

FUNCTIONAL CHANGES IN HUMAN LEUKEMIC CELL LINE HL-60

A Model for Myeloid Differentiation

PETER E. NEWBURGER, MARGARET E. CHOVANIEC, JOEL S.
GREENBERGER, and HARVEY J. COHEN

From the Division of Pediatric Hematology and Oncology, Sidney Farber Cancer Institute and Children's Hospital Medical Center; the Department of Radiation Therapy, Joint Center for Radiation Therapy, Sidney Farber Cancer Institute; and the Department of Pediatrics, Harvard Medical School, Boston Massachusetts 02115

ABSTRACT

Polar solvents induce terminal differentiation in the human promyelocytic leukemia cell line HL-60. The present studies describe the functional changes that accompany the morphologic progression from promyelocytes to bands and polymorphonuclear leukocytes (PMN) over 9 d of culture in 1.3% dimethylsulfoxide (DMSO). As the HL-60 cells mature, the rate of O_2^- production increases 18-fold, with a progressive shortening of the lag time required for activation. Hexosemonophosphate shunt activity rises concomitantly. Ingestion of paraffin oil droplets opsonized with complement or Ig increases 10-fold over 9 d in DMSO. Latex ingestion per cell by each morphologic type does not change significantly, but total latex ingestion by groups of cells increases with the rise in the proportion of mature cells with greater ingestion capacities. Degranulation, as measured by release of β -glucuronidase, lysozyme, and peroxidase, reaches maximum after 3–6 d in DMSO, then declines. HL-60 cells contain no detectable lactoferrin, suggesting that their secondary granules are absent or defective. However, they kill staphylococci by day 6 in DMSO. Morphologically immature cells (days 1–3 in DMSO) are capable of O_2^- generation, hexosemonophosphate shunt activity, ingestion, degranulation, and bacterial killing. Maximal performance of each function by cells incubated in DMSO for longer periods of time is 50–100% that of normal PMN. DMSO-induced differentiation of HL-60 cells is a promising model for myeloid development.

KEY WORDS phagocytosis · superoxide ·
granulocyte · lysosome · lactoferrin

A human promyelocytic leukemia cell line, HL-60, was recently established from the peripheral blood of a patient with acute promyelocytic leukemia (9). It has maintained continuous growth in

suspension culture in the absence of added conditioned medium or colony-stimulating factor for over 18 mo and is tumorigenic in athymic nude mice (10). The majority of HL-60 cells are promyelocytic in morphology and histochemistry, but 4–15% of them show morphologic characteristics of more mature myeloid cells: myelocytes, meta-

myelocytes, band forms, and polymorphonuclear leukocytes (PMN) (9).

Initial studies by Collins et al. reported the induction of morphologic differentiation by *in vitro* exposure to dimethylsulfoxide (DMSO) or other polar solvents (9). Accompanying this morphologic change were decreases in [³H]thymidine incorporation and in proliferation. The resulting terminally differentiated cells, but not their promyelocytic precursors, ingested *Candida albicans*.

The present study examines in detail the functional correlates to DMSO-induced morphologic differentiation of the HL-60 cell line, including: (a) generation of superoxide (O₂⁻), (b), hexosemonophosphate shunt (HMPS) activity, (c) ingestion, (d) lysosomal enzyme content and release by degranulation, and (e) bacterial killing. The sequential development of these functional characteristics of normal human granulocytes, coupled with DMSO-induced morphologic maturation, indicates the value of this system for the analysis of the biochemical events in myeloid differentiation.

MATERIALS AND METHODS

HL-60 cells (generously provided by Dr. R. C. Gallo, National Cancer Institute, Bethesda, Md.) were passaged twice weekly in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% heat-inactivated fetal calf serum (Colorado Serum Co., Denver, Colo.) in 7% CO₂ humidified atmosphere as previously described (12). For each experiment, a single flask of cells was serially subdivided and aliquots resuspended in medium with or without 1.3% DMSO (Fisher Scientific Co., Pittsburgh, Pa.) at various time points. At the time of harvesting for assay, cells had received either no exposure to DMSO ("day 0") or had been continuously incubated in DMSO for 1, 3, 6, or 9 d ("day 1", "day 3", etc). Normal human PMN were purified by sedimentation in Dextran 500 and Ficoll-Paque (both from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), as previously described (12), from the peripheral blood of one investigator and contained <1% mononuclear cells and less than 5% eosinophils. Before assay, cells were washed and resuspended at 2-5 × 10⁷/ml in cold phosphate-buffered saline (PBS), pH 7.4, and 0.01-ml samples were cytocentrifuged for differential counts.

