Differentiation of Promyelocytic (HL-60) Cells into Mature Granulocytes: Mitochondrial-specific Rhodamine 123 Fluorescence

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ABSTRACT Rhodamine 123, a fluorescent dye which binds as a result of the transmembrane potential, was used to stain the mitochondria of HL-60 cells, a cell line established from human promelocytic leukemia cells. The DMSO-induced differentiation of promyelocytic cells into mature granulocytes caused a fourfold decrease in fluorescence intensity that paralleled the disappearance of S-phase and G_2M cells. This suggests that upon myeloid differentiation whereby the cells enter an irreversible quiescent state, the mitochondrial mass of the cells has decreased. This suggestion is corroborated by electron microscopy, which shows a decrease in the number of mitochondria, and by decreases in total mitochondrial protein and cytochrome oxidase activity. The respiratory rate of isolated mitochondria did not change, suggesting that the transmembrane potential remained the same. Undifferentiated cells in exponential phase of growth exhibit an intracellular heterogeneity of fluorescence intensity. This heterogeneity appears to have a cell age basis, as late S/G_2M cells, obtained by centrifugal elutriation, yielded twice the fluorescence intensity of early G_1 cells.

The cells of the granulocyte series of the bone marrow undergo a multi-step differentiation from stem cells to, sequentially, myeloblasts, promyelocytes, myelocytes, metamelocytes, band neutrophils, and, finally, segmented neutrophils (1). Cells in the first three stages proliferate and hence are "in cycle," while cells in the last three stages enter an irreversible state of nonproliferation and hence are "out of cycle" (2). Cells in the last three stages are functionally "mature" granulocytes (1). The biochemical characteristics of each of these cell types is difficult to determine due to their low numbers in the marrow, which necessitates enrichment by flow cytometric sorting, or other means (3).

Collins et al. (4) established a cell line in tissue culture with cells from a female patient with acute promyelocytic leukemia. This cell line, HL-60, has been shown to terminally differentiate into functionally mature granulocytes when exposed to a variety of polar compounds that also induce differentiation in Friend erythroleukemia and mouse myeloid leukemia cells (5). Surprisingly, while dimethyl sulfoxide (DMSO) induces differentiation into metamelocytes and band neutrophils, the tumor-promoting phorbol ester 12-0-tetra-decanoylphorbol 13-acetate induces differentiation along another pathway into macrophages (6). This differentiation can occur in the absence of DNA synthesis (7). HL-60 cells induced to differentiate with DMSO have many of the functional characteristics of normal peripheral blood granulocytes, such as phagocytosis, complement receptors, chemotaxis, and the ability to reduce nitroblue tetrazolium (8). There have been numerous empirical reports, based on electron microscopy, indicating that, when normal promyelocytes differentiate, there is a decrease in the number of mitochondria (see, for example, reference 9), yet no quantitative values are available. Cationic fluorescent probes that are selectively accumulated by mitochondria have revealed much information about the architectural arrangement of mitochondria in living cells (10). One would expect that profound cellular changes, such as differentiation along a multi-step pathway, would lead to dramatic changes in the mitochondria. The elegant flow cytometric study of the increased rhodamine 123 uptake by human lymphocytes stimulated by phytohemagglutinin to undergo blastogenic transformation (11) indicates that quantitative measurements of mitochondrial-specific probes are possible. In this study we report that when HL-60 cells are induced to differentiate by DMSO there is a decrease in the rhodamine 123 fluorescence. Furthermore, the fluores-

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cence heterogeneity of exponential-phase undifferentiated cells has a cell age basis, as late S/G_2M cells have twice the fluorescence of early G_1 cells.

