

**NORMAL FUNCTIONAL CHARACTERISTICS OF CULTURED
HUMAN PROMYELOCYTIC LEUKEMIA CELLS (HL-60) AFTER
INDUCTION OF DIFFERENTIATION BY DIMETHYLSULFOXIDE**

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Mature human myeloid cells (granulocytes) maintain a number of important functional characteristics including those providing a defense against invading microorganisms. Circulating granulocytes accumulate at sites of microbial invasion by chemotaxis, a process directing migration along a chemical gradient provided by microbial products (1). The presence of complement receptors on membranes of certain granulocytes (2, 3) may facilitate recognition and subsequent phagocytosis of opsonized microorganisms (4). Once phagocytosis has occurred, a complex oxidative metabolic process involving the rapid generation of superoxide anion (O_2^-) and its interaction with certain granulocytic enzymes results in killing of the ingested microorganisms (5, 6, 7).

We have recently developed a unique human cell line (designated HL-60) from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. These cultured cells are predominantly promyelocytes and display distinct morphological and histochemical myeloid characteristics (8). When incubated for 5-7 d with appropriate concentrations of dimethylsulfoxide (Me_2SO), the majority of the cultured cells differentiate into morphologically mature myelocytes, metamyelocytes, and banded and segmented neutrophils (9). Our initial studies demonstrated that these induced cells were capable of phagocytizing certain microorganisms (9). We report here that the cultured HL-60 cells despite their leukemic origin and karyotypic abnormalities (8) are induced by Me_2SO to display functional characteristics commonly associated with normal peripheral blood granulocytes including response to chemoattractants and development of complement receptors. Moreover, phorbol myristate acetate (PMA) which induces phagocytosis-associated oxidative metabolism in normal granulocytes (10, 11) similarly induces the Me_2SO -treated HL-60 cells to generate superoxide anion and reduce nitroblue tetrazolium (NBT) dye.

Materials and Methods

Cells. The HL-60 cell line has been maintained in continuous suspension culture for over 2 yr and 100 passages as previously described (8). The experiments reported here were performed on cells between passage 10 and 40. Me_2SO -induced cells were obtained by seeding HL-60 cells at $2.5 \times 10^5/ml$ in growth media and culturing for 6 d in the presence of 1.12% Me_2SO (9). Control cells included K-562, an undifferentiated cell line derived from a patient with chronic myeloid leukemia in blast crisis (12). These cells were grown under standard conditions in RPMI-1640 media (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% heat inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). Peripheral blood granulocytes from normal donors were obtained by the Ficoll-dextran method

(13) and consisted of >95% fully mature segmented polys. All cells were washed twice in plain RPMI before functional assays were performed. Differential counts of cell suspensions were performed on cytopsin slide preparations stained with Wright-Giemsa as previously described (9).

Chemotaxis. The assay was based on the modified Boyden chamber procedure (14). Cells were added to the upper well of a blind well chamber (model F013WLB00101, Neuro Probe, Inc., Bethesda, Md.) at a concentration of 2.5×10^6 cells/ml of Gey's balanced salt solution containing 2% bovine serum albumin. The potent chemoattractant f-met-leu-phe (15) (Andrulis Research Corp., Bethesda, Md.) was added to the lower well at a concentration of 10^{-9} M diluted in phosphate-buffered saline. (A) 13-mm chemotactic membrane (Nucleopore Corp., Pleasanton, Calif.) containing an average pore diameter of $5.0 \mu\text{m}$ separated the two compartments. The membranes were then removed, stained with Wright-Giemsa and examined microscopically for cells that had migrated through the pores to the underside. The number of cells in four successive low power fields ($\times 160$) were then counted and averaged in triplicate experiments.

Complement Receptors. Complement receptors were determined by the erythrocyte antibody complement-(EAC) rosetting technique (16) with sheep erythrocytes (RBC) sensitized with rabbit anti-sheep RBC IgG (Grand Island Biological Co.) with DBA2 mouse serum (Associated Biomedical Systems, Buffalo, N. Y.) as the source of complement.