Oxidative Metabolism

O₂⁻ production was measured quantitatively in pooled cells by a previously described continuous spectrophotometric assay of superoxide dismutase-inhibitable cytochrome *c* reduction (7, 12). Cells were stimulated by phorbol myristate acetate (PMA) 1 μg/ml (Consolidated Midland Corp., Brewster, N. Y.) or by opsonized (fresh

human serum) zymosan 4 mg/ml (OpZ) (ICN K & K Laboratories Inc., Plainview, N. Y.). Assays were performed at 37°C in a double-beam spectrophotometer. Both sample and reference cells contained ferricytochrome *c* (50 nmol), cells (2.5 × 10⁶), and either PMA (1 μg) or OpZ (4 mg) in 1 ml total volume of Krebs-Ringer's phosphate buffer (pH 7.4). The reference cell contained, in addition, superoxide dismutase (0.01 mg) (Sigma Chemical Co., St. Louis, Mo.). The rate of O₂⁻ production was calculated by dividing the linear change in A₅₅₀ by the molar extinction coefficient for the reduction of ferricytochrome *c* (ΔεM = 21,000) (15). O₂⁻ generation was measured qualitatively in individual cells by observing the reduction of soluble yellow nitroblue tetrazolium (NBT) (Sigma Chemical Co.) to insoluble blue formazan. Cells (2.5 × 10⁶/ml) and NBT (0.1%), with or without PMA (1 μg/ml), were incubated in PBS at 37°C for 10 min, washed three times in cold normal saline containing 1 mM *N*-ethylmaleimide (Sigma Chemical Co.), resuspended in 5% human serum albumin (Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.), smeared on glass slides, and counterstained with safranin. 100 consecutive cells were then observed by light microscopy for blue formazan deposits.

We adapted the method of Stossel et al. (25) for the measurement of HMPS activity. Cell suspensions (2.5 × 10⁶ in 1 ml of Krebs-Ringer's phosphate buffer) were incubated with 5 mM glucose containing 0.1 μCi/μmol of either [1-¹⁴C]- or [6-¹⁴C]glucose (New England Nuclear, Boston, Mass.), and released ¹⁴CO₂ was trapped in hyamine hydroxide (New England Nuclear) for scintillation counting. Glucose oxidation was examined both in resting cells and in cells stimulated by PMA (1 μg/ml) or methylene blue (0.1 mM).

Ingestion

Ingestion of sonicated particles of paraffin oil emulsion containing Oil red O dye and either albumin or *Escherichia coli* lipopolysaccharide (Difco Laboratories, Detroit, Mich.) was measured as described by Stossel and Stossel et al. (23, 24). The former particles were opsonized with Ig by incubation in heat-inactivated rabbit antihuman albumin serum (kindly provided by Dr. A. Davis, Center for Blood Research, Boston, Mass.), and the latter were opsonized primarily with complement by incubation in fresh rabbit serum. The quantity of paraffin oil ingested was calculated from the A₅₂₅ of Oil red O in dioxane (Mallinkrodt Inc., St. Louis, Mo.) extracts of washed cell pellets, and the known A₅₂₅ in dioxane of the solution of Oil red O in paraffin oil used for the preparation of the emulsions. Ingestion of latex beads (0.8 μm Diam) (Sigma Chemical Co.) was measured by substituting latex beads (2 × 10⁶ beads/ml) for PMA in the NBT reduction assay above. Under the light microscope, 100 cells from each group were observed for nuclear morphology and the number of ingested beads

(distinguishable from adherent beads by the ring of blue formazan around those digested).

