

Isoenzymes of Lactate Dehydrogenase in Human Leukemic Cells in Culture Treated with Inducers of Differentiation

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ABSTRACT The human leukemic cell lines HL60 and K562, were induced to differentiate terminally by chemical agents. The isoenzyme patterns of lactate dehydrogenase (LD) in the cells before and after differentiation were determined electrophoretically on agarose gels. In general, treatment of the leukemic cells with inducers of differentiation resulted in a quantitative shift of the isoenzyme pattern towards anodic or cathodic forms. This was correlated with the conversion of the chemically treated cells to morphologically more normal cells, as verified by light microscopy and/or synthesis of hemoglobin. The LD isoenzyme patterns of the chemically differentiated cells were: (a) characteristic for the particular cell type obtained rather than for the nature of the inducer used; and (b) not similar to those of normally differentiated cells of the corresponding lineage, indicating that incomplete differentiation had occurred.

The glycolytic enzyme lactate dehydrogenase (LD), which catalyzes the reversible conversion of pyruvate to lactate, exists in multiple molecular forms called isoenzymes. Five LD isoenzymes have been demonstrated in human tissues, cells, and serum. They are designated LD-1 to LD-5. LD-1 is the fastest in migrating toward the anode and LD-5 the slowest. LD isoenzymes have been reviewed previously (7, 9).

Alterations of LD isoenzyme patterns have been observed during development, under changing biological conditions, and frequently in neoplastic tissues (7, 13). In several instances, changes in serum LD specific isoenzyme patterns correlated well with either tumor growth or regression (12). Thus, analysis of LD isoenzyme patterns may be useful in determining whether a chemotherapeutic agent affects a neoplastic tissue.

The HL60 cell line, derived from a patient with acute promyelocytic leukemia, proliferates continuously in suspension culture and consists predominantly of promyelocytes (5). These cells can be induced to terminally differentiate to morphologically mature granulocytes by incubation with a wide variety of compounds, including dimethylsulfoxide (DMSO), hexamethylene bisacetamide (HMBA), and retinoic acid (2, 3, 6). The induced HL60 cells have many of the functional characteristics of normal peripheral blood granulocytes, including phagocytosis, complement receptors, chemotaxis, and increased oxidative metabolism (16, 17). Other differences be-

tween HL60 and DMSO-treated HL60 cells have been reported on cell surface proteins (11) and chromatin denaturation profiles (19). However, it has been suggested that DMSO-differentiated HL60 cells do not reach full maturity (16, 17) because they do not contain detectable lactoferrin, a marker for specific granules of mature granulocytes (17), and because they do not synthesize and secrete plasminogen activator (16).

When cultures of HL60 cells are treated with nanomolar concentrations of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), >95% of the cells are converted to macrophage-like cells, as judged by their morphology, ability to adhere and phagocytize, and increased enzymic activities typical of macrophages (23, 28).

The human leukemic cell line, K562, derived from a patient with chronic myelogenous leukemia (15), has been shown recently to express the fetal i antigen (10) and synthesize glycophorin A (14), a sialoglycoprotein present on erythrocyte precursors and mature red cells. Furthermore, upon induction with sodium butyrate or hemin, K562 cells synthesized hemoglobin (1, 24), the type of which depends on the chemical used to induce synthesis (4). However, the presence of myeloid markers on K562 cells (8) has raised the possibility that these cells can also differentiate along other than erythroid lineages, and it has been observed that treatment of K562 cells with phorbol diesters can induce partial differentiation to myelo-

monocytic cells (reported in reference 4). Treatment of cultures of K562 cells with nanomolar concentrations of TPA converted a portion of the cells to strongly attached cells, which, in contrast to control (untreated) cells, were characterized by a larger number but smaller size of well defined mitochondria; disorganized endoplasmic reticulum; cytoplasmic vacuolization; increased ability to phagocytize latex-beads, and specific changes in the gel electrophoretic profiles of chromosomal proteins (P. Pantazis and J. Dahlberg; and P. Pantazis and W. M. Bonner, unpublished data).