Superoxide Production and NBT Reduction. Generation of superoxide was measured by the ability of this anion to reduce ferricytochrome c, the reduced ferrocycytochrome molecule having a higher absorbance at 550 nm (6, 7). The stimulus for generation was provided by PMA obtained from the National Institutes of Health carcinogen repository and stored at -20 in 100% acetone at a concentration of 500 $\mu\text{g}/\text{ml}$. 1 ml of phosphate-buffered saline (pH 7.4) containing 5×10^6 cells, 0.12 mM ferricytochrome c (Sigma Chemical Co., St. Louis, Mo.) and, where appropriate, 100 ng freshly diluted PMA with or without 60 μg superoxide dismutase (SOD) (Sigma Chemical Co.) was incubated for 20 min at 37°C . The reaction was then stopped by submerging the tubes in an ice bath, the cells were pelleted by centrifugation at 4°C at 200 g for 5 min, and the absorbance of the supernate at 550 nm was then determined in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The quantity of superoxide generated was expressed as the $\Delta A/20 \text{ min}/5 \times 10^6$ cells which is inhibited by 60 μg SOD.

For NBT reduction studies 1 ml of cells suspended at 2×10^6 cells/ml of RPMI media supplemented with 20% fetal calf serum was incubated for 20 min with an equal vol of 0.2% NBT (Aldrich Chemical Co., Milwaukee, Wis.) dissolved in phosphate-buffered saline in the presence and absence of 200 ng freshly diluted PMA. The percent of cells containing intracellular reduced blue-black formazan deposits was then determined on Wright-Giemsa stains of cytopsin preparations of the incubated cells.

Results and Discussion

Chemotaxis. Me_2SO -induced HL-60 cells, which consist predominantly of metamyelocytes and bands rather than segmented neutrophils, the final cells in the myeloid series (9), consistently showed a greater response to the chemoattractant f-met-leu-phe than either uninduced HL-60 or K-562 cells (Table I). Moreover, these relatively immature Me_2SO -treated cells often responded better than more fully mature peripheral blood neutrophils (Table I). This may reflect a greater adaptability of the cultured, induced HL-60 cells to the artificial in vitro chemotactic assay conditions. The migrating cells in the uninduced promyelocytic culture (Table I) upon close microscopic examination appear to be more mature myeloid cells and probably represent the small percentage of myeloid cells spontaneously differentiating beyond the promyelocytic stage that are found in the uninduced HL-60 culture (8). Similar results have been obtained by using f-met-leu (10^{-7} M) (Sigma Chemical Co.) as the chemoattractant. (17).

Complement Receptors. HL-60 cells induced with Me_2SO consistently showed an

TABLE I
Chemotaxis Assay of Cultured Human Leukocytes

Cells	Mature myeloid cells*	Migrating cells/4LPF ($\times 160$)	
		-Chemoattractant	+Chemoattractant‡
	%		
HL-60-uninduced	8-12	8 \pm 3	38 \pm 8
HL-60-induced§	75-85	25 \pm 10	346 \pm 150
Peripheral blood granulocytes	>95	17 \pm 8	220 \pm 86
K-562	0	6 \pm 4	7 \pm 3

* Myelocytes, metamyelocytes, banded and segmented neutrophils on Wright-Giemsa stained preparations of cell suspensions.

‡ 10^{-9} M f-met-leu-phe.

§ Induced with 1.12% Me₂SO as described in Materials and Methods.

|| Numbers equal the average number of migrating cells \pm the observed range in triplicate experiments.

TABLE II
Complement Receptors on Human Leukocytes

Cells	Mature myeloid cells*	EAC rosettes
		%
HL-60-uninduced	8-12	7 \pm 2§
HL-60-induced‡	75-85	24 \pm 9
Peripheral blood granulocytes	>95	<3
K-562	0	<3

* Myelocytes, metamyelocytes, banded and segmented neutrophils.

‡ Induced with 1.12% Me₂SO as described in Materials and Methods.

§ Numbers represent average percent of rosetting cells \pm the observed range in triplicate experiments.

increased percentage of EAC rosetting when compared with uninduced cells and with normal peripheral blood granulocytes (Table II). Because the HL-60 induced cells consist predominantly of relatively immature granulocytes (9) although the Ficoll-dextran isolated peripheral blood granulocytes are almost exclusively fully mature segmented polys, these results are consistent with previous findings that receptors for certain complement components present on immature granulocytes are lost as the granulocytes become fully mature (2, 3). Further studies with EAC complexes prepared from purified components of human complement are necessary to verify these results (18).