Degranulation

Cell suspensions (2×10^7 /ml) in PBS with 0.5 μ g/ml cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.) were incubated 45 min at 37°C with OpZ 6 mg/ml, centrifuged for 5 min at 300 g at 4°C, and the supernatant fluid was assayed for enzyme released. Whole cells were lysed with 0.05% Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.) and assayed for total enzyme content. All enzyme assays were performed in 0.05% Triton X-100. The lysozyme assay measured the change in light scattering (OD_{450}) resulting from the lysis of *Micrococcus lysodeikticus* (20). The β -glucuronidase assay measured the formation of *p*-nitrophenol from *p*-nitrophenyl- β -D-glucuronide (16). The G6PD assay followed the change in A_{340} accompanying the reduction of NADP in the presence of glucose-6-phosphate (5). The peroxidase assay, modified from Bretz and Baggiolini (6), continuously measured the initial linear increase in A_{450} at 25°C upon addition of a 10- μ l sample to a cuvette containing 0.08 M H_2O_2 and 0.32 mM 3,3'-dimethoxybenzidine (Eastman Kodak Co.) in 0.99 ml of the 0.1 M citrate buffer, pH 5.5. Lactoferrin extraction and immunodiffusion assay were performed as described by Bennett and Kokocinsky (4), using goat antihuman lactoferrin (Atlantic Antibodies, Westbrook, Maine) and standards of human colostrum lactoferrin (Sigma Chemical Co.).

Bacterial Killing

ATCC-25923 *Staphylococcus aureus* (kindly provided by Dr. Donald Goldmann, Children's Hospital, Boston, Mass.) was opsonized in fresh human serum, sonicated for 15 s (setting 40 on a Sonifier, Branson Sonic Power Co., Danburg, Conn.), and adjusted to $OD_{450} = 0.5$ in PBS. Mixtures of bacteria and cells, both at 5×10^6 /ml

in PBS containing 5 mM glucose and 10% fresh human serum, were sampled before and after shaking for 90 min at 37°C. Samples were diluted first in distilled H_2O to lyse cells, then in PBS before plating and counting colonies after overnight incubation.

RESULTS

Morphologic Changes

HL-60 cells incubated in 1.3% DMSO underwent progressive morphologic change from predominantly promyelocytes to metamyelocytes, bands, and PMN. By day 9, only 38% of the cells had completed maturation to the band or PMN stage, but further incubation produced cell death without further increase in the proportion of mature cells. The differential counts shown in Table I were typical of all the experiments described below. Viability, determined by trypan blue exclusion, was 85–90% for HL-60 cells unexposed to DMSO and 75–85% for cells in 1.3% DMSO from day 1 through day 9 of incubation.

Oxidative Metabolism

Normal human PMN generate virtually no O_2^- in the resting state. After activation by PMA or OpZ, they show a gradual rise to a maximal constant rate of O_2^- production (reference 5; also P. E. Newburger and H. J. Cohen, unpublished data).

As shown in Fig. 1, HL-60 cells responded similarly in the cytochrome *c* reduction assay. In the course of differentiation the rate of O_2^- production increased 18-fold, with a progressive shortening of the lag time required for activation. Both the rate and lag time approached but did not

TABLE I
HL-60 Cells Incubated in DMSO: Differential Counts and NBT Reduction

Cell type	Differential Counts					NBT reduction
	Promyelocytes	Myelocytes	Metamyelocytes	Bands	PMN	
	% total cells					
HL-60 day 0	96	2	1	1	0	10
day 1	98	1	1	0	0	24
day 3	78	16	6	1	0	84
day 6	41	30	15	12	1	97
day 9	6	34	22	29	9	97
Normal PMN	—	—	—	2	98*	99

HL-60 cells incubated for the indicated number of days in 1.3% DMSO and normal human peripheral blood PMN were harvested and tested for NBT reduction as described in Materials and Methods. Cytocentrifuge smears were stained with Wright's-Giemsa's stain.

* Includes 1–5% eosinophils.

