Development of the Superoxide-generating System during Differentiation of the HL-60 Human Promyelocytic Leukemia Cell Line*

Peter E. Newburger, Cheryl Speier, Niels Borregaard, Christopher E. Walsh, John C. Whitin, and Elizabeth R. Simon§

From the Department of Pediatrics, University of Massachusetts Medical School, Worcester, Massachusetts 01605 and the Departments of Medicine and Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

Utilizing the induced differentiation of HL-60 promyelocytic leukemia cells as a model of myeloid maturation, we examined the development of the superoxide-generating system, focusing on NADPH oxidase activity, membrane depolarization, and cytochrome b content. NADPH oxidase activity, measured as NADPH-dependent superoxide production, increased with both spontaneous and N,N-dimethylformamide-induced differentiation. Activity in particulate fractions from induced HL-60 cells and human peripheral blood polymorphonuclear leukocytes was proportional to their relative rates of superoxide production, but activity from uninduced cells was surprisingly high: one-third that from induced cells, despite only 7% their rate of superoxide generation. NADPH oxidase activities in phagocytic vesicles from induced HL-60 cells and polymorphonuclear leukocytes were equal, indicating the equivalence of the enzyme system in active portions of their cell membranes. Separation by centrifugal elutriation of the HL-60 cell population into fractions of varying maturity confirmed the relationship of NADPH oxidase activity to advancing differentiation in both dimethylformamide-induced and spontaneously maturing cells. Membrane potential change, an early event related to activation of the oxidase, was followed by 3,3'-dipropylthiodicarbocyanine dye fluorescence. The depolarization response increased dramatically in both magnitude and initial rate of change during differentiation. The cells' cytochrome b content increased 3-fold with induction of differentiation, in proportion to the change in NADPH oxidase activity.

Phagocytic cells such as PMN and macrophages generate O$_2^-$ as an essential component of their microbialidal system (1). The one-electron reduction of molecular oxygen to O$_2^-$ is catalyzed by an NADPH oxidase (2-4) that is inactive in resting cells but rapidly activates upon exposure of cells to a variety of soluble or particulate stimuli (1). The exact structure of the NADPH oxidase remains unknown, but there is evidence to suggest that it is a transmembrane (2) electron-transport system with flavoprotein (4-6), cytochrome b (4-7), and perhaps quinone (8) components.

Most studies of the O$_2^-$generating system have utilized mature peripheral blood or exudate PMN. The development of O$_2^-$generating activity during myeloid maturation has received little attention. Zakhireh and Root (9) examined the ability of bone marrow myeloid precursors to reduce NBT dye, a process related to O$_2^-$ production (10), and noted activity only in cells at or beyond the metamyelocyte stage of maturation. However, this histochemical study was not quantitative.

The induced differentiation of the HL-60 promyelocytic leukemia cell line provides an alternative model of myeloid differentiation (11-16). This continuous cell line, derived from a patient with acute promyelocytic leukemia (11), undergoes differentiation in vitro to myelocytes, metamyelocytes, band forms, and PMN upon incubation with polar organic solvents (12) such as dimethyl sulfoxide or DMF and a wide variety of other agents (16). Differentiated HL-60 cells are capable of most PMN functions: chemotaxis, ingestion, respiratory burst activity, and bacterial killing (13-15). Our previous studies (13) showed that HL-60 cells incubated with dimethyl sulfoxide increased their rates of O$_2^-$ production in response to PMA from 0.4 nmol/min/10$^6$ cells at day 0 of incubation to 1.9 at day 3, 7.4 at day 6, and 7.1 at day 9. Concomitantly, the lag time for O$_2^-$ production (a measure of the time required for activation from the resting state) shortened from 97 s at day 0 to 75 at day 3, 61 at day 6, and 54 at day 9. Corresponding reference values for PMN were 11 nmol of O$_2^-$/min/10$^6$ cells and 42 s.