In this study HL60 cells were converted to granulocyte-like or macrophage-like cells, whereas, K562 cells were converted to hemoglobin-synthesizing or adherent cells by various inducers of differentiation. The LD isoenzyme patterns of the chemically treated cells were compared with those of the untreated cells. Differences were observed in the patterns of each cell type. In addition, the inducer used seemed to affect the LD pattern observed.

MATERIALS AND METHODS

Chemicals

Sterile DMSO solution (15% DMSO, lot No. 96674) in Eagle's basal medium was obtained from Microbiological Associates (Walkerville, Md.). HMBA was a gift from Dr. W. M. Bonner. HMBA, made 100 mM in RPMI 1640 medium, filtered and stored at -20°C , was used as a stock solution. TPA (P-L biochemicals, Milwaukee, Wis.) was made 500 mM in acetone and stored at -20°C . Microquantities of stock TPA solution were diluted with sterile RPMI 1640 medium before addition to the cultures. Stock solution of 1 M sodium butyrate, pH 7, was made from concentrated butyric acid (Fisher Scientific Co., Fair Lawn, N. J.), neutralized with concentrated NaOH, and filtered. Benzidine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.), glacial acetic acid (Fisher), and hydrogen peroxide (30% H_2O_2 ; Fisher) were freshly mixed before use for the benzidine reaction. Giemsa and methylene blue staining solutions were from Harleco, American Hospital Supply Corp., Gibbstown, N. J.

Cells and Cultures

HL60 and K562 cells were provided by Drs. R. C. Gallo and S. J. Collins. The cells were grown in a 5% humid incubator in RPMI 1640 medium (National Institutes of Health Media Unit, Bethesda, Md.) and supplemented with 10% fetal calf serum (Flow Laboratories, McLean, Va.). Cells were seeded every 4–5 d at an initial concentration of 2×10^5 cells/ml in Corning T-25 flasks (Corning, N. Y.). Differentiation of HL60 cells to granulocytes was done in the presence of DMSO, HMBA, and retinoic acid, whereas differentiation to macrophage-like cells was accomplished with TPA. Similarly, cultures of K562 cells treated with sodium butyrate and TPA were converted to hemoglobin-synthesizing and attached cells, respectively. The treatment periods as well as the concentrations of chemicals used for the treatment of HL60 and K562 cells are presented in Table I.

Whole blood from a normal donor was centrifuged to pellet the cells which were then washed five times in phosphate buffered saline (PBS, pH 7.2). This cell pellet, comprised mainly of erythrocytes, was used to determine the LD isoenzyme pattern. Contamination with cells other than erythrocytes was negli-

gible and therefore ignored. To obtain granulocytes or monocytes, whole blood was mixed with 5 vol of 0.83% NH_4Cl and cells were pelleted at low speed. This step was repeated twice so that only a small number of contaminating erythrocytes were observed in the remaining cell population, which consisted mainly of lymphocytes and granulocytes. Separation of these two populations was accomplished by centrifugation in a Ficoll-hypaque solution. More than 85% of the pelleted cells were granulocytes. Monocytes were obtained as described by Stobo (27).

Viability and Differentiation Tests

The viability of untreated and chemically treated HL60 and K562 cells was determined by the trypan blue dye exclusion method. Differential counts of control and HL60 cells treated with DMSO, HMBA, and retinoic acid were estimated on microscope slides prepared with a Shanon-Elliott Cytospin centrifuge (Surrey, England) and stained with Giemsa's or methylene blue. The percentage of spontaneously induced (control) and butyrate-induced K562 cells which synthesized hemoglobin was calculated using a hemacytometer, according to the benzidine method of Orkin et al. (19), as modified by Scher and Friend (25). A minimum of 300 cells were scored for each experiment. TPA-treated flasks of HL60 and K562 cells were rinsed four times with RPMI 1640 to remove loosely attached cells. Strongly attached cells were removed by trypsinization and counted in a Coulter Counter, model B (Coulter Electronics, Hiale, Fla.). For photography, TPA-attached cells were rinsed vigorously with PBS before fixation and stained on the flask with Giemsa's or methylene blue.

