

## CONCISE REPORT

# Nonspecific Esterases of Leukemia Cell Lines: Evidence for Activation of Myeloid-Associated Zymogens in HL-60 by Phorbol Esters

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**Myeloid leukemia cell line HL-60 contains fluoride-sensitive, myeloid-associated isoenzymes of nonspecific esterase that increase in activity when cultures are treated with phorbol ester. These isoenzymes are not detectable in B-lymphoblast cell line KLM-2, either in control or in**

**phorbol-ester-treated cultures. No increased de novo synthesis of the isoenzymes is detectable in HL-60 treated with phorbol ester. The data suggest stimulated conversion of a preformed, myeloid-associated zymogen in HL-60.**

**T**HE ABILITY of phorbol esters to induce macrophage differentiation of myeloid leukemia cell line HL-60 is well documented<sup>1,2</sup> and has also been reported for a B-lymphoblast line, KLM-2.<sup>3</sup> Monocytoid cytochemical nonspecific esterase reactivity was a key differentiation marker in those studies. Other studies have demonstrated that monocytes contain a set of fluoride-sensitive isoenzymes of nonspecific esterase with a near-neutral isoelectric point (neutral isoenzymes).<sup>4</sup> The neutral isoenzymes were found in 10 of 16 acute myeloid leukemias, but not in any of 30 lymphoid/stem cell leukemias or lymphoblast prepara-

tions.<sup>4,5</sup> We have investigated the response of these myeloid-associated isoenzymes to phorbol-ester treatment of HL-60 and KLM-2.

### MATERIALS AND METHODS

Cell line HL-60<sup>6</sup> was generously provided by Dr. Robert Gallo (National Cancer Institute, Bethesda, MD) and KLM-2<sup>3</sup> by Dr. Jun Minowada (Roswell Park Memorial Institute, Buffalo, NY). For phorbol-ester treatment, sealed 200-ml suspension cultures were grown to log phase in RPMI 1640 medium, with 10% fetal calf serum and 50 µg gentamycin sulfate/ml, pooled, and divided into 2 equal lots. For HL-60, 12-0-tetradecanoylphorbol 13-acetate (TPA, Sigma, St. Louis, MO) in acetone was added to one lot at final concentrations of 10<sup>-8</sup> M TPA and 0.03% acetone. The control lot (with no TPA) was brought to 0.03% acetone. Each lot was redistributed into T flasks, cultured, and harvested at 24-hr intervals from 0 to 72 hr. For KLM-2 the final concentrations in one lot were 1.7 × 10<sup>7</sup> M (100 ng/ml) TPA and 0.05% acetone. The control lot was brought to 0.05% acetone. Each was redistributed into T flasks and harvested immediately or after a 72-hr incubation. Harvested cells were counted, washed, and stored, and extracts were prepared as previously described.<sup>4</sup> Wright's and nonspecific esterase stain of

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**Table 1. Response of Nonspecific Esterase (NSE) in HL-60 and KLM-2 to Phorbol Esters**

Culture and Treatment	Hours of Incubation	Cells/ml (Hundred Thousands)	NSE (% Monocytoid* Cells)	mU/mg Protein		
				αNAE†	αNBE‡	
HL-60	0	10.9	8	32	41	
	24	16.8	8	33	40	
	Acetone Control	48	16.0	16	35	51
		72	17.0	11	47	65
		0	10.9	8	27	39
TPA-treated	24	8.7	50	53	105	
	48	9.2	≥50‡	56	142	
	72	10.1	≥50	86	190	
	0	5.7	0§	13	17¶	
KLM-2	0	5.7	0	13	17	
	Acetone Control	72	8.6	0	14	20
		0	5.7	0	13	17
TPA-treated	72	7.9	0	14	17	

\*NSE cytochemistry of culture cells, expressed as percentage of cells with monocytoid staining characteristics (strong, fluoride-sensitive αNAE reactivity). Results for αNBE were within 5%.

†αNAE, α-naphthyl acetate esterase; αNBE, α-naphthyl butyrate esterase.

‡Estimate for clumped cells on these smears.

§Scan of > 1,000 cells. All cells from each culture displayed negligible-to-weak NSE activity.

¶αNBE activity was nonlinear. Values given for each culture are from assays of equal levels of extract protein.

slide preparations,<sup>4</sup> enzyme assay of cell extracts,<sup>7</sup> and isoelectric focusing (IEF)<sup>4</sup> procedures have been described.