Thus, having demonstrated the maturation of the superoxide-generating system and its activation mechanism during induced differentiation of HL-60 cells, we sought to examine the biochemical basis of these changes. The present study

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1 The abbreviations used are: PMN, polymorphonuclear leukocytes; di-S-C$_2$(5), 5,5'-dipropylthiodicarbocyanine; DMF, N,N-dimethylformamide; O$_2^-$, superoxide; PBS, Dulbecco's phosphate-buffered saline without Ca$^{2+}$ or Mg$^{2+}$ (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$; pH 7.4); PMA, phorbol myristate acetate; NBT, nitroblue tetrazolium.
foci on the development of NADPH oxidase activity, cytochrome b content, and membrane depolarization.

**EXPERIMENTAL PROCEDURES**

The following materials were purchased from the indicated suppliers: cytochrome c (type VI), nitroblue tetrazolium, catalase, superoxide dismutase, 0.8–µm diameter latex beads, salinomycin A, and Triton X-100 from Sigma; dextran 500 and Ficoll-Paque (Ficoll-Hypaque) from Pharmacia; DMF, certified ACS grade, from Fisher Scientific Co.; Fair Lawn, NJ; PMA from Consolidated Midland Chemicals, Brewster, NY; Dulbecco's modified Eagle medium with 4.5 g/liter of d-fetal calf serum, newborn calf serum solution, antibiotic-antimycotic solution (10,000 units/ml of penicillin base, 10,000 µg/m of streptomycin, and 22 µg/ml of amphotericin B) from Hanks' balanced salt solution from GBHC Laboratories, Grand Island, NY; and ITS premix (100 µg of insulin, 100 µg of transferrin, and 100 µg of sodium selenate) from Collaborative Research, Waltham, MA. These and all other reagents were obtained at the highest level of purity and used without further purification. Catalase (64,000 units/mg of beef liver) from Worthington was dialyzed overnight against distilled water. The fluorescent probe di-S-C₆(5) was the generous gift of Dr. Alan Waggoner, Carnegie-Mellon University, Pittsburgh, PA.

**Cell Preparation—**HL-60 cells, generously provided by Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD), were grown, unless otherwise noted, in Dulbecco's modified Eagle medium with 10% (v/v) heat-inactivated fetal calf serum and 2% antibiotic-antimycotic solution in 75-cm² or 175-cm² tissue culture flasks at 37 °C in a humidified incubator with 5% CO₂ as previously described (13). For cytochrome b experiments, fetal calf serum was replaced by 5 µg/ml boric solution in 75-cm² or 175-cm² tissue culture flasks at 37 °C in a hypotonic lysis of erythrocytes. Differentiation was induced by a modification described by Breitman et al. (17). Differentiation was induced by 0.3% dimethyl sulfoxide (DMSO) in the presence of 100 µg/ml of actinomycin D. The distribution of cells among the fractions was not otherwise noted, in Dulbecco's modified Eagle medium with 10% (v/v) heat-inactivated fetal calf serum and 1% (v/v) antibiotic-antimycotic solution (10,000 units/ml of penicillin base, 10,000 µg/ml of streptomycin, and 22 µg/ml of amphotericin B) (31). Routine cell recoveries showed that 98% purity as previously described (18) by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of erythrocytes.

**Centrifugation Elutriation—**Uninduced or induced HL-60 cells were separated on the basis of size and density with a Beckman JE-6B elutriation system (Beckman, Palo Alto, CA). We used a modification of the protocol described by Meistrich (19) for thymocytes and by Collins and Foster (20) for HL-60 cells. A total of 10⁹ cells were loaded into a JE-6B rotor spinning at 2800 rpm (4 °C), then fractions were collected at increasing flow rates by eluting with Dulbecco's phosphate-buffered saline (18) with 5 mM glucose and 0.9% NaCl. The buffer also contained 500 units/ml of catalase to inhibit NADPH oxidase activity, as previously described (6) by continuous spectrophotometric assay of cytochrome c reduction, followed at 550 nm in a Perkin Elmer model 552 double-beam spectrophotometer. Both soluble and particulate cell content was determined. Each cell fraction plus 50 µg cytochrome c, 1 mM NADPH, and 1 mM NaN₃ in 0.9 ml of 1.3 mM KF buffer, pH 7.0, at 28 °C. The reference cuvette contained 20 µg/ml of superoxide dismutase in addition. Triton X-100 (0.01%) was added to both cuvettes as indicated.