MATERIALS AND METHODS

Cell Culture: HL-60 cells (kindly supplied by Dr. Robert C. Gallo, Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD) were maintained in continuous suspension culture in RPMI-1640 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, MD) in a humidified 5% CO_2-air atmosphere, as described by Collins et al. (5). Cells were counted in a hemocytometer chamber and viability assessed by trypan blue dye exclusion. Exponential-phase, undifferentiated cultures typically have <1% nonviable cells. For morphological assessment of cells, cytospin slide preparations of aliquots of cell suspensions were prepared with a Shandon-Elliot Cytospin centrifuge and stained with Wright-Giemsa's.

Induction of Differentiation: Cells were suspended in growth media containing 1.5% DMSO (Sigma Chemical CO., St. Louis, MO), for periods of up to 7 d, whereupon >96% of the cells are viable, and >90% of the cells have the appearance of mature granulocytes (metamyelocytes and neutrophils).

Electron Microscopy: The cells were fixed in 2% glutaraldehyde–0.1 M phosphate buffer (4°C), postfixed in 2% osmium tetroxide–0.1 M phosphate buffer (4°C), dehydrated in a graded series of acetone, and embedded in Spurr's resin. Thin sections (600–800 Å) stained with uranyl acetate and lead citrate were examined with a Hitachi HU-12 electron microscope.

Cytochrome c Oxidase: The oxidation of cytochrome *c* was measured by the decrease in absorbance at 550 nn, according to the procedure of Applemans et al. (12). Protein was estimated by the method of Lowery et al. (13).

Respiratory Rates: The respiratory rate of isolated mitochondria was measured as the amount of oxygen uptake with succinate as the substrate by a polarographic method with a Clarke-fixed voltage electrode, according to Rogers and Higgins (14). Mitochondria were also isolated according to Rogers and Higgins (14).

Cell Cycle Fractionation: Cells in the log-phase of growth were fractionated according to their size and density (relative cell age from G_1 to G_2M) using a Beckman Elutriator rotor (Beckman Instruments, Spinco Div., Palo Alto, CA). The counter-current centrifugation in the Elutriator rotor was performed according to McEwen et al. (15).

Flow Cytometric Analysis: DNA content: cells (2×10^6/ml) were stained with propidium iodide by the method of Fried et al. (16), and the DNA-propidium fluorescence was measured with a Coulter EPICS-V multiparameter analyzer/sorter (Coulter Electronics, Hialeah, FL). This machine uses straight-through laser optics which yields coefficients of variation for G_1 distributions of HL-60 cells of 1.8–2.2%. Fluorescence intensity is recorded as channel number in a linear manner.

Rhodamine content: cells (2×10^6/ml) in growth media were incubated at 37°C for 10 min with 10 μg/ml rhodamine 123 (Laser-grade, Eastman Organic Chemicals, Rochester, NY), washed and resuspended twice in phosphate-buffered isotonic sodium chloride, pH 7.0, and analyzed for fluorescence using an internal standard of fluorescent polystyrene beads for machine calibration of fluorescence intensity from day to day. The flow cytometer is adjusted for each sample so that the beads have a maximum intensity at channel 90 with an amplification of 1, then the amplification is changed to 10 to analyze the 10-fold less fluorescent rhodamine-stained cells. The 488-nm emission line of the argon laser is used for excitation, and a 510-nm long-pass emission filter is positioned in front of the fluorescence detector.

Cell Cycle Distribution: The overall DNA content distributions (obtained as described above) were analyzed for the proportions of cells in G_1, S, and G_2M according to a model we have previously described (17), using a computer program which yields cell cycle distributions as well as rates of DNA synthesis that we have also previously described (17).