For double-labeling of cytosolic proteins of HL-60, harvested cells were washed and 0.5–0.7 g wet weight was resuspended at 0.25 g/ml of minimal Eagle's medium containing either 1  $\mu$ Ci of <sup>3</sup>H-leucine (for phorbol-ester-treated cells) or 0.25  $\mu$ Ci of <sup>14</sup>C-leucine (for acetone-only-exposed cells). The cell suspensions were incubated for 2 hr at 37°C in 5% CO<sub>2</sub> in O<sub>2</sub>. Equal wet weights of differentially labeled, age-matched cell suspensions were mixed, washed twice with minimal Eagle's medium, and processed as described above.

To purify neutral species from HL-60, combined labeled extracts were dialyzed against 10 mM 4-morpholineethanesulfonic acid<sup>4</sup> buffer with 10% glycerol and applied to a 0.9 × 4.5 cm column of DEAE-cellulose (Whatman) in the same buffer. Fractions of 1.0 ml were collected, and the retained fraction was eluted with a NaCl gradient. Alpha-naphthyl-butyrate esterase activity was monitored in the eluate. Peak fractions were concentrated for IEF and radiolabel measurement. Gel segments (2 mm) were digested with 0.5 ml of 30% H<sub>2</sub>O<sub>2</sub> at 80°C and assayed for <sup>3</sup>H and <sup>14</sup>C emissions in a scintillation counter by using Aquasol fluor. <sup>3</sup>H/<sup>14</sup>C ratios were plotted to detect bands with increased ratios, indicative of proteins putatively induced by phorbol ester treatment.

## RESULTS

Cultures of HL-60 treated with TPA ceased cell division and, during the 72 hr, became firmly adherent to the substratum or clumped. Their morphology changed dramatically, from blastic and promyelocytic to monocytoid and macrophage-like. Cells with cytochemically strong, fluoride-sensitive esterase increased significantly in number (Table 1). Cells in control cultures remained dispersed and divided until plateau concentrations were reached. These control cultures developed moderate numbers of morphologically and cytochemically monocytoid cells, along with a moderate increase in esterase activity and neutral isoenzyme levels. Esterase activity in TPA-induced cultures increased 2–3-fold over that of age-matched controls (Table 1), and a significant relative increase in fluoride-sensitive neutral isoenzymes was evident (Fig. 1).

Cultures of KLM-2 treated with TPA continued to divide and reached plateau concentrations similar to those of the acetone control cultures (Table 1). Few adherent cells were observed in either treated or control cultures, but cells in each formed clumps in suspension. No cells with monocytoid nonspecific esterase cytochemistry were observed, nor did nonspecific esterase activity increase in either culture (Table 1). Zymograms revealed no detectable neutral isoenzymes in either culture at 0 or 72-hr incubation (Fig. 1). A duplicate experiment yielded similar results.

In one experiment, differentially labeled crude extracts of HL-60 were subjected directly to IEF. There was no evident increase of <sup>3</sup>H over <sup>14</sup>C incorporation in the neutral isoenzyme region of the gel from 0 through 72-hr incubation. In a second experiment, the neutral isoenzymes were concentrated by ion-exchange

