Proliferation of Mitochondria during the Cell Cycle of the Human Cell Line (HL-60)

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ABSTRACT Using rhodamine 123 to stain mitochondria of the human cell line HL-60, we have followed their increase over the cell cycle by flow cytometry. A near-linear synthesis of mitochondrial mass was shown to occur over the cell cycle. A comparison with the cell’s DNA synthesis pattern obtained by the same technique established a common time-base. The mitochondrial synthesis curve changes with culture age. As a control, the dye was tested for its binding specificity and for its use to resolve mitochondria microscopically. Its stoichiometric range was established and, above 0.25 μg/ml, it was shown to reduce growth rate and cell viability in culture.

Although a considerable amount of information has been developed on the constituents and genetics of mitochondria, the exact nature of their growth and proliferation is only vaguely understood. The simplest aspect of their biology, namely how they increase in number, volume, or surface area as a function of time in the cell cycle, has been difficult to evaluate because of their plastic form, the variability in their size, and the extent to which they fuse or fragment (1–6). Because of this, a simple count of their number per cell as such is recognized as quantitatively useless (7). The classic experiments by Luck (8) strongly supported a mechanism favoring increase by division of existing mitochondria rather than by de novo synthesis or formation from other cellular membranes. In addition to this aspect of the problem, the course of mitochondrial DNA synthesis over the cell cycle has been reported as either linear or discontinuous in a variety of cell types (5, 9–13). The degree of constancy of mitochondrial DNA, i.e., the copy number per cell and how it changes with the cell cycle, is difficult to standardize (7). This is even more so in the variety of cells that have access to aerobic and anaerobic modes of growth (7). Such data confuse the issue and preclude any generalization.

The recent discovery of a supravital mitochondrial specific stain, namely the fluorescent laser dye rhodamine 123 (14), and the development of flow cytometry (15) offer the possibility of monitoring the synthesis of mitochondria as a function of the cell cycle. The present report concerns an examination of this synthetic process in the human leukemia cell, HL-60 (16). This process was also monitored as a function of culture age. The present report concerns an examination of monitoring the synthesis of mitochondria as a function of cell cycle. The present report concerns an examination of monitoring the synthesis of mitochondria as a function of cell cycle.

MATERIALS AND METHODS

The human leukemia cell line HL-60 originally isolated by Collins et al. (16) was used. Cultures were grown in plastic flasks in an air-CO2 incubator (37°C) at pH 7.4 on alpha modification of Eagle’s medium with Eagle’s salts minus ribosides and deoxyribosides plus glutamine with 15% fetal calf serum (Flow Laboratories, Inc., McLean, Va.) and sodium bicarbonate. Culture growth was monitored by determining cell number. Number and mean cell volume were determined using a Coulter counter model Z (Coulter Electronics Inc., Hialeah, Fla.). Stocks were passed at weekly intervals and maintained under standard conditions. Experiments were started from either exponential or stationary phase stocks.

Cells were stained in culture medium by use of an aqueous rhodamine 123 (Eastman Kodak Company, Rochester, N.Y.) stock solution of 1 mg/ml added into medium to make the concentrations specified in each case. After staining for 1 h, they were washed twice in the culture medium. Propidium iodide staining for DNA used a modification of the protocol of Crissman et al. (17). Mitochondria were prepared by differential sedimentation using a standard method (18), and they were stained by successive additions of rhodamine 123 until they could be differentiated by fluorescence microscopy. They were not washed. The microscope used was a Zeiss Universal fitted with a tungsten-halogen light source for vertical excitation with broad-band filtered light from 420 to 490 nm. Observations were made at 500 nm and longer.

Photographs were made using a Nikon FE 35-mm camera coupled to the Zeiss Universal microscope. Tri X film was used and developed to an ASA of 1,600 with Dfamin (Acufine, Inc., Chicago, Ill.). Control spectral fluorescence curves on aqueous solutions of the dye and of dye plus mitochondria yielded both emission (maximum, 522 nm) and excitation (maximum, 497 nm) spectra that were nearly identical, falling within 3 nm. Although fluorescence enhancement between the dye and the mitochondria was not measured, it was estimated to be low, if present, although a marked partition of the dye into mitochondria can be observed.