RESULTS

Preliminary Experiments

In preliminary experiments it was found that, after 7 d of exposure to DMSO, >90% of HL-60 cells reduced nitroblue tetrazolium, hence >90% of the cells differentiate under these culture conditions (8). Whereas control cells have the large rounded nuclei with prominent nucleoli, typical of immature, undifferentiated granulocytes (Fig. 1*A*), the DMSO-treated cells have acquired the segmented, polymorphological nuclei typical of mature, differentiated granulocytes (Fig. 1*B*). Furthermore, these electron micrographs revealed an apparent decrease in both the number and the size of the mitochondria (for example, see Fig. 1). Morphometric calculations on mitochondria were performed with a Zeiss MOP-3 Image Analyzer (Carl Zeiss, Inc., New York, NY). The total area of the mitochondrial cross sections observed in 100 cells was 26,025 mm^2 and 6,760 mm^2 for control and differential cells, respectively. This is a ratio of 3.85 for control vs. differentiated. Preliminary flow cytometry measurements of intracellular fluorescence after incubation with rhodamine 123 revealed a fourfold decrease in the average intensity per cell after 7 d of exposure to DMSO. The addition of 1 mm of sodium cyanide, a mitochondrial inhibitor, caused an almost immediate and total loss of rhodamine fluorescence, hence the binding of this dye seems to be dependent upon the maintenance of an active mitochondrial transmembrane potential. Furthermore, the addition of 1.5% DMSO 1 h before the addition of rhodamine had no effect on subsequent rhodamine uptake, nor did DMSO cause a decrease in the fluorescence of cells previously stained with rhodamine.

Although electron microscopy seems to indicate fewer and smaller mitochondria in the differentiated cells, it is difficult to obtain accurate quantitative numbers. Also, one might postulate that the smaller mitochondria of the differentiated cells might have a lower transmembrane potential, hence would bind less rhodamine. Measurements of cytochrome *c* oxidase activity, a crude estimate of mitochondrial mass, and protein content estimates of isolated mitochondria preparations predict an almost fourfold decrease in the total mitochondrial mass of the differentiated cells. The activity of cytochrome *c* oxidase

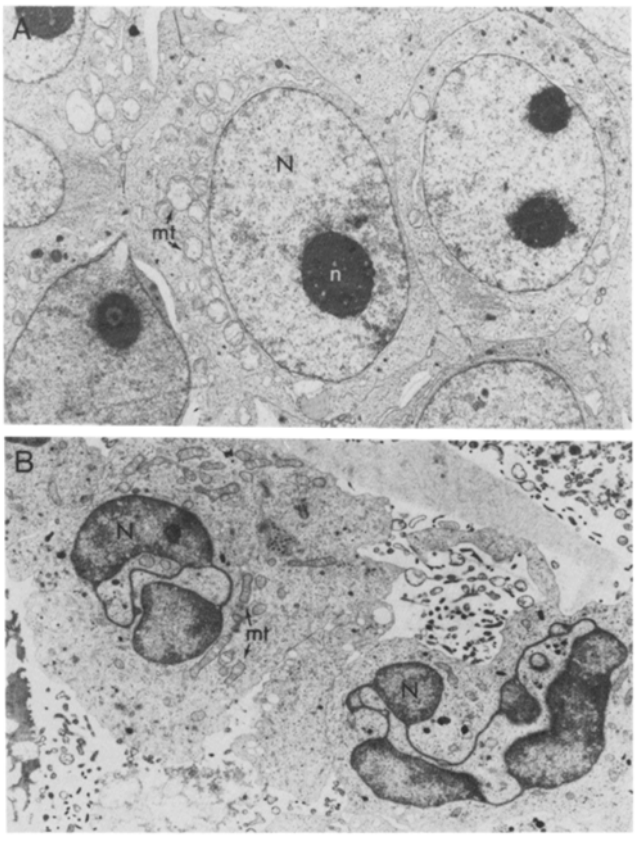

FIGURE 1 Electron micrograph of: (*A*) Control cells. (*B*) 7-Day DMSO-treated cells. *N*, Nucleus. *n*, Nucleolus. *mt*, Mitochondria. X 8,400.

