using a nondenaturing gel assay system without (A) and with (B) poly(Glu:Tyr) as substrate as described in Experimental Procedures. Tyrosine kinase activity in untreated cells (*lanes 1* and *5*), and cells treated for 48 hr with either $4 \times 10^{-7}$ M A23187 (*lanes 2* and *6*), $2.5 \times 10^{-8}$ M RA (*lanes 3* and *7*), or RA and A23187 (*lanes 4* and *8*).

factor (27, 28). In the present investigation, we have documented the synergistic induction of differentiation after treatment of HL-60 cells with combinations of suboptimal concentrations of RA and A23187. This induction was dose dependent for RA and specific for RA-treated cells since DMSO, IFN-γ, or 1,25(OH)$_2$D$_3$ failed to elicit a synergistic response in combination with A23187. In order to explore whether protein kinase C- or calmodulin-dependent reactions were involved in the induction of differentiation by RA and A23187, H-7, an inhibitor of protein kinase C (29), and the calmodulin antagonists, W-7 (30) and trifluoperazine (31) were used. None of these agents at minimally toxic concentrations could block the induction of differentiation by RA and A23187, indicating that activation of protein kinase C or stimulation of calmodulin-dependent pathways was not directly involved in the process of differentiation. Our results are consistent with a recent report by Matsui *et al.* (32), wherein the calmodulin antagonists W-7 and trifluoperazine failed to block the differentiation of HL-60 cells treated with either RA, 1,25(OH)$_2$D$_3$, or TPA.

The c-*myc* protooncogene is greatly amplified in HL-60 cells (2, 3) and a marked reduction in c-*myc* expression was observed after the induction of differentiation by DMSO, 1,25(OH)$_2$D$_3$, IFN-γ, or TPA (4–6). Kelly *et al.* (33) have shown a substantial cell cycle-specific induction of c-*myc* RNA in response to mitogens and growth factors, and a significant reduction in c-*myc* mRNA levels was evident in human lymphoma (Daudi) cells treated with IFN-β in response to the growth inhibitory properties of this lymphokine (34). In a report by Beimling *et al.* (35), the translation product of c-*myc*, a protein of 64 kDa, was found to be associated with the nuclear matrix in human colon carcinoma cells. These investigations suggest that c-*myc* may regulate the ability of cells to traverse the cell cycle and enter the G$_1$ phase and may be closely associated with the initial events leading to cellular differentiation or proliferation. The early reduction in c-*myc* expression preceding the varying extents of morphologic differentiation produced by treatment of HL-60 cells with either RA, A23187, or the combination of the two agents agrees with this concept and suggests that c-*myc* expression is closely coupled to differentiation in this cell system.

Several lines of evidence implicate tyrosine kinases in the regulation of proliferation of normal and malignant cells. Receptors for growth factors such as epidermal growth factor and platelet-derived growth factor possess tyrosine kinase activity (36–38). Recent studies (39, 40) have indicated that, although total tyrosine kinase activity in leukemic and lymphocytic cells appears to be unchanged, differences in individual tyrosine kinase activities or the substrates phosphorylated by these kinases may be associated with the regulation of growth, as illustrated by the inhibition of topoisomearse I activity after its phosphorylation by a tyrosine kinase from liver or pp60$^{src}$ (41). The present observation, that a membrane-bound tyrosine kinase activity is associated with the appearance of the differentiated phenotype, suggests that it may either play a role in the differentiation process or mediate membrane-dependent processes associated with the mature cell type.

In summary, the present study has shown that the calcium ionophore, A23187, and RA act synergistically on HL-60 cells to enhance their differentiative response, and that this effect is exemplified by an early reduction in c-*myc* expression and the appearance of a differentiation-associated tyrosine kinase activity. Further studies to characterize the latter activity and its regulation during differentiation are in progress.