Cytospectrometry was carried out using the analytical components of two different cell sorter systems, i.e., the Coulter TPS1, with excitation at 488 nm and emissions at 510 nm and higher, and the Coulter EPIC, using a 5-W argon laser.
exciting at 488 nm with emissions at 510 nm and greater. These instruments measure and record distributions of cell number vs. forward light scatter and/or fluorescence intensity as a dilute suspension of cells passes through a laser beam. These variables can be examined simultaneously by setting a gate or window on one while measuring the other. By setting the gating control, the total distribution can be subdivided into an arbitrary number of gates and these used to obtain distributions for the other variable. The term gate in this report implies the use of a subdivision of a total distribution (light scatter) to examine the second variable (fluorescence) independently. Cells were examined by flow fluorometry over culture ages from 22 to 96 h.

To generate the three-dimensional graph, data from the flow microfluorometer were analyzed and drawn by an IBM 360/91 computer. Comparable analyses were carried out on all data used for the determination of cell cycle synthesis curves.

**RESULTS**

To test the feasibility of determining the average per cell mitochondrial change over the cell cycle by fluorescence flow cytometry, several controls were necessary. (a) Microscopic examination of all cells used was made to ensure that localization of the dye was consistent with mitochondrial morphology. (b) Microscopic observations were carried out on isolated mitochondria to confirm the specificity of rhodamine 123. (c) The stability of the staining was tested by successive washing. (d) A test of the binding characteristics of the dye to mitochondria in situ at various concentrations was made. (e) A determination was made of the effect of the dye on the growth characteristics of HL-60 cells in culture. (f) The light-scatter profiles were analyzed to establish a relationship between the cell volume as measured by a Coulter counter and by light scatter. These were combined with the DNA synthesis pattern displayed by the cells to establish a common cell cycle time-base. After evaluating these conditions, cytofluorometry was carried out on cells stained in culture with rhodamine 123. The general procedure used was the measurement of fluorescence intensity during gating on light scatter.

Fig. 1a is a phase-contrast photomicrograph of a compressed HL-60 cell. Such observations were made in red light, 598 nm, to prevent photodynamic effects at shorter wavelengths. Fig. 1b is a photograph of the same cell taken with rhodamine 123 fluorescent emission. Correspondence can be seen between the two micrographs, particularly in the size, location, and morphology of elements considered to be mitochondria. Fig. 1c and d are photomicrographs of the isolated mitochondria stained with rhodamine 123. They show photographs of mitochondria adsorbed to the cover slide. The background results from unattached mitochondria in Brownian movement.

Estimates of the strength of binding of the stain to the cells were made by staining a population and measuring the mean fluorescence by flow microfluorometry. After four 20-min washes with culture medium, the mean fluorescence of the population had not changed. With prolonged washing the fluorescence dropped to 72% of the control.

To test the relationship between the extent of staining and the concentration of stain, the following experiment was done. A culture of cells in mid-exponential phase was divided into aliquots and these were stained with increasing concentrations of the dye, i.e., from 0.25 to 96 μg/ml with 300,000 cells/ml. These aliquots were washed and the fluorescence profiles were obtained on each with the flow cytometer. The mean fluorescence was determined on each fluorescence vs. number distribution, and these values are plotted against the concentration in Fig. 2. This graph is a result of two ranges of concentration, one at the low range, 0.25–8 μg, and one at the high range, 7–98 μg. As indicated, above 2 μg/ml the fluorescence stays relatively constant up to 16 μg/ml. In this range, mitochondria are the only elements stained. General staining of other parts of the cell is observed above 16 μg/ml, and the entire cell is stained at higher concentrations. As a consequence of these findings, staining was standardized by using a fixed number of cells, 3 x 10⁶/ml, at an initial concentration of 5 μg of stain/ml of culture medium for a duration of 1 h. Stained cell preparations were monitored microscopically for specific localized fluorescence before each analysis by flow cytometry. In addition to this relationship, a separate study showed that stained

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**FIGURE 1** (a) A compressed live human leukemia cell (HL-60) by phase-contrast microscopy. (b) The same cell stained with rhodamine 123 and seen by emission fluorescence at 510 nm and longer. An epi illumination of 488 nm to 420 nm was used. Bar, 10 μm. The arrows in a and b point to a recognizable mitochondrion. (c) Phase-contrast image of isolated mitochondria and (d) rhodamine 123-stained fluorescent image of the same field.

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1 Cascarano, J., D. Montisano, and T. W. James. Manuscript in preparation.
FIGURE 2  Mean population fluorescence of rhodamine-stained aliquots of HL-60 cells plotted against log concentration of dye. Two concentration ranges were used: 0.25-8 μg/ml (O) and 3-96 μg/ml (+).