decreased from 3.64 nmol of substrate oxidized per minute per 10^8 cells to 1.05 nmol of substrate oxidized per minute per 10^8 cells, after 7 d of exposure to DMSO. For the same cells, the total protein in the mitochondria isolated from 10^9 cells decreased from 38.4 to 10.2 mg. Estimates of respiratory rates predict no change in the transmembrane potential as the cells differentiate. The respiratory rates with succinate as the substrate were 86 ng atoms of oxygen consumed per minute per milligram of mitochondrial protein for control cells and 88 ng of oxygen consumed per minute per milligram of mitochondrial protein for differentiated cells. Thus, from these preliminary experiments we conclude that there is a decrease in the total mitochondrial mass after 7 d of exposure to DMSO, and that the fluorescence intensity of rhodamine 123 is a measure of mitochondrial mass. Flow cytometry measurements of rhodamine binding would seem ideally suited to study changes in mitochondrial mass as cells differentiate, as electron microscopy does not readily permit the examination of large numbers of cells, and protein and enzyme measurements do not provide values for individual cells in a possibly heterogeneous population.

Differentiation of HL-60 Cells

DMSO-induced differentiation of HL-60 cells into mature granulocytes takes ~7 d (5) and results in their leaving the cell cycle in a progressive manner, as revealed by the decrease in the numbers of cells with DNA contents corresponding to S-phase and G_2M cells. This is illustrated in Fig. 2, which depicts the DNA distributions of day-0, day-3, and day-5 cells. The percentages of S + G_2M cells were 48, 44, 38, 26, 15, 4, 3, and 2 for days 0 to 7, respectively (days 1, 2, 4, 6, and 7 are not shown in Fig. 2). It should be stressed that, when cells leave the cycle and enter into an irreversible nondividing state, they do so after mitosis, and hence have a "G_1 DNA content." Thus, the 5-day cells depicted in Fig. 2 are not "G_1 cells" but "irreversible-state cells with a G_1 DNA content."

Rhodamine Fluorescence during Differentiation

The rhodamine fluorescence distributions over the entire differentiation period were determined (Fig. 3). The distribution for day 1 was similar to day 0, and the distributions of day 6 and day 7 were similar to that of day 5, hence these are not shown. The means of the intensity distributions, in channel numbers, were 66, 65.5, 65, 43, 36, 22, 18, and 16 for days 0 to 7, respectively. The decreases in rhodamine fluorescence as the cells progressively differentiate (Fig. 3) appear to correlate with the decreases in the proliferative compartments depicted in Fig. 2. This is illustrated in Fig. 4, which represents the mean rhodamine intensities of the data of Fig. 3 as a function of the percentages of cells that have a G_1 DNA content (Fig. 2), from left to right, for days 0, 1, 2, 3, 4, 5, 6, and 7 after the addition of DMSO. As can be seen, there is very little diminution of rhodamine fluorescence up to day 2 (third data point from left), but thereafter the fluorescence intensity decreases in a fairly linear fashion (dashed line, linear correlation coefficient is 0.990), up to day 7 (Fig. 4). By day 7, 98% of the cells have a G_1 DNA content, and the mean rhodamine fluorescence has declined to only about one-fourth of that at day 0.

Cell Cycle Staging of Cells Separated by Centrifugal Elutriation

The intercellular heterogeneity depicted in Fig. 3 for exponential-phase cells (0-day) might have a cell age basis in the sense that mitotic cells would be expected to have twice the number of mitochondria of early G_1 cells. However, it is not clear whether biogenesis of new mitochondria occurs in a linear

