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Interactions of Rhodamine 123 with Living Cells Studied by Flow Cytometry[1]

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ABSTRACT

The cationic fluorochrome rhodamine 123 (R123), reported to bind specifically to mitochondria of living cells, was presently investigated with respect to its uptake by a variety of cell types in various functional states and the subsequent effect of the dye on cell growth. The emission spectrum of R123 taken up by cells undergoes a 12-nm red shift, suggesting formation of a complex. Cells accumulate R123 rapidly; near maximum binding is reached after 5 to 10 min, regardless of the temperature (0–37°) of incubation. There is a dose-dependent relationship between R123 concentration in the medium and the dye accumulation in the cell that covers the range of 0.1 to 10.0 and 0.1 to 5.0 μg of R123 per ml under equilibrium and nonequilibrium conditions, respectively. Some leakage of the dye from cells occurs, following their transfer into dye-free medium. Despite the leakage, the intracellular dye can be detected after at least two cell divisions, thus indicating that: (*a*) the R123-labeled cells divide; (*b*) during division, labeled mitochondria are distributed into the daughter cells; and (*c*) R123 may be used as a cell tracer.

Cell death often is accompanied by a transient increase in R123 fluorescence. Dead cells exhibit either uniform, strong fluorescence or show a patchy labeling pattern suggesting swollen mitochondria. With time (4 to 8 hr), dead cells lose ability to retain R123 and lyse. Uptake of R123 by living cells is increased during the transition from quiescence into the cycle, and a decrease is seen when Friend leukemia cells undergo erythroid differentiation; in all cases, changes in R123 uptake are correlated with changes in cellular RNA content. Simultaneous cell staining with R123 and ethidium or propidium provides a rapid assay of the viability of the cells and their metabolic state, *i.e.*, as related to proliferation or motility.

Pulse-labeling of cells with up to 10 μg of R123 per ml has no significant effect on their immediate growth and cloning efficiency. In the continuous presence of R123, however, cells become specifically arrested in the G_{1A} compartment, *i.e.*, in early G_1 phase. Detailed analysis of the cell cycle kinetics reveals that cell progression through all phases is slowed 4 hr after addition of R123. Cell exit from G_{1A}, however, is affected as early as 2 hr following addition of R123, and with time the cells are unable to leave this compartment at all. Uncharged rhodamine dyes (rhodamine 110 and rhodamine B) do not accumulate in mitochondria and are without effect on the cell cycle. The cytostatic effect of R123 is discussed in light of the dye specificity for mitochondrial membranes and the disruption of cell energy metabolism, resulting in the inability of the cells to attain a critical content of essential components (*i.e.*, ribosomal RNA), necessary for cell entrance into the prereplicative (G_{1B}) compartment of G_1 phase.

INTRODUCTION

Certain permeant cationic fluorochromes such as cyanine (3, 16, 20) or rhodamine (6, 15, 16) dyes are taken up specifically by mitochondria of living cells. Among these dyes, R123[2] has been the most extensively studied. Uptake of these fluorochromes (per unit of mitochondrial membrane) is believed to reflect the transmembrane potential (1, 18, 19, 21, 25). While the fluorescence intensity of individual mitochondria within a given cell appears to be uniform, large intercellular variations are observed in many cell systems (3, 15–17). Cell-to-cell differences may be a result of either different numbers of mitochondria per cell, a difference in mitochondrial potential, or both. In either case, cellular staining with these fluorochromes appears to be related to the metabolic state, *i.e.*, energy requirements of the cell. Thus, activation of quiescent cells in confluent cultures (3), stimulation of lymphocytes (6, 20), or cell progression through the cycle (15), are all characterized by a change in binding of these probes. Since some of these dyes are nontoxic, they were proposed as supravital mitochondrial probes (6, 15, 18) that may be useful in the analysis of different functional states of the cell, *e.g.*, proliferation, differentiation, or motility. In early studies using UV microscopy, visual estimation of the dye binding were reported, but there were few attempts recently to quantitate the dye uptake (3, 6, 15).