**References**


1. Hemmi, H., and T. R. Breitman. Induction of differentiation of the human promyelocytic cell line HL-60 and primary cultures of human leukemia cells: a model for clinical treatment, in: *Developments in Cancer Chemotherapy* (R. I. Glazer, ed.). CRC Press, Boca Raton, FL, 247–280 (1984).
2. Dalla Favera, R., F. Wong-Staal, and R. C. Gallo. *Onc* gene amplification in promyelocytic cell line HL-60 and primary leukemia cells of the same patient. *Nature (Lond.)* **299**:61–63 (1982).
3. Collins, S., and M. Groudine. Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukemia cell line. *Nature (Lond.)* **298**:679–681 (1982).
4. Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo. Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **79**:2490–2494 (1982).
5. Reitsma, P. H., P. G. Rothberg, S. M. Astrin, J. Trial, Z. Bar-Shavit, A. Hall, S. L. Teitelbaum, and R. C. Gallo. Regulation of *myc* gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature (Lond.)* **306**:492–494 (1983).
6. Watanabe, T., E. Sariban, T. Mitchell, and D. Kufe. Human c-*myc* and N-*ras* expression during induction of HL-60 cellular differentiation. *Biochem. Biophys. Res. Commun.* **126**:999–1005 (1985).
7. Müller, R., T. Curran, D. Müller, and L. Guilbert. Induction of c-*fos* during myelomonocytic differentiation and macrophage proliferation. *Nature (Lond.)* **314**:546–548 (1985).
8. Sariban, E., T. Mitchell, and D. Kufe. Expression of the c-*fms* proto-oncogene during human monocytic differentiation. *Nature (Lond.)* **316**:64–66 (1985).
9. Woolford, J., V. Rothwell, and L. Rohrschneider. Characterization of the human c-*fms* gene product and its expression in cells of the monocyte-macrophage lineage. *Mol. Cell. Biol.* **5**:3458–3466 (1985).
10. Grunberger, G., Y. Zick, S. I. Taylor, and P. Gorden. Tumor-promoting phorbol ester stimulates tyrosine phosphorylation in U-937 monocytes. *Proc. Natl. Acad. Sci. USA* **81**:2762–2766 (1984).
11. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.)* **308**:693–698 (1984).
12. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.* **257**:7847–7851 (1982).
13. Mori, T., Y. Takai, B. Yu, J. Takahashi, Y. Nishizuka, and T. Fujikura. Specificity of the fatty acyl moieties of diacylglycerol for the activation of calcium-activated, phospholipid-dependent protein kinase. *J. Biochem.* **91**:427–431 (1982).
14. Kaibuchi, K., Y. Takai, M. Sawamura, M. Hoshijima, T. Fukikura, and Y. Nishizuka. Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J. Biol. Chem.* **258**:6701–6704 (1983).
15. Zawalich, W., C. Brown, and H. Rasmussen. Insulin secretion: combined effects of phorbol ester and A23187. *Biochem. Biophys. Res. Commun.* **117**:448–455 (1983).
16. Robinson, J. M., J. A. Badwey, M. L. Karnovsky, and M. J. Karnovsky. Superoxide release by neutrophils: synergistic effect of a phorbol ester and a calcium ionophore. *Biochem. Biophys. Res. Commun.* **122**:734–739 (1984).
17. Penfield, A., and M. M. Dale. Synergism between A23187 and 1-oleoyl-2-acetylglycerol in superoxide production by human neutrophils. *Biochem. Biophys. Res. Commun.* **125**:332–336 (1984).
18. Glazer, R. I., M. S. Chapekar, and M. C. Knode. Measurement of membrane-bound tyrosine kinase activity during differentiation of HL-60 leukemia cells by immune interferon and tumor necrosis factor. *Biochem. Biophys. Res. Commun.* **140**:908–915 (1986).
19. Ansley, H., and L. Ornstein. Enzyme histochemistry and differential white cell counts on the Technicon hemalog D, in *Advances in Automated Analysis* (Technicon Corp.), Vol. 1. Futura Publishing Co., Mount Kisco, NY, 437–446 (1972).
20. Breitman, T. R., S. E. Selonick, and S. J. Collins. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA* **77**:2936–2940 (1980).
21. Auffray, C., and F. Rougeon. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107**:303–314 (1980).