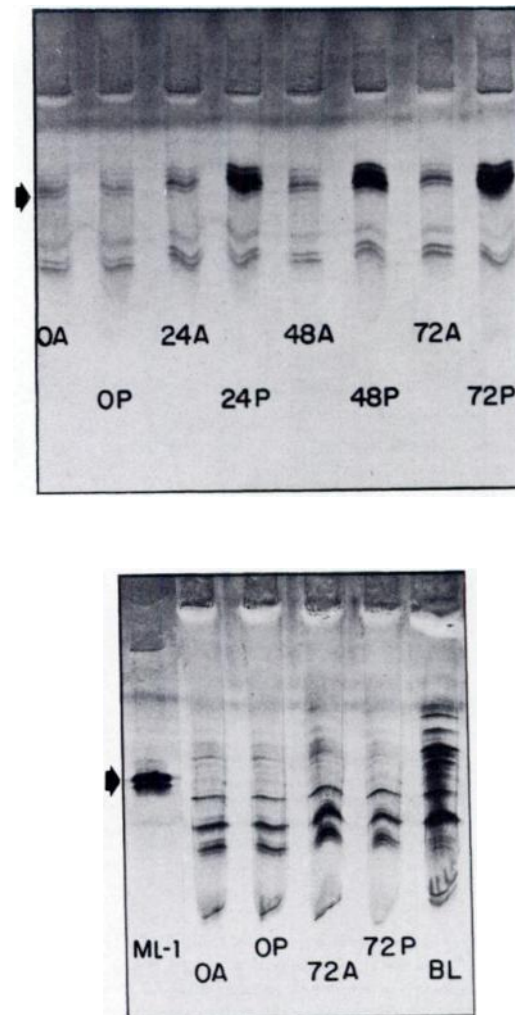


Fig. 1. Nonspecific esterase zymograms of HL-60 (top) and KLM-2 (bottom) cultures treated with phorbol esters. For each gel, the IEF range was pH 3.5 (bottom) to 8.5 (top). P, phorbol ester-treated; A, acetone control. Prefixed number indicates hours of incubation. Nonspecific esterase activity is revealed by alpha naphthyl acetate-fast garnet GBC stain. HL-60 extracts were normalized to 25  $\mu$ g of applied protein to demonstrate marked stimulation of neutral isoenzymes in phorbol-ester-treated cultures over age-matched controls (bold arrow). KLM-2 extracts were applied at maximum loading of 250  $\mu$ g, yet no neutral species are detectable in treated or control cultures. Prominent neutral isoenzymes are seen in the positive control—acute myeloid leukemia cell line ML-1<sup>9</sup> (bold arrow)—but not in the B-lymphocyte-negative control (BL).

chromatography from the differentially labeled extracts at 0 and 48 hr. These formed a modest peak at 0 hr but were markedly increased in activity and zymogram intensity at 48 hr (Fig. 2). Peak fractions subjected to IEF again showed no increased <sup>3</sup>H over <sup>14</sup>C incorporation (Fig. 2). This double-label method is capable of detecting  $\geq 0.02\%$  net synthesis of creatine kinase over background in estrogen-induced rat uterus cells.<sup>8</sup>