Volumes of isolated rat liver mitochondria obtained by hematocrit sedimentation when resuspended and measured in a fluorometer yield fluorescence intensities linearly proportional to their packed volume.

Studies of the chronic effect of the dye on the growth of cells established that maximum growth rate is profoundly affected, being sustained only below 0.25 μg/ml (Table I). At the same time, growth measured as total yield of cells declines with increasing concentration of dye. Between 0.25 and 0.75 μg/ml, the percent yield relative to controls drops from 75 to 22%, and at 2 μg/ml the yield was 5% of the control. The mean cell volume measurements show a change but only at the highest concentration where cytolysis is occurring.

Because the determination of the increase in number of mitochondria over the cell cycle depends on the strength of the correlation between light scatter and cell cycle age, we have done two types of experiments to establish a relationship between the light-scatter distribution and the synthesis curves of mitochondria and DNA. By selecting an increasing series of gates from the total light-scatter distribution, each level of cellular fluorescence can be examined across the profile. This was accomplished by monitoring the individual fluorescence distributions at a series of light-scatter gates. Fig. 3 is a three-dimensional plot of the rhodamine fluorescence vs. cell number obtained and displayed for each light-scatter gate. The normalizations of these data rest first on obtaining a light scatter vs. cell number plot on the whole population. Using this light-scatter range, a fluorescence profile is obtained on the population for a fixed time interval and the total number of cells is noted. This number is equivalent to the number of cells under the light-scatter profile obtained for the same time interval, indicating that all cells in the sample have taken up the dye. This distribution is used to set range and number of light scatter gates that will be used. Each gate is then monitored for the same time interval. Consequently, the sum of the number of cells in each gate closely approximates the number found on the total fluorescence distribution. Thus, each fluorescence vs. number distribution shown in Fig. 3 is proportional to that gate's fraction of the culture. The means of each distribution as plotted in Fig. 3 yield the double-dashed lines which show the mitochondrial synthesis curve. Fig. 4 is a simple plot obtained from the same kind of data but of propidium iodide-DNA fluorescence from the same culture. The portions of the plot corresponding to G1, S, and G2 + M levels of DNA are readily identified. This analysis established that the light-scatter gates are closely related to the cell cycle age in the population. The same gating procedure was applied to a culture stained at various stages with rhodamine 123 to determine whether the mitochondrial increase changes as a function of the culture age. Fig. 5 is a series of such plots obtained on cells at different culture ages. In this experiment, the curves for mitochondrial increase as measured by rhodamine 123 fluorescence cover culture ages from 22 to 96 h. The slopes appear to be essentially linear, while the magnitude of the initial and final gates reflect the fact that cell volume changes with culture age. The linearity appears to be lost in the 70-h and the 96-h cultures, which have an exponential character. This is more obvious when they are plotted on semilog paper. Using these same culture data, one can generate Fig. 6; it shows the total mean fluorescence of each of these populations from Fig. 5 plotted against the mean cell (Coulter) volume. Because the mean cell volume changes with culture age, the rather good correlation between the total fluorescence and the mean cell volume adds evidence to the stoichiometric characteristic of the measurement.

Experiments were designed to test for the equal distribution of stained mitochondria to daughter cells at the time of cell division.

| Culture no. | Dye | Maximum growth rate (h^-1) | Yield (% of control) | Mean cell volume (μm^3) |
|-------------|-----|---------------------------|----------------------|------------------------|
| 1           | Control | 0.031                  | 100                  | 2.000                  |
| 2           | 0.25    | 0.031                  | 75                   | 2.200                  |
| 3           | 0.75    | 0.031                  | 22                   | 1.900                  |
| 4           | 1.00    | 0.008                  | 10                   | 1.900                  |
| 5           | 2.00    | 0.008                  | 5                    | 1.700                  |
DISCUSSION

Our effort to determine the pattern of mitochondrial increase per cell over the cell cycle consisted of establishing the mitochondrial specificity and stoichiometry of the fluorescent dye rhodamine 123 and then determining the mean fluorescence of a series of cell size classes that in turn are a function of the cell cycle age.

It has been shown that rhodamine 123 is specific for mitochondria as evaluated by microscopic observations (14). We have confirmed this for HL-60 cells and present photographs of observations on the cells and on isolated mitochondria. The validity of the specificity is also supported by our finding that in vivo mitochondria display a saturation plateau at dye concentrations between 2 and 16 μg per 5 × 10⁵ cells/ml. At lower concentrations the mitochondria stain faintly, whereas above the 16-μg level other parts of the cell show fluorescence (Fig. 2). The constancy of the fluorescence intensity in the middle range suggests that the binding specificity is distinctly different for mitochondria than for other cell organelles. The faint staining at the lower levels suggests that they are the first organelles to bind the dye. The stability of the fluorescence with washing of the cells also supports this contention of specificity and stoichiometry.