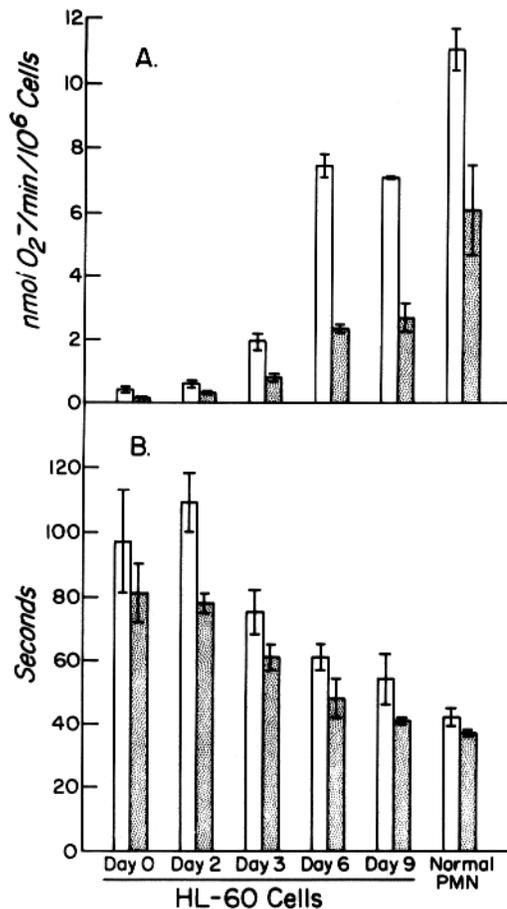


FIGURE 1 O₂⁻ generation by HL-60 cells and normal PMN. HL-60 cells were cultured for the indicated times in 1.3% DMSO. (A) shows linear rates of O₂⁻ production. (B) shows the lag time for the activation of the O₂⁻ generation system. Cells were stimulated by PMA (clear bars) or OpZ (stippled bars). Bar heights represent means of triplicate determinations; error lines, SEM.

equal those of PMN. Cells washed in 1.3% DMSO before harvesting were not detectably different from unexposed cells.

The cytochrome *c* reduction assay quantitatively measures pooled O₂⁻ production by all cells. To determine what fraction of cells was generating the O₂⁻, we also utilized a NBT reduction assay that allows qualitative examination of each cell. HL-60 cells incubated for 1 d in DMSO (when no morphologic change has yet taken place) showed twice the proportion of PMA-stimulated O₂⁻-producing cells as those unexposed to DMSO. By days 3 and 5, 84 and 97% of cells, respectively, reduced NBT upon PMA stimulation. Unstimu-

lated cells reduced no NBT. Because exposure to PMA causes morphologic degeneration (18), it was not possible to determine the stage of differentiation of the active cells.

HMPS activity (Table II) was measured by the increase in oxidation of [1-¹⁴C]glucose upon PMA stimulation. PMA-stimulated [6-¹⁴C]glucose oxidation (i.e., Krebs' cycle) was far lower in magnitude. As the HL-60 cells morphologically differentiated, HMPS activity increased more than 20-fold. Maximum activity, reached by day 6, equalled that of normal PMN. Methylene-blue-stimulated HMPS activity rose only threefold over the same time period: day-0 cells released 191 ± 14 cpm/30 min/10⁶ cells (mean ± SEM) and day-6 cells 578 ± 16.

Ingestion

As for O₂⁻ measurement, we utilized assays of ingestion by pooled cells (paraffin oil droplets) and individual cells (latex beads). Ingestion of complement- and of Ig-opsonized droplets (Fig. 2) doubled by day-2 of DMSO incubation and by day-9 was six times that of unexposed cells. At day 9, the peak ingestion rate for HL-60 cells was 75% that of normal PMN. All cells ingested Ig-opsonized droplets 20–30% as rapidly as complement-opsonized droplets.

Latex bead ingestion (Table III) also reached maximum at day 9. The proportion of mature HL-60 cells (metamyelocytes, bands, and PMN) ingesting latex and the mean number of beads ingested per cell did not change significantly over the course of incubation in DMSO. Active HL-60 bands and PMN ingested as many beads as normal PMN. The safranin counterstain did not distinguish promyelocytes from myelocytes, so the ap-

TABLE II
Hexosemonophosphate Shunt Activity

Cells	[1- ¹⁴ C]glucose oxidation	[6- ¹⁴ C]glucose oxidation
HL-60 day 0	65 ± 1	15 ± 9
day 1	167 ± 17	ND*
day 3	1,330 ± 60	ND
day 6	1,900 ± 10	127 ± 58
day 9	1,800 ± 360	ND
Normal PMN	1,700 ± 240	331 ± 19

Results are expressed as the mean ± SEM of triplicate determinations of the difference between ¹⁴CO₂ release (cpm/30 min/10⁶ cells) by PMA-stimulated cells and ¹⁴CO₂ release by resting cells.