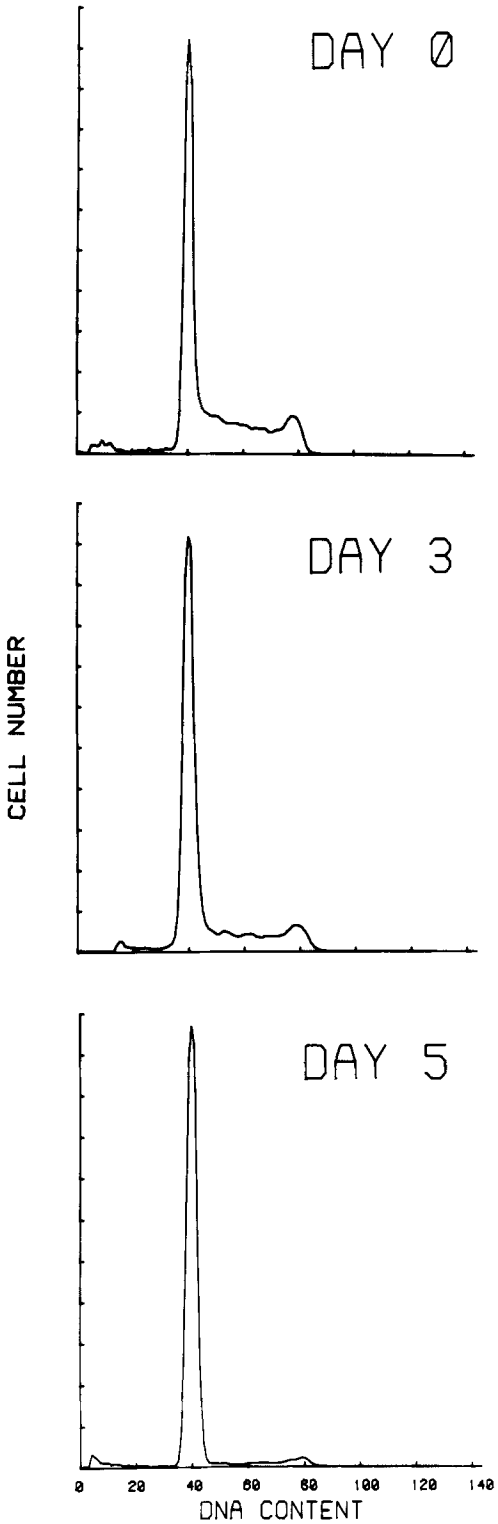

FIGURE 2 Exit of HL-60 cells from the cell cycle after induction by DMSO. At 24-h intervals from 0 to 7 d after exposure to DMSO, portions of cells were analyzed for DNA content as described under Materials and Methods. The model channel numbers for G_1 and G_2M cells were, respectively, 40 and 80. The S-phase cells are distributed between channels 40 and 80. The number of cells in the maximum channel was 10,000 for each determination. The DNA distributions for 1, 2, 4, 6, and 7 d are not shown, for brevity's sake.

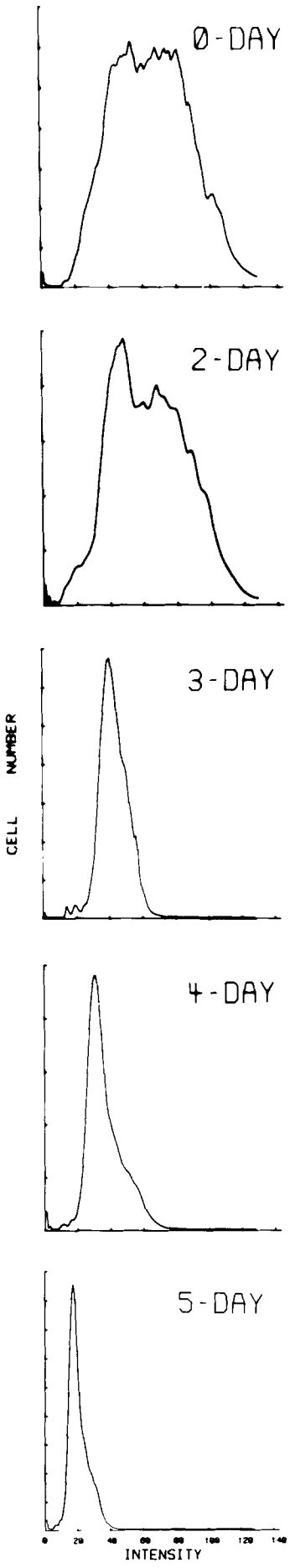

FIGURE 3 Decreasing rhodamine 123 fluorescence of differentiating cells. At daily intervals from 0 to 7 d after exposure to DMSO, portions of cultures were treated with rhodamine 123 according to Materials and Methods. Portions of the same cultures used in Fig. 2 "percent of cells with a G_1 DNA" were used. The fluorescence distributions for 1-, 6-, and 7-d cultures are not shown, for brevity's sake.