Gel Electrophoresis of LD Isoenzymes

Aliquots of cell suspensions containing 1×10^6 cells were centrifuged to a pellet and the supernate was discarded. Subsequently, the cells were washed once in PBS and suspended in PBS diluted with distilled water (1:1). The cell suspension was subjected to one cycle of freezing and thawing which caused lysis of the cells. The cell fragments were then separated by centrifugation. Four- μl samples from the clarified supernate were electrophoresed on agarose gels according to a procedure for the quantitative determination of LD isoenzymes (20), as recently modified (21). Briefly, the main features of the procedure are (a) an electrophoretic separation of LD isoenzymes on microscope slides covered with agarose-gel, (b) a colorimetric demonstration of the enzymatic activity by a tetrazolium reduction procedure, and (c) a quantitation of the isoenzyme zones by densitometric scanning. A Joyce-Loebl densitometer (Chromoscan model; Joyce-Loebl Ltd., Team Valley, Gateshead, England) equipped with an integrator was used for estimation of the percentage distribution of LD isoenzymes.

RESULTS

Cytotoxic Effect of Inducers of Differentiation

Viability of HL60, DMSO- and HMBA-treated HL60, K562, and butyrate-treated K562 cells remained consistently $>96\%$, as determined by the trypan blue method, for the periods of treatment shown in Table I. Retinoic acid had a more cytotoxic effect on HL60 cells with $\sim 70\text{--}75\%$ viable cells in the culture at the fourth d of treatment. However, retinoic acid was the most potent inducer of myeloid differentiation (Table I), in agreement with a previous report (2). It seemed that the more potent the inducer of differentiation the higher its cytotoxic

TABLE I
Treatment of Leukemic Cells (HL60, K562) with Various Inducers of Differentiation and Results of Differentiation

Cells induced	Inducer used	Concentration of inducer used	Period of treatment	Type of cultured cells	Percentage of differentiated cells*
HL60	DMSO	170	6	suspended	80–85 (5–15)‡
HL60	HMBA	2.5	6	suspended	90–95 (5–15)
HL60	Retinoic acid	1×10^{-3}	4	suspended	90–95 (5–15)
HL60	TPA	20×10^{-6}	4	adherent	85–90§
K562	Sodium butyrate	1–2	5	suspended	65–75
K562	TPA	20×10^{-6}	4	adherent	8–10

* Determined by light microscopy of cells stained with Giemsa's or methylene blue, with the exception of K562 cells which after treatment with sodium butyrate were assayed by the benzidine method.

‡ Numbers in parentheses indicate percentage of spontaneously differentiated cells in untreated (control) cultures.

§ Percentages of adherent cells were calculated from flasks which were washed four times with medium before trypsinization.

effect on treated cells. TPA had no cytotoxic effect on cells because the viability of treated cells was consistently >97%, for TPA concentrations between 2 and 20 nM. Higher concentrations of TPA in the cultures resulted in a decrease of viability of both HL60 and K562 cells (unpublished observations). In this study, HL60 and K562 cells were treated with 20 nM TPA for 4 d. Treatment of the cells with TPA for >4 d resulted in an increasing detachment of attached cells. This was not due to the deaths of attached cells per se because the percentage of viable cells in suspension remained unaltered up to 6 d. Perhaps prolonged TPA-treatment resulted in a loss of ability of cells to adhere.

Ability of Inducers to Induce Differentiation

Treatment of HL60 and K562 cells with inducers of differentiation converted these cells into more differentiated cells (Fig. 1). Treatment of HL60 cells with DMSO resulted in the appearance of granulocytes in the cultures (Fig. 1, *A* and *B*), as was first observed by Collins et al. (6). The capability of each inducer to produce differentiated cells is shown by the percentage of differentiated cells which appeared after each treatment (Table I). The effectiveness of each inducer to form granulocytes was concomitant with its ability to decrease proliferation of the cells (data not shown). The ability of each inducer to convert HL60 cells to various percentages of the granulocytic cell types has been reported (2). Treatment of HL60 cells with TPA converted >90% of the cells into attached cells by day 4 (Fig. 1 *C*), in agreement with a previous report (24). The growth rate of the HL60 cells in the culture upon addition of TPA was zero, because the number of cells at the time of TPA addition was not altered after incubation of the cells in the presence of TPA. Similar results have already been reported (22).