* ND, not determined.

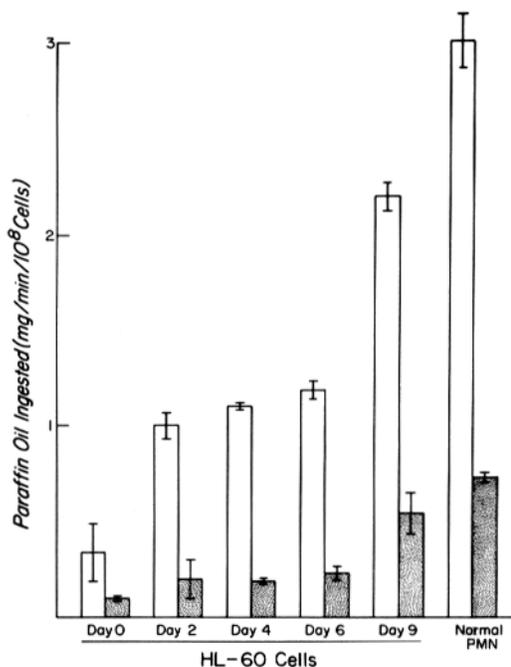


FIGURE 2 Ingestion of paraffin oil droplets by HL-60 cells and normal PMN. HL-60 cells were cultured for the indicated times in 1.3% DMSO. Clear bars show ingestion rates for droplets of emulsion containing lipopolysaccharide, opsonized with fresh serum (complement). Stippled bars represent ingestion of droplets containing albumin, opsonized with heat-inactivated anti-albumin serum (Ig). There was no ingestion of unopsonized droplets. Bar heights show means, and error lines show SEM of triplicate determinations.

parent increase in both proportions of active immature cells and number of beads per cell may reflect undetected maturation from promyelocytes to myelocytes.

Degranulation

Table IV shows the results of enzyme assays of lysed whole cells and the percentages of enzyme activity released during OpZ-induced degranulation. As HL-60 cells matured in DMSO, lysozyme content doubled, β -glucuronidase increased 50%, and G6PD tripled, but peroxidase fell 60%. Enzyme release by degranulation increased three- to fourfold at either day 3 (lysozyme and peroxidase) or day 6 (β -glucuronidase), then declined. Maximal enzyme content and release approached those of normal PMN. Release of G6PD, a cytoplasmic enzyme which served as a control for nonspecific "leakiness," decreased as the cells differentiated.

The release of similar proportions of all four enzymes from day-0 cells suggests that lysosomal enzyme release from these undifferentiated cells may represent leaking rather than degranulation.

Lactoferrin is a marker specific to human secondary (specific) granules (16). HL-60 cells contained no detectable lactoferrin at any stage of maturation. The lower limit of detection by precipitin line in the immunodiffusion system was 0.01 mg/ml, ~1% the content extracted from normal PMN at 5×10^8 /ml. The precipitin line for normal PMN did not interact with the HL-60 day-9 or day-3 extracts (i.e., did not deviate as it approached those wells), indicating that the HL-60 cells had <1% the lactoferrin content of normal PMN.

Bacterial Killing

After 3 d of incubation in DMSO, HL-60 cells killed opsonized *S. aureus* and by day 6 approached the efficiency of normal PMN (Fig. 3).

DISCUSSION

These studies indicate that DMSO induces functional as well as morphologic (10) maturation in human promyelocyte leukemia cell line HL-60. These cells dramatically increased their capacity to generate superoxide, activate the HMPS, ingest particles, degranulate, and kill bacteria. Maximal performance of each function was 50–100% that of normal PMN.

As the HL-60 cells matured, the rate of O_2^- generation increased and the lag time required for activation of the system decreased. Because activation requires ongoing energy production (8), the shortening of the lag time could represent an increased energy supply (e.g., a rise in available ATP), increased production of an activating system (e.g., a kinase), or synthesis of a more easily activated oxidase. Because the O_2^- generating system is membrane-bound (2), an alternative explanation could be that DMSO-induced differentiation produces a change in membrane structure of fluidity that facilitates both the activation process and the enzyme activity of the oxidase.