fashion from beginning to end of the cell cycle, or whether it occurs in a discontinuous fashion (with starts, pauses, and stops). To address this question, exponential-phase cells were separated into 10 fractions by centrifugal elutriation. The DNA distributions of fractions numbered 4, 7, 8, 9, and 10, in order of elutriation, are shown in Fig. 5 *A*, *B*, *C*, *D*, and *E*, respectively. The cell cycle distributions obtained by computer analysis of the DNA distributions (17) corresponded to percentages of $G_1/S/G_2M$ of 92/8/0 (Fig. 5 *A*), 31/62/7 (Fig. 5 *B*), 17/68/15 (Fig. 5 *C*), 11/47/42 (Fig. 5*D*), and 9/29/62 (Fig. 5 *E*). Thus, these fractions correspond to early G_1 (Fig. 5 *A*), mid-G_1/early S (Fig. 5 *B*), late G_1/middle S (Fig. 5 *C*), late G_1/late S (Fig. 5 *D*) and late S/G_2M (Fig. 5 *E*).

Rhodamine Fluorescence of Cells Separated by Centrifugal Elutriation

The corresponding rhodamine fluorescence of the five selected centrifugal elutriation fractions are depicted in Fig. 6. It can be seen that there is much less intercellular heterogeneity in each of the centrifugal elutriation fractions (with the possible exception of Fig. 6 *B* which corresponds to mid-G_1/early S) than was obtained with unfractionated exponential-phase cells (Fig. 3, day 0). For example, the distributions depicted in Fig. 6 had coefficients of variation of 16.7, 24.2, 12.9, 11.5, and 19.0 (for Fig. 6 *A–E*, respectively), while exponential-phase cells had a corresponding value of 58.1 (Fig. 3, day-0). Furthermore, the mean intensity of the late S/G_2M fraction (Fig. 6 *E*) occurs at channel 78, which is about twice that of the early G_1 fraction (Fig. 6 *A*), which occurs at channel 38. When the mean rhodamine fluorescence intensities of all of the centrifugal elutriation fractions were plotted against the fraction number, a line with a linear correlation coefficient of 0.96, a value usually taken to indicate linearity, was obtained (data not shown). However, it should be stressed that sedimentation rates are not precisely related to cell cycle position, hence a linear rate of mitochondrial biogenesis cannot be inferred from our data. These experiments depicted in Figs. 5 and 6 were repeated four times with essentially the same results.

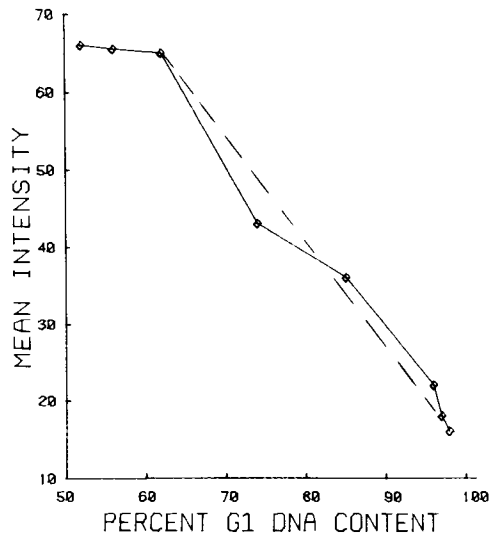

FIGURE 4 Relationship between rhodamine 123 fluorescence and exit from the cell cycle. The data of Fig. 2 (percent of cells with a G_1 DNA content) and Fig. 3 (mean of rhodamine fluorescence) were used. The data for 2- to 7-d have a linear correlation coefficient of 0.990.