Because fluorescence of individual cells stained with R123 can be easily measured by flow cytometry (6), quantitation of dye uptake per cell is possible for large populations of cells. In the present paper, we provide quantitative data on the interactions between R123 and different cell types. Special attention is given to the kinetics of binding and its relationship to cell viability, quiescence, and differentiation, and the the long-term effects of this fluorochrome on cell clonogenicity and cell cycle progression.

MATERIALS AND METHODS

Cells

Lymphocytes. Blood was collected by venipuncture from healthy individuals. The cultures of lymphocytes were prepared in Eagles' basal medium and incubated in the presence and absence of phytohemagglutinin (Grand Island Biological Co., Grand Island, N. Y.) as described (6, 11).

FL Cells. The Friend virus-infected murine leukemic cell line GM-

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[2] The abbreviations used are: R123, rhodamine 123; CHO, Chinese hamster ovary; AO, acridine orange; FL, Friend leukemia; RB, rhodamine B; R110, rhodamine 110.

86, derived from clone 745, was obtained from the Institute for Medical Research (Camden, N. J.). The cells were maintained in Eagle's basal medium with 2.5×10^{-2} M 4-(2-hydroxyethyl)-1-piperazinethylsulfonic acid buffer (Grand Island Biological Co.) and 15% fetal calf serum as described (24). Erythroid differentiation was induced by addition of 230 mM dimethyl sulfoxide; the number of differentiated cells was evaluated by the benzidine method (23).

L1210 Cells. Cells were grown in suspension as described (24). Prior to addition of rhodamine dyes, the cultures were split 1:3 on 3 successive days to ensure asynchronous growth. Analysis of the R123 effect on cell kinetics was determined in a stathmokinetic experiment as described (13). Details of this experiment are given in the legends to Charts 6 to 8.

CHO Cells. CHO cells were maintained as monolayer cultures in F-12 (GIBCO) as described (24). The cultures were routinely passaged at a ratio of 1:20 twice weekly. Asynchronously growing cells were plated at a concentration of 100 cells/35-mm-diameter well (Costar, Cambridge, Mass.). Following reattachment, R123 was added at appropriate concentrations for 24 hr. Cultures were then rinsed and refed with fresh medium. Following growth for an additional 6 days, the cultures were fixed with Carnoy's fixative and stained with crystal violet. Colonies of 50 or more cells were counted, as described (13). The studies were carried out in triplicate. The plating efficiency was 70 to 90% for asynchronously growing, control CHO cells.

R123 Uptake

R123 (laser dye purity) was obtained from Eastman Organic Chemicals, Rochester, N. Y. The stock solution (1 mg/ml) was made in distilled water. Further dye dilutions were done in culture media or in buffered salt solutions, as indicated in the respective legends to charts and tables. R123 uptake was studied either under equilibrium or after preincubation of the cells with the dye, followed by rinsing and resuspension in R123-free media. In the latter case, the fluorescence was measured 1 hr after cell resuspension in the absence of R123 unless otherwise indicated. In some experiments cells were counterstained with ethidium bromide or propidium iodide (both from Polysciences, Inc., Warrington, Pa.) at a final concentration of 10^{-5} M. In all incubations, there were 1 to 2×10^6 cells/ml of the respective dye solutions. Variation of cell number within the range of 0.2 to 2.0×10^6/ml did not change their stainability. In some experiments, to eliminate dead cells, the cells suspensions were incubated with a freshly made mixture of 0.25% trypsin and DNase I at 100 μg/ml (both from Worthington) at 37° for 30 min. Broken and dead cells or isolated nuclei are dissolved by these enzymes while living cells remain. Cell viability was also estimated by the standard test of trypan blue exclusion.