O_2^- production in HL-60 cells was not limited to bands and PMN. At day 3, only 7% of the cells had matured beyond the myelocyte stage, yet 84% reduced NBT, a reaction that depends upon O_2^- generation in intact cells (3). Morphologically immature cells also activated the HMPS, ingested, and degranulated. These results confirm the data

TABLE III
Ingestion of Latex Beads

	Promyelocytes and Myelocytes				Metamyelocytes			Bands and PMN		
	Proportion ingesting beads	Beads per cell		Proportion ingesting beads	Beads per cell		Proportion ingesting beads	Beads per cell		
		Range	Mean ± SEM		Range	Mean ± SEM		Range	Mean ± SEM	
HL-60 day 0	1/98	1	1	1/2	6	6	—	—	—	
day 1	1/99	1	1	1/1	7	7	—	—	—	
day 3	3/87	1-5	3 ± 1	3/10	11-20	13 ± 4	2/3	18, 40	29	
day 5	8/76	1-6	4 ± 1	8/19	5-17	11 ± 2	2/3	30, 45	38	
day 9	8/52	1-7	5 ± 1	13/34	2-20	12 ± 1	6/13	15-53	29 ± 6	
Normal PMN	—	—	—	—	—	—	89/100	1-76	23 ± 2	

For each morphologic type, statistical comparison of the number of beads per cell by the Wilcoxon test (13) showed $p \geq 0.10$ for day 0 or 1 vs. day 9 and day 9 vs. normal PMN.

TABLE IV
Enzyme Content of Cells and Release During Degranulation

Cell type	β -glucuronidase		Lysozyme		Peroxidase		G6PD	
	Whole cells	% release	Whole cells	% release	Whole cells	% release	Whole cells	% release
HL-60 day 0	1.02 ± 0.01	8.93 ± 0.20	0.824 ± 0.022	7.16 ± 0.87	2.43 ± 0.10	5.56 ± 0.01	0.298 ± 0.013	7.18 ± 0.19
day 1	0.821 ± 0.002	12.6 ± 0.03	0.691 ± 0.052	7.44 ± 0.20	1.51 ± 0.02	5.92 ± 0.56	0.306 ± 0.005	3.61 ± 0.31
day 3	1.21 ± 0.01	18.2 ± 0.60	0.941 ± 0.20	31.4 ± 2.3	1.47 ± 0.06	18.3 ± 1.0	0.581 ± 0.013	1.82 ± 0.05
day 6	1.58 ± 0.01	26.1 ± 0.20	1.65 ± 0.04	19.9 ± 0.8	1.21 ± 0.04	17.5 ± 1.3	0.945 ± 0.095	1.19 ± 0.04
day 9	1.44 ± 0.01	19.5 ± 0.01	1.62 ± 0.11	15.6 ± 1.3	0.992 ± 0.028	11.9 ± 0.5	1.03 ± 0.02	1.71 ± 0.01
Normal PMN	3.02 ± 0.03	33.2 ± 0.20	1.99 ± 0.04	35.8 ± 0.4	2.76 ± 0.09	26.3 ± 0.7	1.91 ± 0.09	<0.1

Results are expressed as the mean ± SEM of triplicate or quadruplicate determinations. Enzyme activity expressions are β -glucuronidase: $A_{410}/3 \text{ h}/10^7$ cells; lysozyme: $\Delta\text{OD}_{450}/\text{min}/10^7$ cells; peroxidase: $\Delta A_{450}/\text{min}/10^6$ cells; and G6PD: $\text{nmol NADPH}/\text{min}/10^6$ cells.

indicating that bone marrow myeloid precursors can reduce NBT (27) and ingest paraffin droplets (1) and that other morphologically immature leukemia cell lines generate O_2^- (12).

We used glucose oxidation as a measure of HMPS activity to examine changes in the respiratory burst. The observed rise could also conceivably reflect changes in glucose transport or storage pools, in glutathione levels, or in HMPS enzyme activities. If so, however, one would expect similar changes to take place regardless of the mode of stimulation. The much greater magnitude of the increase in PMS-stimulated glucose oxidation relative to that in oxidation induced by methylene blue suggests that at least the former represents a real change in oxidative metabolism.