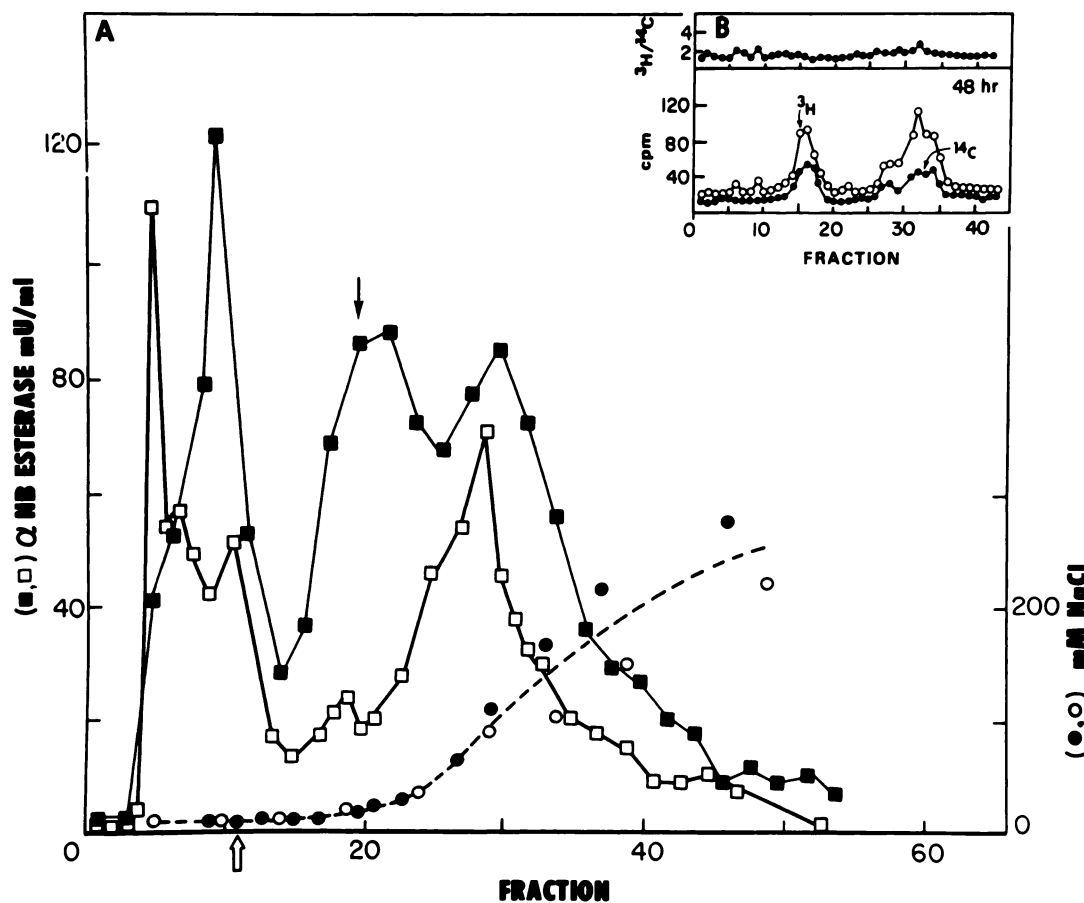


Fig. 2. Purification of differentially radiolabeled neutral species from age-matched HL-60 extracts. Open symbols, 0-hr cultures; closed symbols, 48-hr cultures. Combined extracts were subjected to ion-exchange chromatography with a NaCl gradient (started at open arrow). The neutral species are eluted at 20–40 mM NaCl, as demonstrated by  $\alpha$ -naphthyl butyrate ( $\alpha$ NB) esterase assay and zymogram analysis. These form a modest peak at 0 hr, but are markedly increased in activity at 48 hr. (Inset) The peak fraction of neutral isoenzymes (black arrow) was concentrated and subjected to IEF, pH 3.5–9.5. Whereas two prominent peaks of radiolabel incorporation are seen in the 48-hr concentrate, the ratio of  $^3\text{H}/^{14}\text{C}$  incorporation remains constant throughout. The neutral species are contained in the peak at fractions 28–33. The 0-hr concentrate yielded similar results.

#### DISCUSSION

Fluoride-sensitive, neutral nonspecific esterase isoenzymes of monocytes are detectable in about two-thirds of acute myeloid leukemias, but have not been detected in lymphoid or stem cell leukemias.<sup>4,5</sup> HL-60 produces moderate levels of neutral isoenzymes. Crude extract nonspecific esterase activity was stimulated 2–3-fold in HL-60 by phorbol ester induction of monocyte/macrophage differentiation and was associated with increased levels of fluoride-sensitive neutral isoenzymes. Other investigators have reported that the B-lymphoblast line, KLM-2, is induced to macrophage differentiation by phorbol ester treatment<sup>3</sup> and that induced cells developed monocytoid cytochemical nonspecific esterase reactivity. We did not detect monocytoid cytochemistry or neutral isoenzymes in either treated or control KLM-2 cultures. Our concentrations of phorbol esters were comparable to those used by

other researchers, but we did not use colony-stimulating factor supplement, which has been reported to increase the proportion of cytochemically positive cells from 25% to 50%.<sup>3</sup> Nevertheless, our results speak against bona-fide esterase changes resulting from phorbol ester treatment of this lymphoblast cell line.

Despite marked stimulation of neutral isoenzyme activity in HL-60 by phorbol esters, no increased de novo synthesis of these myeloid-associated species was detectable. These results are consistent with stimulated conversion of myeloid zymogens in HL-60 to active neutral isoenzymes during macrophage induction by phorbol esters.

#### REFERENCES

1. Rovera G, Santoli D, Damsky C: Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc Natl Acad Sci USA* 76:2779, 1979

2. Territo M, Koeffler HP: Induction by phorbol esters of macrophage differentiation in human leukaemia cell lines does not require cell division. *Br J Haematol* 47:479, 1981
3. Hiraoka A, Kubota K, Preisler HD, Minowada J: Macrophage differentiation of human precursor B-cell line by phorbol ester and colony stimulating factor. *Blood* 60:997, 1982
4. Yourno J, Burkart P, Lizzi F, Tartaglia A: Enzymologic classification of acute leukemias: Nonspecific esterase markers distinguish myeloid and lymphoid varieties. *Blood* 60:304, 1982
5. Yourno J, Burkart P, Lizzi F, Tartaglia A: Enzymologic classification of acute leukemias: Zymogram analysis of nonspecific esterase in the differentiation of myeloid and lymphoid/stem cell types. (submitted for publication)
6. Collins SJ, Gallo RC, Gallagher RE: Continuous growth and differentiation of human myeloid leukemia cells in suspension culture. *Nature* 270:347, 1977
7. Mastropaolo W, Yourno J: An ultraviolet spectrophotometric assay for  $\alpha$ -naphthyl acetate and  $\alpha$ -naphthyl butyrate esterases. *Anal Biochem* 115:188, 1981
8. Kumar A, O'Connor DL, Seeger JI, Beach TA, Dickerman HW: Purification and characterization of creatine kinase, an estrogen-induced uterine protein (IP) from immature rats. *Biochem Biophys Res Commun* 111:156, 1983
9. Minowada J, Koshiba H, Sagawa K, Kubonishi I, Lok MS, Tatsumi T, Han T, Srivastava BIS, Ohkuma T: Marker profiles of human leukemia and lymphoma cell lines. *J Cancer Res Clin Oncol* 175:1, 1981