Cell Staining with AO

To analyze effects of R123 on cell cycle progression, the cells were preincubated with R123 for various periods of time and then stained with AO. It was observed in pilot experiments that prior exposure of cells to up to 50 μg of R123 per ml does not affect subsequent cell stainability with AO. R123 accumulated in mitochondria is rapidly released from the cells during procedures preceding cell staining with AO, *i.e.*, treatment with Triton X-100 or fixation. Thus, it was possible to analyze the cell cycle distribution of R123-treated cells without interference by this dye on measurements of cellular DNA or RNA.

Simultaneous staining of DNA and RNA with AO was described in detail (10, 11, 22). The stoichiometry of DNA and RNA staining has been demonstrated in several cell systems (2). Staining specificity was controlled by DNase I and RNase digestions (11, 22).

To analyze the effect of R123 on the cell cycle in more detail, including quantitation of cells in mitosis, the cells were stained with AO following removal of RNA and partial denaturation of DNA *in situ* as described (9, 12). In this technique, cell pretreatment at low pH followed by staining at pH 2.6 results in partial DNA denaturation, the extent of which was shown to be proportional to the degree of chromatin condensation (8, 12). The differential staining of native *versus* denatured DNA occurs because AO intercalates into double-stranded DNA and fluoresces green, whereas dye interaction with denatured DNA results in metachromatic red fluorescence (9). This technique discriminates functionally distinct compartments of G_1 phase (7, 8, 13), and was applied to characterize the effects of R123 on cell progression at various cell cycle points.

Fluorescence Measurements

Cell fluorescence and light scatter were measured in the FC 200 Cytofluorograf (Ortho Diagnostic Instruments, Westwood, Mass.) interfaced to a Data General minicomputer. The data on R123 binding were obtained using 488 nm excitation and green fluorescence detection at 515 to 575 nm with forward light scatter measured as a second parameter.

In the case of AO-stained cells, the red ($F_{>600}$; measured in a band of 600–650 nm) and green (F_{530}; at 515 to 575 nm) fluorescence emissions were separated optically and measured by separate photomultipliers. The width of the green fluorescence pulse was also measured and used to distinguish single cells from cell doublets.

Fluorescence spectra were recorded using a thermostated SLM 4800 spectrofluorometer (SLM, Urbana, Ill.). The spectra presented in Chart 1 were not corrected for the emission monochromator or photomultiplier tube response. To obtain the net spectrum of the dye accumulated in cells, the emission spectra of the cell suspensions were corrected by subtraction of the supernatant spectrum following cell centrifugation. The cell concentration was kept constant (8.5×10^5/ml ± 10%) and cells were stirred during the measurement.

RESULTS

Fluorescence Spectra of Free and Cell-bound-R123. The excitation spectrum of R123 has a maximum at 500 nm; the molar extinction coefficient $\epsilon^{500}_{max} = 7.5 \times 10^4$ cm^{-1} mol^{-1} in water (Chart 1). The maximum of the emission spectrum varies, depending on dye concentration (not shown). At low concentration (2.6×10^{-8} M or 10 ng/ml), the maximum is at 525 nm, and it approaches 545 nm at 1.3×10^{-3} M. In contrast, the absorption spectrum does not change with dye concentration. The computer simulation of spectral changes indicates that the