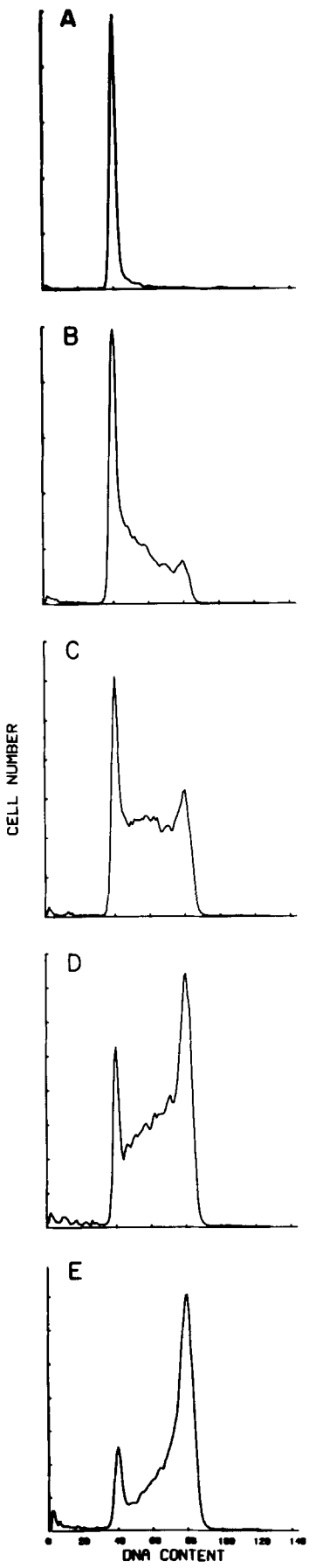

FIGURE 5 Cell cycle distributions of log-phase undifferentiated cells separated by centrifugal elutriation. Approximately 10^8 cells from log-phase cultures not exposed to DMSO were separated into 10 fractions by centrifugal elutriation, and portions of each fraction were analyzed for DNA content to determine the cell cycle phases represented. The fractions depicted are 4 (*A*), 7 (*B*), 8 (*C*), 9 (*D*), and 10 (*E*), in order of elutriation. Fractions 5 and 6 are not shown, for brevity's sake.