Phagocytic vesicles were prepared and assayed by modifications of previously described techniques (23, 24). Briefly, cells at 10⁹/ml and 0.8-µm diameter latex beads (preincubated with 1 µg/ml of PMA and thirite washed) at 10⁶/ml were incubated for 7 min at 37 °C in 11.2% (v/v) sucrose, pH 7.4, Hanks' balanced salt solution (31) containing 1 mM NaN₃. The incubation was terminated by addition of cold 1 mM EDTA in Hanks' balanced salt solution; then cells were recovered by centrifugation at 125 × g for 5 min at 4 °C, resuspended in 11.2% sucrose, pH 7.4, and sonicated as described above. The sonicate medium was brought to 46% sucrose by addition of 60% (w/v) sucrose, overlaid in a centrifuge tube with 35% (w/v) followed by 11.2% (v/v) sucrose, and centrifuged at 24,000 × g for 20 min. Phagocytic vesicles, harvested from the upper interface, were diluted with distilled water to 0.25 M sucrose. The vesicle preparation was assayed for protein content by the Lowry method (21) and for NADPH-dependent superoxide dismutase-inhibitable cytochrome c reduction as described above for particulate fractions, except for the use of 0.05 M KP buffer, pH 7.5.

NBT slides were prepared as previously described (13) by incubating cells and PMA-coated latex beads under conditions identical to that for preparing phagocytic vesicles, with the addition of 0.04% (w/v) NBT. The cell pellet was smeared on glass slides and counterstained with 1% (w/v) eosin. Two hundred phagocytic vesicles were scored for the number of associated latex beads and the presence and localization of reduced NBT (blue formazan deposits).

**Membrane Depolarization—**The membrane potential change in response to PMA stimulation was measured as the change in emission fluorescence of the dye di-S-C₆(5) as previously described (25). Cells (2 × 10⁶) were equilibrated with 2 × 10⁻⁶ M di-S-C₆(5) in 0.95 ml of Krebs-Ringer's phosphate buffer, pH 7.4, at 37 °C with continuous stirring. The buffer also contained 500 units/ml of catalase to inhibit peroxide-mediated dye degradation (26). Activation was initiated by the addition of microsyringe of 1.0 µg of PMA in 0.05 µl of the same buffer. Continuous measurement of the dye fluorescence was performed on an in-line Oxiclear gas purifier (VWR Scientific Co., subsidiary of Univar Corporation, Boston, MA) was blown over the surface (27). Anaerobicosis was confirmed by inhibition of PMA-stimulated cytochrome c reduction. After obtaining the anaerobic versus aerobic difference spectrum, the sample cuvette was opened, a few grains of

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sodium dithionite, and a dithionite-reduced versus oxidized difference spectrum obtained (7). Finally, 0.1 ml of 1% (v/v) Triton X-100 was added to both cuvettes and the spectrum repeated. Alternatively, cells were incubated for 10 min at 37 °C in PBS plus 5 mM glucose in cuvettes with or without 667 ng/mg of cell protein of antimycin A, then spectra recorded. Antimycin A was first dissolved at 10 mg/ml in absolute ethanol, then diluted to 0.2 mg/ml in 1 mg/ml of bovine serum albumin in 0.025 M KP, pH 7.5, then further diluted 1:80 to 1:100 in the sample cuvette. The reference cuvette received an equivalent volume of ethanol in albumin-KPi prepared as for antimycin A. All spectra were performed from 400 to 800 nm on a double beam Perkin-Elmer model 576 or model 552 spectrophotometer.