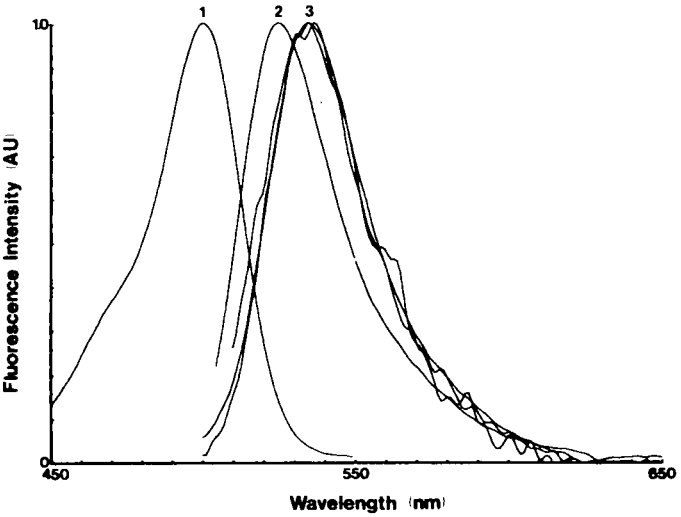

Chart 1. Excitation (*1*) and emission (2) spectra of free R123 (2.6×10^{-8} M) in Hanks' buffered saline solution. Emission spectra of R123 taken up by L1210 cells (*3*) were recorded after cell incubation with 0.1, 1.0, and 10.0 μg of R123 per ml; the relative fluorescence intensities of these suspensions were 1, 4, and 23, respectively. All spectra were normalized to 1.0 (arbitrary unit).

observed concentration-dependent red shift of the emission is the result of an inner filter effect rather than excimer formation. The emission spectrum of the R123 accumulated by the cells as compared with the free dye shows a red shift, the extent of which (12 nm) does not change with dye concentration or degree of cell labeling. This change may be interpreted as reflecting either complex formation or chemical modification of the dye rather than as a result of the inner filter effect or excimer formation.

Kinetics of R123 Uptake. Cells exposed to R123 accumulate the dye rather rapidly (Chart 2). Most of the R123 is taken up during the initial 5 min of incubation, approaching a plateau after 10 min. Although FL cells bind more R123 than L1210 cells, the kinetics of binding is similar for both cell lines. Comparable levels of fluorescence are obtained following cell incubation at 0 and 24°. The reaction, however, is somewhat slower at 0°. The kinetics of R123 uptake at 37° is similar to that seen at 24° (not shown).

A close relationship is observed between the concentration of R123 in the culture medium and the degree of its accumulation in the cell (Chart 3). Under equilibrium conditions, the linear dose dependency covers the range of 0.1 to 10.0 μg of R123 per ml. Within this range, a 10-fold increase in R123 concentration results in a 4- to 5-fold increase in cell fluorescence. However, when cells are incubated with the dye, rinsed, and then measured (Chart 3, nonequilibrium staining), the amount of dye retained is proportional to its concentration only within the range of 0.1 to 5.0 μg/ml. At higher concentrations, cells retain rather constant amounts of the dye after their transfer to the dye-free media.

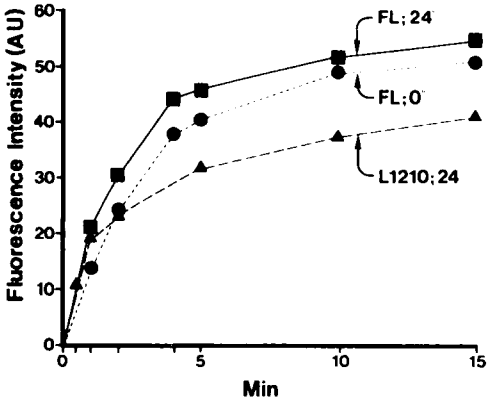

Chart 2. Kinetics of R123 uptake by FL or L1210 cells exposed to 10 μg of R123 per ml at 0 or 24° and measured under equilibrium with the dye.

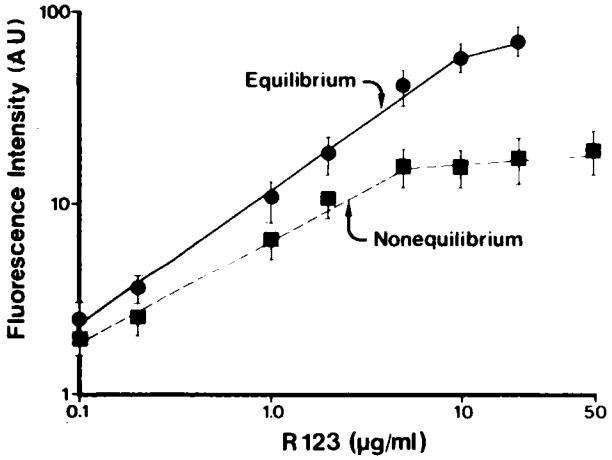

Chart 3. Intensity of fluorescence of FL cells incubated with various concentrations of R123 for 15 min, measured either in equilibrium with the dye, or after rinsing and resuspension in dye-free medium.