If one assumes the intensity of fluorescence to be directly proportional to the volume of the mitochondria as has proved the case with rat liver mitochondria,¹ our cytofluorometric data leave little doubt that the synthesis of mitochondria over the cell cycle is continuous. Several factors require that caution be exercised in giving a more precise interpretation to the data.

FIGURE 4  DNA synthesis curve of HL-60 cells obtained by propidium-iodide-DNA fluorescence, using the same technique as in Fig. 3. The line is the projection of the distribution means of the gate-fluorescence plane. The bars are the standard deviation of each distribution.

FIGURE 5  Mitochondrial synthesis at a series of different culture ages, obtained by the method in Fig. 3.

fission. They were done in the following way. A large culture of cells was stained according to the standard procedure and then washed under sterile conditions, thus giving the cells an acute exposure to the stain. They were then incubated for continued growth. The culture was sampled daily for flow cytometry. Each sampling monitored the fluorescence, giving the distribution of fluorescence per cell as a function of the total light scatter. Because the mean fluorescence would be expected to halve if the dye were distributed to each cell at the time of division, measurements were made at intervals approximating the generation time. Our results deviate from this prediction. This can be explained by an imbalance between mitochondrial proliferation and cell growth during the culture cycle. This is supported by the data in Fig. 5, which show a gradual decrease in the average fluorescence with culture age. Keeping in mind that the dye does not appear to be readily removed by washing and that the rate of loss of fluorescence is greater than would be predicted by cell division dilution, a contradiction is evident that deserves more extensive exploration and will be discussed below.

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One of these is the function relating light scatter to cell size and thus cell age, and the other is the relationship of mitochondrial fluorescence to the mass, volume, or membrane surface area of the organelle.

Although there is a direct proportionality between cell volume and cell cycle age, the correlation includes several statistical variables that are difficult to evaluate (19). In this present study we have obtained population distributions of (Coulter) cell volume, light scatter (20), and rhodamine 123 fluorescence on the same culture. By simply normalizing these distributions, we can show them to be very similar. This is consistent with the hypothesis that rhodamine 123 fluorescence is a monotonous function of cell volume, and this is correlated to cell cycle age by a quasilinear association.

The function relating cell age to light scatter has been strengthened by showing that the DNA synthesis curve over the cell cycle takes on the typical G1, S, G2 + M form when light scatter is the gating base used. This allowed us to employ a common function, i.e., the light scatter, to evaluate the increase in mitochondrial fluorescence against the base used for the DNA profile giving a common age reference for the two processes.

By these criteria it is reasonable to state that the synthesis curves of Figs. 3 and 5 are linear. These data are consistent with those of Attardi et al. (5) in which the electron micrograph profiles of cell, cytoplasm, and mitochondria were measured on sections of synchronized HeLa cells. Although their methodology was considerably different from that used in this study, the results are similar. Furthermore, our data on the culture age were used to show that mean cell volume (Coulter) plotted against mean cell fluorescence at each age would generate a general relationship between cell volume and mitochondrial mass. The plot of these means at each culture age against each other yields a straight line as shown in Fig. 5. Larger cells contain a greater amount of fluorescent mitochondria than small cells, supporting the widely held idea that there is a correspondence between volume and respiratory capacity of a cell. Curiously, the least square line that has a 0.98 correlation coefficient extrapolates to zero fluorescence at a volume approximately equal to the cell's nuclear volume.

Our attempts at monitoring the division dilution of acutely stained mitochondria by flow microfluorometry have made us aware of the contradiction that the fluorescence is more than halved after one generation's time even though washing the cells does not markedly decrease the dye concentration. In other work we have noted that there is a disproportionate mitochondrial increase relative to cell division in the early portion of a culture cycle, whereas the opposite occurs in the late portion of the culture cycle. These preliminary observations suggest a mitochondrial proliferation rate that differs from the cell growth rate over the culture cycle. We consider this phenomenon the most likely basis for the decrease in the dye concentration.