The latex bead experimental data indicate that the rise in ingestion resulted from increased numbers of active mature cells rather than an increase in the capability of each morphologic cell type. The development of full ingestion capacity may reflect formation of surface complement and Ig receptors or changes in cytoskeleton motility permitting pseudopod and phagosome formation.

The ability of OpZ to stimulate degranulation and O_2^- generation in less mature cells implies the presence of complement receptors at least by day-3 of culture in DMSO. More detailed studies will be required to find out whether any change in receptor number or affinity takes place during maturation of HL-60 cells. Cytoskeletal changes alone could produce the rise in ingestion capacity and, indeed, must be invoked for the ingestion of latex beads, which required no opsonization.

Degranulation reached maximum at 3-6 d in DMSO, then decreased. The absence of lactoferrin suggests that HL-60 cells may produce defective secondary (specific) granules or perhaps none at all. Degranulation may become less efficient as cells progress from the early stages, in which primarily (azurophilic) granules predominate, to mature stages in which secondary granules become important (22). Because the normal pattern of development of degranulation is not known, an alternative explanation could be simply that early myeloid cells degranulate more efficiently than PMN.

A possible analogy is the Chediak-Higashi syn-

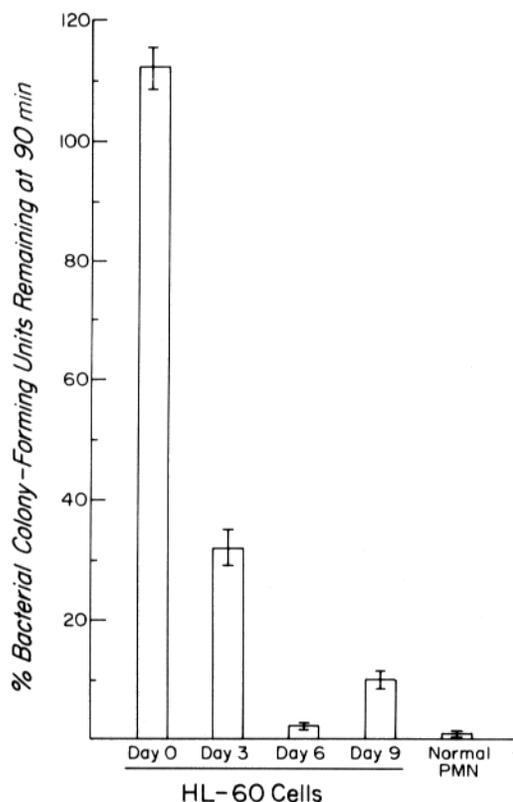


FIGURE 3 Bacterial killing by HL-60 cells and normal PMN. HL-60 cells were cultured for the indicated times in 1.3% DMSO. The number of viable staphylococci was measured before and after incubation for 90 min with each cell type. The proportion of bacteria remaining after incubation with HL-60 day-0 cells was identical to that after incubation with no cells. Bar heights represent the means, and error lines the SEM of quadruplicate determinations.

drome, in which PMN contain normal primary but abnormal secondary granules (17) and show selective impairment of degranulation (26). The bactericidal studies indicate that these cells can kill staphylococci in the absence of lactoferrin. Spitznagel has described a patient with myeloproliferative disease whose PMN contained no lactoferrin or specific granules and killed staphylococci normally but had reduced killing efficiency for *E. coli*, proteus, and enterococci (21).

Another in vitro model of normal granulocyte differentiation is the mouse myeloid leukemia cell line reported by Sachs (19). This mouse cell line differentiates in response to colony-stimulating activity (macrophage and granulocyte inducer),

plant lectins, some steroid hormones, and a variety of organic chemicals including DMSO. Studies with these cells have provided information on the genetic control of morphologic differentiation, lysozyme synthesis, surface receptor formation, and cytoskeleton changes. A human myeloid leukemia cell line dependent on colony-stimulating activity for both growth and differentiation in vitro has recently been described by Koeffler and Golde (14).

Analysis of functional differentiation in HL-60 and other cell lines should be useful to the elucidation of the mechanisms of normal myeloid differentiation and relationships between structure and function, much as studies with the Friend erythroleukemia system (11) have done for erythroid development. Comparison of the differences between models could provide information relating the relative importance of various processes to granulocyte function.

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