A decrease in fluorescence intensity is observed when cells are at first preincubated with R123 and then cultured in the absence of the dyes (Chart 4). The initial decrease, seen during the first 30 min, most probably reflects leakage of the dye from cells to the medium and establishment of a new equilibrium. After 24 hr, cell fluorescence is lowered by nearly 5-fold, which is most probably due to the dilution of R123 in the progeny cells as a result of 2 cell divisions; the doubling time of L1210 cells is about 12 hr (24).

Uptake of R123 and Cell Viability. Experiments have been performed to correlate changes in R123 binding with cell viability. Chart 5 illustrates consecutive changes in R123 uptake observed during cell death. In these experiments, in addition to R123, the cells were counterstained with propidium iodide. The viability tests based on trypan blue exclusion correlated, in general, with estimates of viability by exclusion of ethidium or propidium (not shown). In the present experiments, cells were killed by heat (60°), repeated freezing and thawing, hypotonicity, alcohol, or detergents. Because R123 was reported to stain only living cells, it was expected that the dead cells would not be able to retain the dye. This was not the case, however, and in numerous experiments immediately after cell death a transient increase in R123 fluorescence was observed. Because dead cells become stainable with propidium, they could then be distinguished from living cells by their increased green and red fluorescence values on scattergrams or 2 parameter histograms (Chart 5). The drop in R123 fluorescence was seen 4 to 8 hr after cell death; at that time cells had minimal green fluorescence and increased red fluorescence. Cells killed by detergents or subjected to very low hypotonicity rapidly lost the ability to stain with R123 without passing through a phase of increased stainability with this dye.

All samples subjected to flow cytometry were also examined by light and UV microscopy. Living cells had R123 stainability limited mostly to the oval or rod-like organelles typical of mitochondria as described by others (3, 15–17). Nuclei were negative, but in addition to mitochondria, a weak, diffuse green fluorescence was often seen in the cytoplasm. After counter-

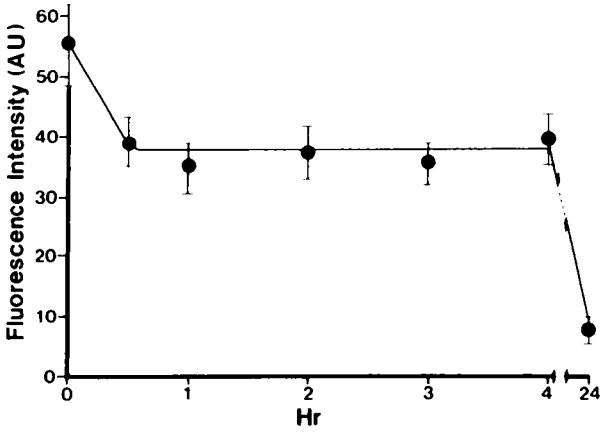

Chart 4. Decrease in fluorescence of L1210 cells incubated with 10 μg of R123 per ml for 15 min, then rinsed and cultured in the absence of the dye.