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REFERENCES

1. Wildman, S. G. 1965. Organelles in Living Plant Cells. Film #5906, University of California Extension Media Center, Berkeley, Calif. 2. Hoffmann, H. P., and C. J. Avers. 1973. Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. Science (Wash. D. C.) 181:749-751.

3. Barnard, T. M. 1974. The mitochondrial of the flagellate, Polysphondylium ulna. J. Ultrastruct. Res. 48:414-419.

4. Atkinson, A. W., Jr., C. P. L. John, and B. E. S. Gunning. 1974. The growth and division of the single mitochondrion and other organelles during the cell cycle of Chorea, studied by quantitative stereology and three dimensional reconstruction. Protoplasma. 81:77-109.

5. Attardi, G., T. Costantino, J. England, D. Lynch, W. Murphy, D. Ojala, J. Posakony, and B. Storrie. 1975. The biogenesis of mitochondria in HeLa cells: a molecular and cellular study. In Genetics and Biogenesis of Mitochondria and Chloroplasts. W. Birky, P. S. Perlman, and T. J. Byers, editors. Ohio State University Press, Columbus, Ohio. 4-65.

6. Stevens, B. J., and E. Moustacchi. 1976. Ultrastructural characterization of mitochondria from a yeast mutant sensitive to "petite" induction (AY227). In Genetics, Biogenesis and Bioenergetics of Mitochondria. W. Blandow, R. J. Schwartz, D. Y. Thomas, K. Wolf, and F. Kaudewitz, editors. Walter De Gruyter, Inc., Elmsford, New York. 137-151.

7. Williamson, D. H. 1976. Packaging and recombination of mitochondrial DNA in vegetatively growing yeast cells. In Genetics, Biogenesis and Bioenergetics of Mitochondria. W. Blandow, R. J. Schwartz, D. Y. Thomas, K. Wolf, and F. Kaudewitz, editors. Walter De Gruyter, Inc., Elmsford, New York. 117-123.

8. Luck, D. L. 1975. Formation of mitochondria in Neurospora: a study based on mitochondrial density changes. J. Cell Biol. 64:461-470.

9. Smith, D. A., D. R. Tauro, E. Schweizer, and H. O. Halvorson. 1968. The replication of mitochondrial DNA during the cell cycle of Saccharomyces lactis. Proc. Natl. Acad. Sci. U. S. A. 65:356-364.

10. Williamson, D. H., and E. Moustacchi. 1971. The synthesis of mitochondrial DNA during the cell cycle in the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta. 24:346-350.

11. Cottrell, S. T., and C. J. Avers. 1970. Evidence of mitochondrial synchrony in synchronous cell cultures of yeast. Biochim. Biophys. Acta. 238:253-260.

12. Wildman, S. G. 1965. Organelles in Living Plant Cells. Film #5906, University of California Extension Media Center, Berkeley, Calif. 2. Hoffmann, H. P., and C. J. Avers. 1973. Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. Science (Wash. D. C.) 181:749-751.

13. Nagley, P. K. S. Sriraktrakul, and A. W. Lunnane. 1977. Structure, synthesis and genetics of yeast mitochondrial DNA. Adv. Microb. Physiol. 16:157-277.

14. Johnson, L. V., M. L. Walsh, and L. B. Chen. 1980. Localization of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. U. S. A. 77:996-999.

15. Melamed, M. R., P. F. Mullaney, and M. L. Mendelesohn. 1979. Flow Cytometry and Cell Sorting. John Wiley & Sons, Inc., New York. 1-716.

16. Collins, S. J., R. C. Gallo, and E. G. Gallagher. 1977. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature (London). 270:347-349.

17. Crumman, H. A., M. S. Oka, and J. A. Steinmann. 1976. Rapid staining methods for analysis of deoxyribonucleic acid and protein in mammalian cells. J. Histochem. Cytochem. 24:64-71.

18. Wilson, M. S., J. C. Casamento, L. L. Wooster, and H. B. Picket. 1978. Quantitative isolation of liver mitochondria by zona centrifugation. Anal. Biochem. 85:255-264.

19. Janas, T. W., P. Hemond, G. Czer, and R. Bohman. 1975. Parametric analysis of volume distributions of Schizosaccharomyces pombe and other cells. Exp. Cell Res. 94:267-276.

20. Saltzman, G. C., P. F. Mullaney, and B. J. Price. 1979. Light-scattering approaches to cell characteristics. In Flow Cytometry and Cell Sorting. R. M. Melamed, P. F. Mullaney, and M. L. Mendelesohn. John Wiley & Sons, Inc., New York. 105-124.