22. Bradford, M. M. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
23. de Pont, J. J. H. H. M., and A. M. M. Fleuren-Jakobs. Synergistic effect of A23187 and a phorbol ester on amylase secretion from rabbit pancreatic acini. *FEBS Lett.* **170**:64–68 (1984).
24. Fain, J. N., S.-Y. Li, I. Litosch, and M. Wallace. Synergistic activation of the rat hepatocyte glycogen phosphorylase by A23187 and phorbol ester. *Biochem. Biophys. Res. Commun.* **119**:88–94 (1984).
25. DiVirgilio, F., D. P. Lew, and T. Pozzan. Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic $Ca^{2+}$ levels. *Nature (Lond.)* **310**:691–693 (1984).
26. Tomida, M., Y. Yamamoto, and M. Hozumi. Stimulation by interferon of induction of differentiation of human promyelocytic leukemia cells. *Biochem. Biophys. Res. Commun.* **104**:30–37 (1982).
27. Olsson, I. L., and T. R. Breitman. Induction of differentiation of the human histiocytic lymphoma cell line U-937 by retinoic acid and cyclic adenosine 3′:5′-monophosphate-inducing agents. *Cancer Res.* **42**:3924–3927 (1982).
28. Olsson, I. L., T. R. Breitman, and R. C. Gallo. Priming of human myeloid leukemic cell lines HL-60 and U-937 with retinoic acid for differentiation effects of cyclic adenosine 3′:5′-monophosphate-inducing agents and a T lymphocyte-derived differentiation factor. *Cancer Res.* **42**:3928–3933 (1982).
29. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**:5036–5041 (1984).
30. Hidaka, H., Y. Sasaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii, and T. Nagata. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc. Natl. Acad. Sci. USA* **78**:4354–4357 (1981).
31. Schatzman, R. L., B. C. Wise, and J. F. Kuo. Phospholipid-sensitive calcium-dependent protein kinase: inhibition by antipsychotic drugs. *Biochem. Biophys. Res. Commun.* **98**:669–676 (1981).
32. Matsui, T., Y. Nakao, N. Kobayashi, T. Koizumi, T. Nakagawa, M. Kishihara, and T. Fujita. Effects of calmodulin antagonists and cytochalasins on proliferation and differentiation of human promyelocytic leukemia cell line HL-60. *Cancer Res.* **45**:311–316 (1985).
33. Kelly, K., B. H. Cochron, C. D. Stiles, and P. Leder. Cell specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell* **35**:603–610 (1983).
34. Jonak, G. J., and E. Knight, Jr. Selective reduction of c-*myc* mRNA in Daudi cells by human $\beta$ interferon. *Proc. Natl. Acad. Sci. USA* **81**:1747–1750 (1984).
35. Beimling, P., T. Benter, T. Sander, and K. Moelling. Isolation and characterization of the human cellular *myc* gene product. *Biochemistry* **24**:6349–6355 (1985).
36. Heldin, C.-H., and B. Westermark. Growth factors: mechanism of action and relation to oncogenes. *Cell* **37**:9–20 (1984).
37. Cohen, S., G. Carpenter, and L. King, Jr. Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation. *J. Biol. Chem.* **255**:4834–4842 (1980).
38. Heldin, C.-H., B. Ek, and L. Ronnstrand. Characterization of the receptor for platelet-derived growth factor on human fibroblasts: demonstration of an intimate relationship with a 185,000 dalton substrate for the platelet-derived growth factor stimulated kinase. *J. Biol. Chem.* **258**:10054–10061 (1983).
39. Wickremasinghe, R. G., A. Piga, A. R. Mire, M. R. Taheri, J. C. Yaxley, and A. V. Hoffbrand. Tyrosine protein kinases and their substrates in human leukemia cells. *Leukemia Res.* **9**:1443–1450 (1985).
40. Piga, A., M. R. Taheri, J. C. Yaxley, R. G. Wickremasinghe, and A. V. Hoffbrand. Higher tyrosine protein kinase activity in resting lymphocytes than in proliferating normal or leukemic blood cells. *Biochem. Biophys. Res. Commun.* **124**:766–773 (1984).
41. Tse-Dinh, Y.-C., T. W. Wong, and A. R. Goldberg. Virus- and cell-encoded tyrosine protein kinases inactivate DNA topoisomerase *in vitro*. *Nature (Lond.)* **312**:785–786 (1984).


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