2–4% of the control K562 cells in the culture were positively stained with benzidine by day 6. However, the number of benzidine positively stained K562 cells was ~70% when sodium butyrate was present in the cultures for 6 d. Despite the fact that a large number of butyrate-treated K562 cells synthesized hemoglobin, we were not able to detect morphological changes by light microscopy (Fig. 1 *D* and *E*) as were seen by Andersson et al. (1), in confirmation of a recent report that various sublines derived from the original K562 cells, when treated with butyrate, are induced to synthesize hemoglobin, although they do not exhibit morphological changes (4). However, nanomolar concentrations of TPA in cultures of K562 cells resulted in the appearance of ~10% strongly attached cells (Fig. 1 *F*), which possessed some morphological, functional, and biochemical properties different from those acquired by control K562 cells (P. Pantazis and J. Dahlberg; and P. Pantazis and W. M. Bonner, unpublished data).

Isoenzymes of Lactate Dehydrogenase

The electrophoretic patterns of LD isoenzymes from control and chemically treated HL60 and K562 cells are shown in Fig. 2. Note that the electrophoretic method used provided a clear resolution of the isoenzymes. In most cases, changes could be detected by direct observation. However, densitometric scanning of these patterns allowed quantitation of the isoenzymes. Each cell line, untreated or treated, was assayed two to four times, in individual experiments. The standard deviation was nearly zero for LD-1 and LD-5, and <2% for LD-2, LD-3, and LD-4. The quantitative results are shown in Table II. They were observed in early passages of HL60 cells. In general,

untreated (control) HL60 cells exhibited low activities of the parental forms LD-1 and LD-5, and high activities of LD-2 and LD-3 isoenzymes. DMSO-treated cells exhibited a clear-cut quantitative shift in the isoenzymes towards the cathodic forms. Similar shifts were observed in the relative activities of the isoenzymes from HL60 cells treated with HMBA (Fig. 2 and Table II). However, retinoic acid resulted in only a slight shift of the LD isoenzymes towards cathodic forms, unlike the shifts observed when HL60 cells were treated with DMSO or HMBA. This was surprising, because retinoic acid is the most potent inducer of HL60 myeloid differentiation (2). Perhaps the zymogram of HL60 cells treated with retinoic acid does not reflect the actual pattern in differentiated cells, because 25–30% of the treated cells were dead due to the high cytotoxicity at the concentrations used. Variations in the relative activities of LD-2, LD-3, and LD-4 perhaps reflect the ability of each inducer to cause differentiation of the HL60 cells (3). It is also likely that activity variations in the LD isoenzymes reflect variations in the cell types obtained after treatment with each inducer of differentiation towards granulocytes (2). In general, the ratios of isoenzyme activities of untreated and treated HL60 cells were found to be different from the ratios of isoenzymes of fresh human granulocytes of a normal donor (Fig. 2 and Table II).

Treatment of HL60 cells with TPA resulted in appearance of attached cells (Fig. 1 *C*), as reported by others (23). The LD isoenzyme of these cells exhibited a quantitative shift towards the cathodic forms. This shift was associated with the morphological changes. However, the isoenzyme pattern of TPA-attached HL60 cells did not resemble the pattern of normal monocytes. This indicated that treatment of HL60 cells with TPA shifted the isoenzyme pattern towards cathodic forms, i.e., a pattern which does not resemble that of normal monocytes.

The ratios of LD isoenzymes in the human proerythroblastic cells, K562, showed a rather Gaussian distribution with the pattern differing from that of HL60 cells (Fig. 2). An increase of more anodic isoenzymes was seen in hemoglobin-synthesizing cells obtained after treatment of K562 cells with millimolar concentrations of sodium butyrate. No LD-5 was detected in

TABLE II
Quantitation of Relative Activity of LD Isoenzymes of HL60 and K562 Cells Before and After Treatment with Inducers of Differentiation

Cells	Treatment	Distribution of LD-Isoenzymes				
		LD-1	LD-2	LD-3	LD-4	LD-5
		%				
HL60	none	11	36	41	11	1
HL60	DMSO	5	24	35	30	6
HL60	HMBA	4	21	46	25	4
HL60	Retinoic acid	6	32	42	16	4
granulocytes (normal)	none	2	9	22	29	38
HL60	TPA	3	18	38	32	10
K562	none	6	23	33	28	10
K562	Sodium butyrate	27	35	32	6	0
K562	TPA	6	24	36	27	7
erythrocytes (normal)	none	44	46	10	0	0
monocytes (normal)	none	8	33	43	16	0

these cells. However, no significant changes were observed in the pattern of attached K562 cells after TPA treatment (Fig. 1 F). This indicated that although TPA-attached cells and TPA-attached HL60 cells were morphologically similar, they did not exhibit similar LD zymograms.