RESULTS

NADPH Oxidase Activity—In order to measure the NADPH oxidase activity responsible for O2 generation, we first assayed NADPH-dependent, superoxide-dismutase-inhibitable reduction of cytochrome c by the “particulate fraction” of HL-60 cells. As shown in Fig. 1, the specific activity of this membrane-enriched preparation from differentiated HL-60 cells (“+DMF”) was twice that of uninduced HL-60 cells (“−DMF”) but still half that of PMN. All preparations showed a similar enhancement of activity by Triton X-100 (Fig. 1, cross-hatched bars). The total NADPH oxidase activities recovered followed the same pattern exhibited by the specific activities. Particulate fraction preparations from uninduced HL-60 cells contained total NADPH oxidase activities of 21 ± 6 nmol of O2/min; induced HL-60, 58 ± 12 nmol of O2/min; and PMN, 79 ± 17 nmol of O2/min. Induced and uninduced HL-60 cell populations are heterogeneous in their stages of differentiation (12). Induction causes a shift to a much higher proportion of mature cell types, but all stages from promyelocyte to PMN are present in both. Centrifugal elutriation provides a means of fractionating these mixtures into subpopulations of greater or lesser maturity. As shown in Table I, elutriation of induced HL-60 cells separated into the early fractions (fractions 1 and 2) a subpopulation of small cells, 80% mature, representing 46% of the cells in the starting mixture. These fractions, enriched for highly differentiated cells, contained NADPH oxidase activity equivalent to that of PMN (for PMN reference value, see Fig. 1). Subsequent fractions with less mature cells showed lower NADPH oxidase activity. Elutriation of uninduced HL-60 cells produced a different cell distribution, with more cells in the later, less mature, large cell fractions. Fraction 8, with only 6% differentiated cells, showed particulate fraction NADPH oxidase activity of only 1.7 nmol of O2/min/mg of protein. The first fractions (fractions 1–3), containing most of the mature cells, showed higher specific activities that probably contributed to the activity of the whole population represented in Fig. 1.

In order to select for NADPH oxidase activity in activated segments of the cell membrane, we prepared phagocytic vesicles from HL-60 cells and PMN. Such preparations contain only the portion of the membrane that has wrapped around an ingested particle to form a phagosome. We utilized latex beads coated with PMA in order to maintain the same activator of O2 generation as in the particulate fraction and whole cell (13) experiments. As shown in Fig. 2, phagocytic vesicles from induced HL-60 cells contained NADPH oxidase activity equal to that of PMN and more than twice that of uninduced HL-60 cells. There was no enhancement of activity by Triton X-100 (cross-hatched bars), indicating that the preparations contained only inside-out (i.e. NADPH-binding site external) membrane (2, 24) without contamination by Triton-enhanceable randomly oriented membrane unassociated with the latex beads. NBT slides of HL-60 cells incubated with PMA-coated latex showed activation only within the phagocytic vesicles. Active vesicles containing both latex and reduced NBT (formazan) were present in 97% of PMN and 79% of induced HL-60 cells (results are the means of 100-cell counts in duplicate experiments). Such active phagocytic vesicles were present in 30% of uninduced cells but, unlike the induced HL-60 or PMN, only 8% of cells contained >20 vesicles per cell and the remaining 22% contained 1 to 20 vesicles per cell, with most cells only having one to five ingested particles. Thus, the phagocytic vesicle activity in uninduced cells represents a selection of membrane from a small minority of active cells, probably those expressing spontaneous maturation.

**Membrane Depolarization**—The fluorescent dye di-S-C6(5) provides an indirect probe of membrane potential in cells too small for direct electrode measurements (25). Continuous flurometric analysis, such as that shown in Fig. 3, can follow

![Fig. 1](http://example.com/fig1.png)  
**Fig. 1 (left).** Particulate fraction NADPH oxidase activity from HL-60 cells and PMN. HL-60 cells, either uninduced (−DMF) or induced (+DMF) by incubation with 60 mM DMF, and PMN were prepared and NADPH-dependent O2 production measured as described under "Experimental Procedures." Open and hatched bars represent, respectively, measurements in the absence and presence of 0.018% (v/v) Triton X-100. Bars and error lines indicate the means ± SE of five experiments.

![Fig. 2](http://example.com/fig2.png)  
**Fig. 2 (right).** Phagocytic vesicle NADPH oxidase activity from HL-60 cells and PMN. HL-60 cells, either uninduced (−DMF) or induced (+DMF) by incubation with 60 mM DMF, were prepared and NADPH-dependent O2 production measured as described under "Experimental Procedures." Open and hatched bars represent, respectively, measurements in the absence and presence of 0.018% (v/v) Triton X-100. Bars and error lines indicate the means ± SE of six experiments.