Superoxide Generation and NBT Dye Reduction. Both uninduced and Me₂SO-induced HL-60 cells were consistently stimulated by PMA to generate superoxide with the level generated by induced cells comparable to that generated by normal peripheral blood granulocytes (Table III). These results are further substantiated by studies with NBT dye reduction. NBT, a water soluble dye, is converted to insoluble intracellular blue formazan by phagocytizing neutrophils (19), a reaction mediated by superoxide (20). Both normal granulocytes and Me₂SO-induced HL-60 cells stimulated by PMA show a higher percentage of cells reducing the dye than the uninduced HL-60 as well as control K-562 cells (Table IV). The intracellular precipitation of the reduced formazan crystals precludes adequate morphological assessment of the particular cells reducing the dye, but the close correlation between the percent of mature cells in a

TABLE III
Superoxide Generation by Human Leukocytes

Cells	Mature myeloid cells*	O ₂ ⁻ generated (A ₅₅₀ ‡/5 × 10 ⁶ cells/20 min)	
		-PMA	+PMA, 100 ng/ml
	%		
HL-60-uninduced	8-12	0.089 ± 0.35	0.228 ± 0.038
HL-60-induced§	75-85	0.062 ± 0.022	1.111 ± 0.086
Peripheral blood granulocytes	>95	0.020 ± 0.008	1.045 ± 0.075
K-562	0	0.028 ± 0.003	0.052 ± 0.018

* Myelocytes, metamyelocytes, banded and segmented neutrophils.

‡ Numbers represent the changes in absorbance at 550 nm of incubated reaction mixtures (see Materials and Methods) which are inhibited by 60 µg of superoxide dismutase.

§ Induced with 1.12% Me₂SO as described in Materials and Methods.

|| Numbers represent the mean with ± the range in triplicate experiments.

TABLE IV
NBT Reduction by Human Leukocytes

Cells	Mature myeloid cells*	Cells reducing NBT‡	
		-PMA	+PMA, 100 ng/ml
	%		%
HL-60-uninduced	8-12	<1	8
HL-60-induced§	75-85	<1	72
Peripheral blood granulocytes	>95	<1	>90
K-562	0	<1	<1

* Myelocytes, metamyelocytes, banded and segmented neutrophils.

‡ Percent of cells containing blue-black formazan deposits as determined on Wright-Giemsa stains of cytopsin preparations of cell suspensions incubated with NBT solution as described in Materials and Methods.

§ Induced with 1.12% Me₂SO as described in Materials and Methods.

culture and the percent of cells reducing NBT (Table IV) suggests that it is the mature myeloid cells rather than the immature promyelocytes that are generating superoxide in both uninduced and Me₂SO-induced PMA stimulated HL-60 cultures.

Thus, the HL-60 leukemia cells, which were derived from a patient who ultimately died from promyelocytic leukemia¹ and which maintain distinct karyotypic abnormalities (8)¹, can be induced under appropriate stimuli to differentiate to morphologically mature granulocytes performing many of the functions commonly associated with normal peripheral blood granulocytes including response to chemoattractants, development of complement receptors, phagocytosis (9), superoxide production, and NBT dye reduction. In related studies, murine myeloid leukemic cells displaying a similar *in vivo* block in differentiation can be induced *in vitro* to mature functioning granulocytes (21). These experiments indicate that certain myeloid leukemic cells maintain the genetic capability to function as normal granulocytes but the expression

¹ Gallagher, R. E., S. J. Collins, K. McCredie, J. Trujillo, M. Ahearn, R. Ting, C. Tai, G. Aulakh, F. Ruscetti, and R. C. Gallo. Characterization of the continuous differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Manuscript submitted for publication.

of these genes *in vivo* is blocked by some unknown mechanism. The etiology of this *in vivo* block in differentiation found in HL-60 as well as other human myeloid leukemia cells is currently being investigated in our laboratory. Moreover, the HL-60 cell line, which is capable of switching on such distinct differentiation markers, provides a model system for studying nuclear and cytoplasmic protein changes as well as variations in messenger RNA expression in differentiating myeloid cells.

Summary

The HL-60 human promyelocytic leukemia cell line can be induced to terminally differentiate to mature myeloid cells sharing a number of functional characteristics with normal granulocytes including response to chemoattractants, development of complement receptors, phagocytosis, superoxide production, and nitroblue tetrazolium dye reduction. Hence the Me₂SO-induced HL-60 cells provide a unique *in vitro* model for studying various important aspects of human myeloid cell differentiation.

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