DISCUSSION

The human promyelocytic and proerythroblastic cells, HL60 and K562, respectively, have been reported to be converted into more mature cells in the presence of various inducers of

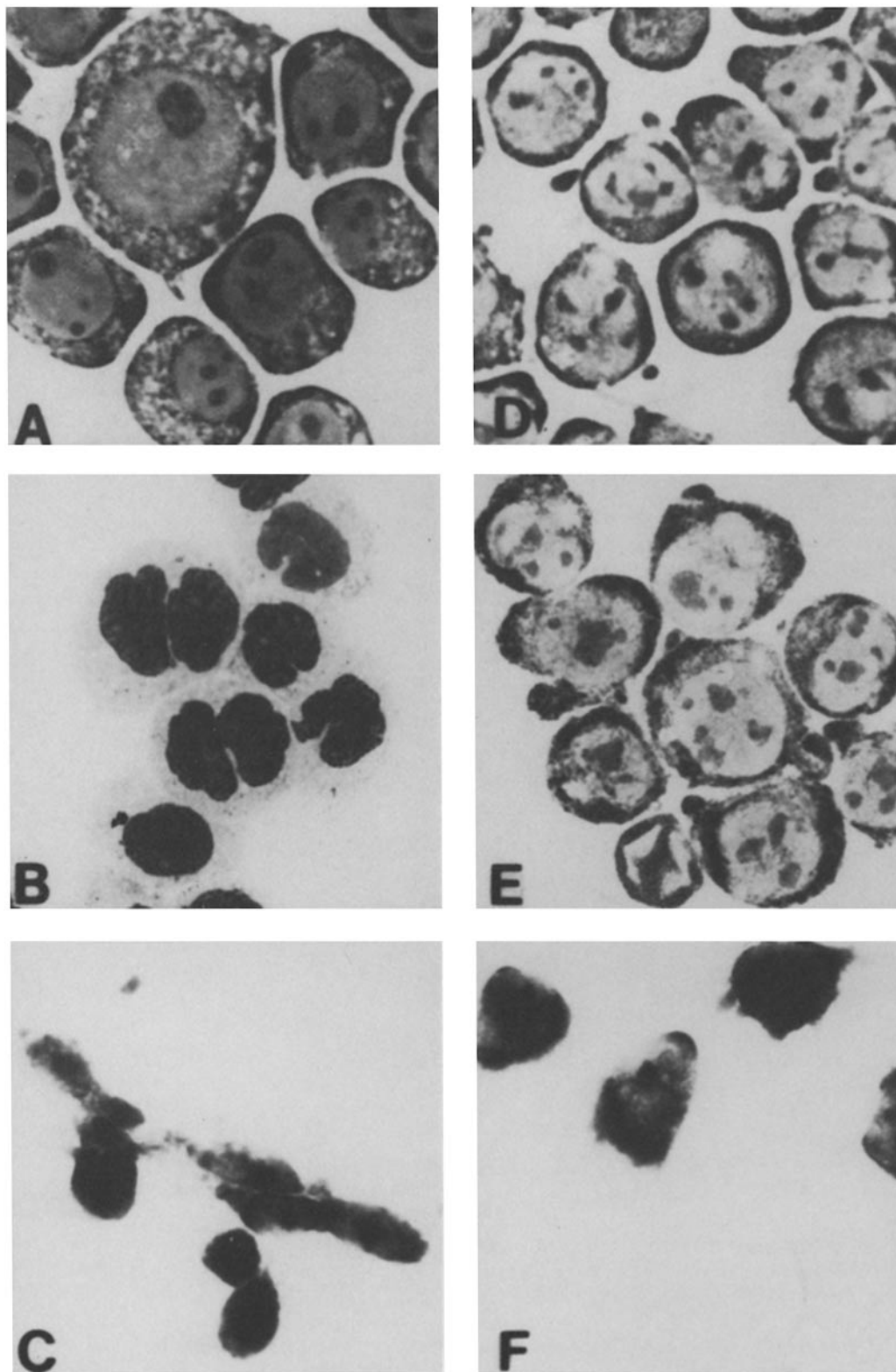


FIGURE 1 Morphology of HL60 and K562 cells before and after treatment with inducers of differentiation. A, untreated HL60 cells; B, DMSO-treated HL60 cells; C, TPA-treated HL60 cells; D, untreated K562 cells; E, K562 cells treated with sodium butyrate; and F, TPA-treated K562 cells. $\times 1,220$.