**Table I**  
**NADPH oxidase activity in elutriation fractions of HL-60 cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Elutration fraction(s)</th>
<th>Differentiated cells</th>
<th>Cell number</th>
<th>% of total cells recovered</th>
<th>nmol O2/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60, +DMF 1+2</td>
<td>80</td>
<td>46</td>
<td>90 ± 22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3+4</td>
<td>57</td>
<td>15</td>
<td>13.4 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2</td>
<td>47</td>
<td>5</td>
<td>7.2 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>10</td>
<td>4.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>21</td>
<td>1.7 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIG. 1 (left). Particulate fraction NADPH oxidase activity from HL-60 cells and PMN. HL-60 cells, either uninduced (−DMF) or induced (+DMF) by incubation with 60 mM DMF, and PMN were prepared and NADPH-dependent O2 production measured as described under "Experimental Procedures." Open and hatched bars represent, respectively, measurements in the absence and presence of 0.018% (v/v) Triton X-100. Bars and error lines indicate the means ± SE of five experiments.

FIG. 2 (right). Phagocytic vesicle NADPH oxidase activity from HL-60 cells and PMN. HL-60 cells, either uninduced (−DMF) or induced (+DMF) by incubation with 60 mM DMF, were prepared and NADPH-dependent O2 production measured as described under "Experimental Procedures." Open and hatched bars represent, respectively, measurements in the absence and presence of 0.018% (v/v) Triton X-100. Bars and error lines indicate the means ± SE of six experiments.
was assayed.

Experimental Procedures." Cell types are indicated on the figure as follows: uninduced HL-60 (---); HL-60, day 3, in 60 mM DMF (----); HL-60, day 7, in DMF (---); and PMN (----).

TABLE I
Membrane potential change in PMA-stimulated HL-60 cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Elutriation fraction(s)</th>
<th>Maximal fluorescence change</th>
<th>Initial rate of fluorescence change</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Pre-elutriation</td>
<td>0.21</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.56 ± 0.17</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>4−7</td>
<td></td>
<td>0.20 ± 0.07</td>
<td>12 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.04 ± 0.01</td>
<td>1.9 ± 0.8</td>
</tr>
</tbody>
</table>

the change in membrane potential that occurs upon stimulation, in this case by PMA added at time 0. Uninduced cells showed no detectable depolarization response. Cells induced to differentiate by incubation with DMF for 3 or 7 days showed progressive increases in the maximum fluorescence change. The kinetics of response for induced HL-60 cells and PMN were similar: an 8-s lag time followed by a rise to a maximum at 3-4 min. As shown quantitatively in Table I, induction of differentiation is accompanied by a dramatic rise in the depolarization response, up to a maximum half that of PMN. Elutriation of HL-60 cells at day 4 of incubation in DMF (i.e., before maximal induction) produced fractions with a similar progression of response. The least differentiated, large cells in fractions 4−7 had a very small degree of depolarization and a slow rate of rise of fluorescence. Progressively smaller, more differentiated cells in fractions 3 and 2 increased both the maximal fluorescence change and its initial rate of increase. Fraction 2 activity was enriched more than 2-fold above the pre-elutriation mixture.

Cytochrome b—Preliminary experiments showed that HL-60 grown in culture medium containing fetal calf serum were contaminated by hemoglobin, detectable on anaerobic versus aerobic difference spectra. The hemoglobin content could not be removed by repeated washing of cells in PBS and increased with the induction of differentiation, suggesting an increased "stickiness" or ability to absorb hemoglobin onto mature cells. Further studies of cytochrome b were therefore performed in HL-60 cells grown for at least 3 weeks in serum-free medium, but even these cells often showed a small hemoglobin absorbance peak on anaerobic versus aerobic difference spectra. Such absorbance was subtracted from that measured in dithionite-reduced minus oxidized difference spectra in order to calculate cytochrome b contents.

Fig. 4 illustrates dithionite difference spectra from uninduced (lower tracing) and induced (upper tracing) HL-60 cells grown for two months in serum-free medium and devoid of hemoglobin. The spectra show the typical α, β, and γ (Soret) peaks of cytochrome b (7, 23) at 558, 520, and 428 nm, respectively. The Soret peak for uninduced cells was shifted to a shorter wavelength. The induced cells contained more cytochrome b, evident in the greater absorbance at each peak. Very gentle cell handling, to avoid lysis or degranulation, prevented the appearance (and interference) of the 474 nm myeloperoxidase peak in this and most other experiments.