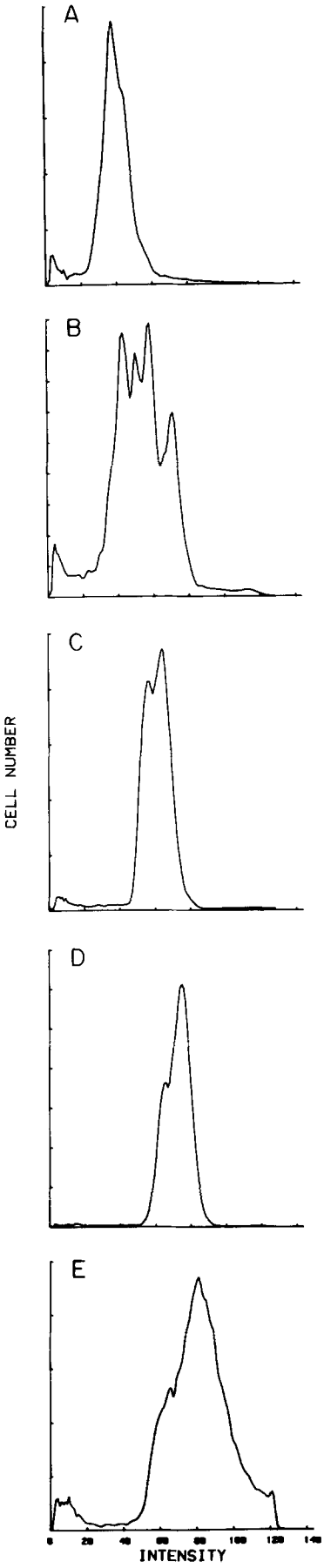

FIGURE 6 Rhodamine fluorescence of exponential-phase undifferentiated cells separated by centrifugal elutriation. Portions of the same cells used for Fig. 5 were stained with rhodamine 123 and analyzed. The elutriation fractions are 4 (*A*), 7 (*B*), 8 (*C*), 9 (*D*), and 10 (*E*).

DISCUSSION

Johnson et al. (10) have shown that several cationic fluorescent dyes, of which rhodamine 123 is one, are able to permeate living cells and are selectively accumulated by the mitochondria in a manner dependent on the high transmembrane potential (inside negative) of these organelles. Hence, all other things being equal, rhodamine 123 fluorescence appears to reflect the total mitochondrial membrane mass. The use of a viable fluorescent dye has obvious advantages over planimetric measurements of electron micrographs of fixed cells. Although no previous data are available for HL-60 cells, a decrease in the putative number of mitochondria has been reported to occur when normal granulocyte precursors differentiate into mature granulocytes (9). Thus our quantitative data would confirm these empirical observations. Darzynkiewicz et al. (11) found that the maximal rhodamine 123 fluorescence was exhibited by lymphocytes 3 d after stimulation, a time when the maximum number of S-phase cells was present. They suggested that there might thus be a correlation between the "rate of DNA synthesis" and rhodamine uptake. However, we have found that the rate of DNA synthesis in these cells is virtually constant throughout the S-phase (data not shown) and have observed no correlation between rhodamine fluorescence and S-phase (Fig. 7). Hence, it seems more likely that the data of Darzynkiewicz et al. (11) reflect the greater number of proliferating cells (in all phases of the cycle) rather than a S-phase dependency. Our data suggest that in proliferating undifferentiated HL-60 cells the biogenesis of new mitochondrial mass (i.e., rhodamine fluorescence) is a function of cell age in the cell cycle. As HL-60 cells differentiate into mature granulocytes, they become nonproliferating by day 7, as evidenced by the virtual absence of S and G_2M phase cells in the DNA distributions and a fourfold decrease in the rhodamine fluorescence (Fig. 4). This is consistent with the idea that after differentiation of myeloid cells into granulocytes the cells are in an irreversible, nonproliferative state (2). However, HL-60 cells can also be caused to differentiate into functional, cytotoxic macrophages by exposure to TPA (12-0-tetradecanoylphorbol 13-acetate), a tumor promoter (18). We have examined HL-60 cells for changes in rhodamine binding after differentiation into functional macrophages and have observed no change in fluorescence intensity (data not shown). Clearly, diverse pathways of differentiation affect mitochondria in different ways.

Levenson et al. (19) have demonstrated that the initiation of the commitment to terminal differentiation of murine erythroleukemia cells, by DMSO, is presumably due to a depolarization of the mitochondrial transmembrane potential. They observed a twofold decrease in the fluorescence of the membrane probe 3,3′-dihexyloxacarbocyanine iodide by 12 h, which was correlated with calcium release by the mitochondria, concomitant with an increase in cytoplasmic calcium levels (18).

Our respiratory rate measurements of isolated mitochondria, while predicting no change in membrane potential, should be taken with caution as they may not be a true reflection of the intracellular metabolic capacities of the mitochondria (20). Nevertheless, these data coupled with preliminary electron microscopy, total mitochondrial protein, and cytochrome *c* oxidase measurements confirm that the observed fourfold reduction in rhodamine fluorescence is due to a fourfold decrease in the total mitochondrial mass, not to a change in mitochondrial function that changes the transmembrane potential.

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