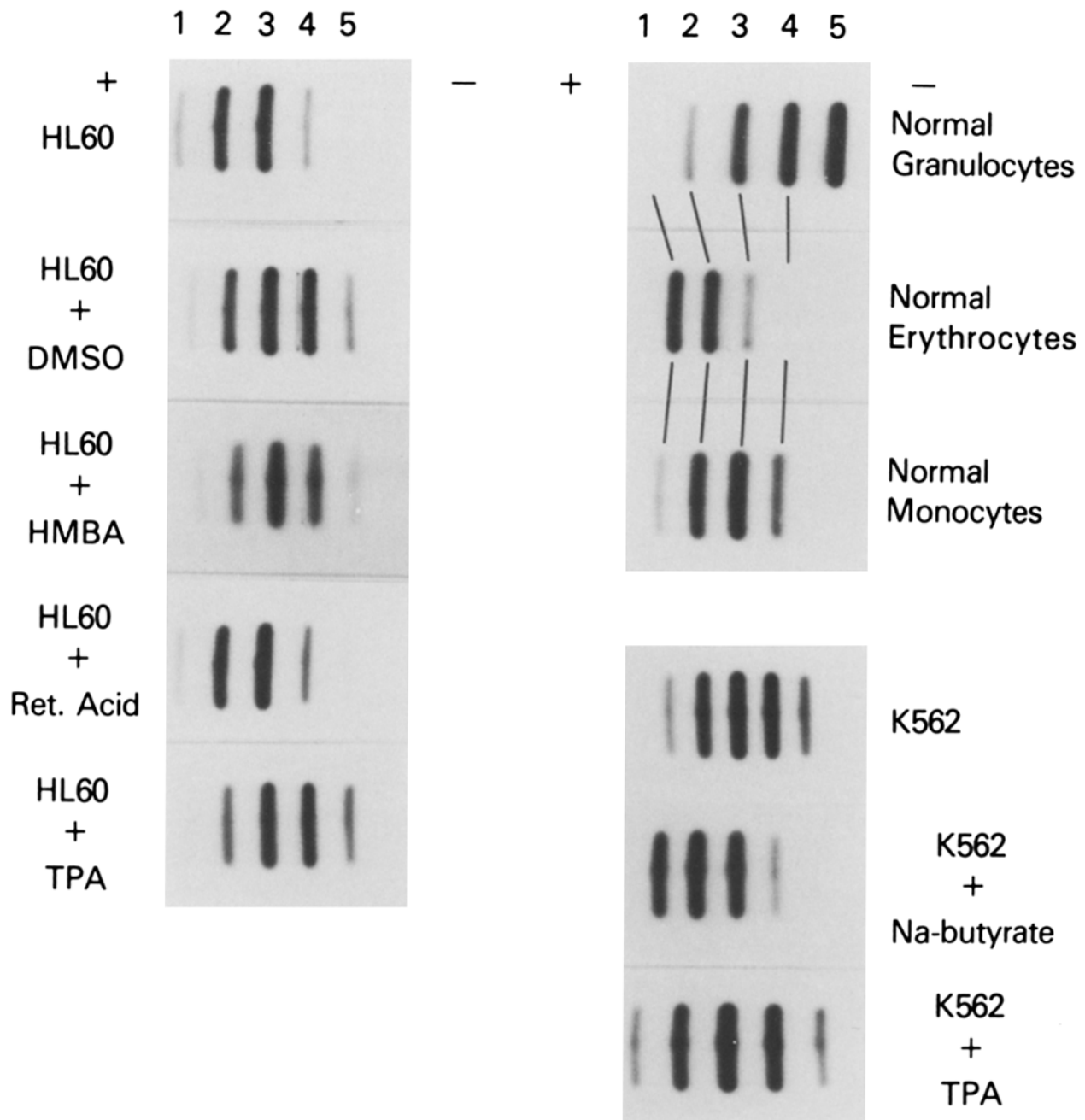


FIGURE 2 Agarose gel electrophoretic patterns of LD isoenzymes of HL60 and K562 cells treated with various chemical agents. Granulocytes, erythrocytes, and monocytes were prepared from normal donors in individual experiments. *Ret. Acid*, retinoic acid.

differentiation (2, 3, 6, 23). This maturation is associated with changes in the morphological (3, 6, 23) and functional (2, 3, 6, 17, 22, 28) properties of these cells. In this study we attempted to correlate shifts in the electrophoretic patterns of LD isoenzymes with the differentiation stage as well as with the cell type obtained after treatment of HL60 and K562 cells with various inducers of differentiation.

Conversion of HL60 cells to more mature cells along the granulocyte pathway has been shown to be accompanied by increased glucose oxidation (16) and production of superoxide anions (2, 3, 16, 17). In this study we showed that conversion of HL60 promyelocytes to metamyelocytes and granulocytes

was accompanied by a shift in the proportions of LD isoenzymes towards more cathodic isoenzymes. However, the chemically matured HL60 cells never acquired the quantitative pattern of LD isoenzymes of normal granulocytes. This could be due to one or more of the following reasons: (a) a population of HL60 cells does not overcome the blockage at the promyelocytic stage after treatment with inducers of differentiation (2, 3, 6); (b) although the number of converted HL60 cells is >80% for all inducers used, only a small fraction of the converted population resembles mature granulocytes (2); and (c) despite their morphological assessment, the "mature" HL60 cells do not acquire all the properties of mature normal granulocytes

(16, 17). Additionally, microvariations in the LD isoenzyme patterns of the converted HL60 cells reflect the ability of each chemical agent to induce differentiation and/or variation in the number of cell types along the granulocytic pathway (2).