Table III presents quantitative measurements of cytochrome b in uninduced and induced HL-60 cells, and in elutriation fractions of cells partway (day 4) into induction. The cytochrome b content rose nearly 3-fold, but the induced cells still contained only 22% as much as PMN. Induced HL-60 grown in serum-free medium produced O₂ at a rate equal to that of cells grown in fetal calf serum and hence 60-70% that of PMN. However, difference spectra of induced HL-60 cells incubated with and without antimycin A, an inhibitor of mitochondrial electron transport, showed 14 ± 3 (mean ± SD)
SE, n = 3) pmol/10^7 cells of detectable cytochrome b. Induced cells and PMN showed no detectable cytochrome b after such incubation. Antimycin A did not inhibit PMA-stimulated O₂⁻ production by any of these cells. Thus, most of the cytochrome b in uninduced HL-60 cells, but not induced cells or PMN, appears to be mitochondrial rather than NADPH oxidase-associated.

Elutriation fraction 3, containing the smallest, most mature cells, contained even more cytochrome b than the mixed population of fully induced HL-60s. Progressively larger, less mature cells in later fractions contained far less.

**DISCUSSION**

The induction of differentiation in HL-60 promyelocytic leukemia cells results in maturation of the O₂⁻ generating system (13, 14). Both the activity of the system, expressed as the rate of O₂⁻ production, and the ability to activate the system from its resting state, measured as the lag time before O₂⁻ production, develop over the course of incubation with polar solvent inducing agents (13). The present studies of the biochemical basis of the development of the O₂⁻-generating system indicate that HL-60 cells contain the O₂⁻-producing NADPH oxidase and that its activity increases with both induced and spontaneous differentiation. The membrane potential change (associated with activation of the O₂⁻-generating system) also increases over the course of differentiation, as does the cells’ content of cytochrome b, an NADPH oxidase component that can be measured independently from the state of activation.

HL-60 cells induced to differentiate by incubation with DMF showed 3-fold increases in both specific and total NADPH oxidase activity in particulate fractions prepared by sonication and differential centrifugation. The relative activities in differentiated cells and PMN were roughly proportional to their relative rates of O₂⁻ production (13) but the activity in uninduced cells was surprisingly high, given their very low rate of O₂⁻ production (13). Possibly, the NADPH oxidase activity is not fully expressed in intact uninduced HL-60 cells due to limitations in substrate supply, lowered affinity for NADPH, or the presence of an intracellular inhibitor. Alternatively, the preparation of the particulate fraction could select for membrane from more differentiated cells; however, the initial sonication produced >95% lysis of both induced and uninduced cells and the eventual particulate fraction yield was similar for both.

The equivalence of NADPH oxidase specific activities in phagocytic vesicles from induced HL-60 cells and PMN indicates that the areas of HL-60 cell membrane engaged in phagocytosis probably contain similar numbers of enzyme units as do PMN membranes. These results also demonstrate the ability of HL-60 cells to activate and translocate the NADPH oxidase to phagocytic vesicles and hence the membrane localization of the enzyme system, as in PMN. The similar lack of latency (i.e. change of detectable activity with addition of detergent) suggests a similar transmembrane orientation of the enzyme (2, 24) in HL-60 cells and PMN. The lower NADPH oxidase activity in phagocytic vesicles from uninduced cells probably represents a lower concentration of enzyme units in active membrane, but could also result from a lower Vₘₐₓ due to maturational differences in an enzyme component or its membrane milieu (4, 28).

The correlation of NADPH oxidase activity with differentiation in elutriation fractions of induced and uninduced HL-60 cells confirms the relationship between enzyme activity and cell maturation by a means independent of the duration of exposure to the inducing agent. This finding in uninduced HL-60 cells indicates increasing activity with spontaneous differentiation. In induced cells, the activity of the most mature fraction reaches that of PMN, thus confirming the equivalence noted in the phagocytic vesicle experiments.