It has been reported recently that TPA-treated HL60 cells develop several of the morphological, biochemical, and functional properties of normal macrophages (23). Additionally, in our study we found that when HL60 cells were converted to macrophagelike cells in the presence of TPA there was an increase in the cathodic LD isoenzymes. However, this isoenzyme pattern was different than the pattern of normal monocytes.

Treatment of the proerythroblast-like cells, K562, with sodium butyrate resulted in the appearance of a high percentage of hemoglobin-synthesizing cells (1, 4). This conversion was accompanied by an increase of the anodic isoenzymes LD-1, LD-2, and LD-3. Also, these isoenzymes were found to be the predominant forms of normal erythrocytes, in agreement with a previous report (26). Thus, butyrate-treated K562 cells are similar to normal erythrocytes with respect to the ability to synthesize hemoglobin and lactate dehydrogenase.

Treatment of K562 cells with TPA resulted in the appearance of a small number of attached cells whose zymogram was similar to that of the untreated cells but different from the zymogram of the macrophagelike cells obtained after treatment of HL60 cells with TPA. This is an indication that from enzymatic viewpoint adherent cells derived from TPA-treated K562 cells are different than macrophagelike cells derived from TPA-treated HL60 cells.

The results of this study showed that changes in the differentiation stage of leukemic cells in vitro were generally accompanied by shifts in the electrophoretic patterns of LD isoenzymes of these cells. As a result, the extent of cell differentiation could be monitored by measuring the quantitative shifts of the enzyme. Also, the results showed that this methodology may be useful for monitoring the effects of therapeutic agents on biological properties rather than on cell phenotype. Using this methodology, we are currently studying changes in the LD isoenzyme patterns of leukemic patients at various stages of the disease before or after treatment with antileukemic agents.

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