Membrane depolarization appears related to the stimulus-response coupling that translates an external phagocytic stimulus to a broad range of cell responses, including the activation of the O₂⁻-generating NADPH oxidase (29, 30). With induced maturation of HL-60 cells, the cell membrane depolarization that occurs upon stimulation rose dramatically both in the magnitude of the maximal membrane potential change and in the initial slope of the response. This physiologic change may be the functional correlate of reported maturational changes in HL-60 cell membrane lipids (31) and surface proteins (32). It may also relate to the previously described shortening of the lag time required for activation of O₂⁻ generation (13). Although the time of the initial and maximal depolarization response did not change with differentiation, the magnitude of the response could influence the rate of more distal steps in the activation process. Alternatively, the membrane potential change and the lag time could represent separate pathways of response to a primary membrane event.

The cytochrome b content of HL-60 cells increased with induction of differentiation, with a 3-fold rise that correvered with that observed for NADPH oxidase activity. However, the larger proportion of mitochondrial (i.e. reduced in the presence of antimycin A) cytochrome b in the uninduced cells indicates that its actual rise is much greater, perhaps more analogous to the 19-fold increase in whole cell O₂⁻ production. This finding raises the question of whether the NADPH oxidase activity in uninduced cells requires very little or no cytochrome b, as observed in a recently-reported chronic granulomatous disease variant (28). Also, the maximal cytochrome b content of even the most mature HL-60 elutriation fraction was still far below that of PMN, out of proportion to their relative O₂⁻-generating capacities. This finding suggests that all of the cytochrome b in PMN may not be metabolically active in O₂⁻ production. Conceivably, it could even be an inducible protein without direct involvement in superoxide generation (28).

A related question raised by the present study regards the intracellular localization of cytochrome b. Recent work by Borregaard et al. (33), utilizing Percoll density gradient centrifugation, found cytochrome b in resting cells associated with a density fraction containing secondary granules. Upon stimulation, the cytochrome b translocated to the plasma membrane fraction. Other studies have argued for both granule and membrane localization (34, 35). However, HL-60 cells contain no detectable lactoferrin, vitamin B₁₂-binding protein, or secondary granules (13, 15). Thus, the cytochrome b in HL-60 cells must be associated either solely with the plasma membrane or with another form of granule, perhaps that reported by Deva et al. (36) to contain gelatinase and to have the same density as secondary granules. A plasma membrane site could represent a primary location or the product of a translocation event (e.g. defective secondary granules could fuse with the cell membrane as rapidly as they are formed).

The results presented here also confirm those of Roberts et al. (37), who observed an inducible rise in HL-60 cytochrome b content and identified it as a very low potential cytochrome ("cytochrome b₄₅₅") distinct from those of mitochondria and endoplasmic reticulum. They observed a similar cytochrome b content in uninduced cells (6 pmol/mg of protein, which equaled 10 pmol/10⁷ cells) and in cells incubated for 7–8 days in dimethyl sulfoxide (38.5 pmol/10⁷ cells). At day 14 in dimethyl sulfoxide they found 105 ± 46 pmol/mg of protein...
(mean ± SE, n = 3) equalling 82 pmol/10^7 cells, but that difference from our data is not significant (p = 0.26 by independent t test) due to the large variance in their data. They may also have observed higher cytochrome b values due to the use of fetal calf serum in the culture medium, as discussed under "Results," although the measured low reduction potential rules out a major hemoglobin contribution. The longer incubation they required to achieve maximal differentiation probably reflects differences in HL-60 strains and the slower induction response to dimethyl sulfoxide relative to DMF (12). They, too, found, by subcellular fractionation, a major proportion of mitochondrial cytochrome b in uninduced cells.

The results presented here also illustrate the utility of centrifugal elutriation for the separation of HL-60 cells of varying maturity. The technique provides a means of testing a series of cells at progressive stages of differentiation but with equal times of incubation with an inducing agent, thus allowing independent assessment of the variables of maturity and chemical exposure. As a preparative method, it can enrich for mature cells from induced cultures or for immature cells from uninduced cultures in order to provide a more homogeneous cell population for further functional or biochemical studies.

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