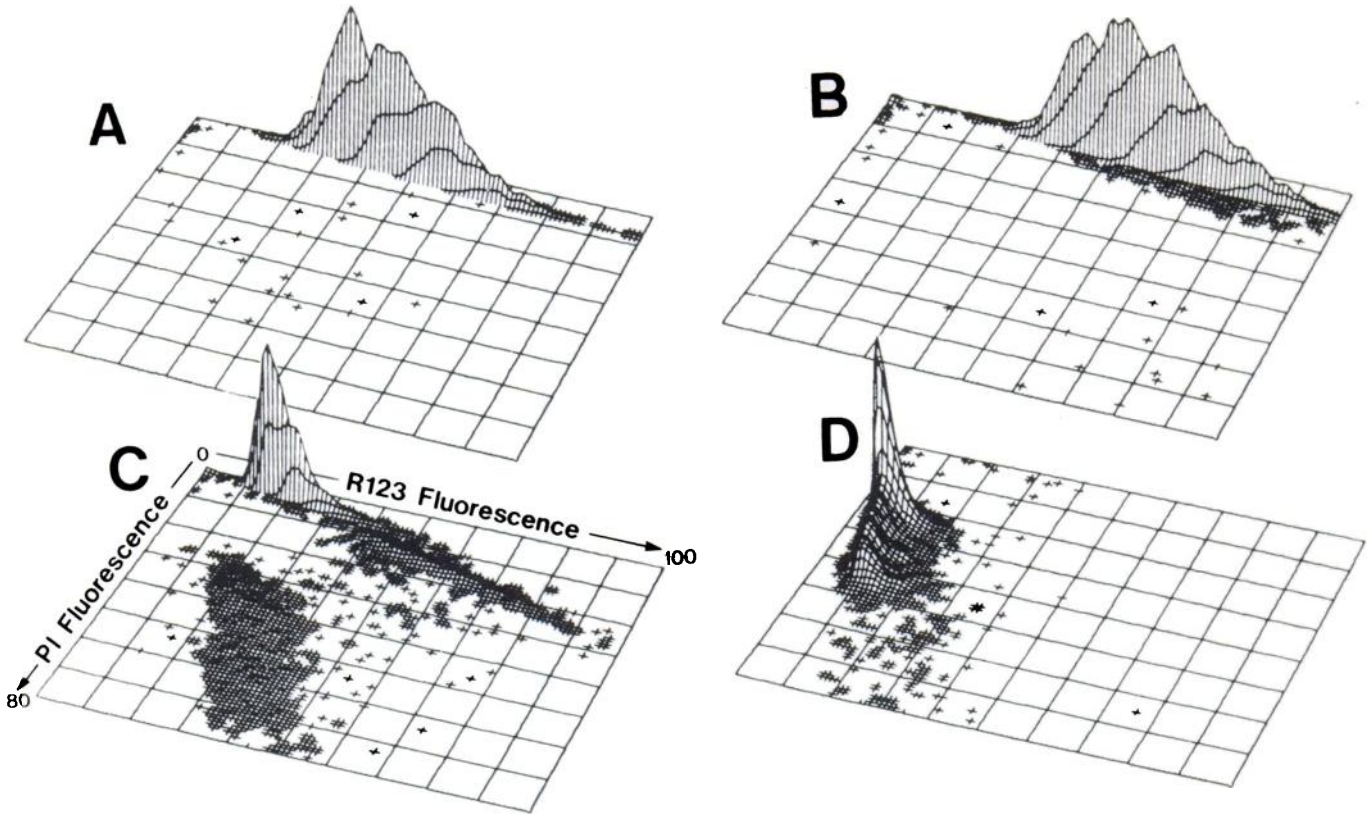

Chart 5. Examples of the cell fluorescence changes after simultaneous staining with R123 and propidium iodide (*PI*) following treatments resulting in cell death. Two-parameter frequency histograms illustrating stainability of L1210 cells with PI and R123. The vertical dimension (*Z* axis) represents cell number, scaled arbitrarily. *A*, cells from exponentially growing cultures. Living cells exhibit high variability of green (R123) and no red [propidium iodide (*PI*)] fluorescence. Few (<1%) dead cells show both R123 and propidium iodide fluorescence. *B*, cells treated with hypotonic solution (culture medium diluted 50% with distilled water) for 10 min. An increase in R123 and a minor increase in propidium iodide fluorescence are seen. *C*, cells treated with a strongly hypotonic solution (medium diluted 4-fold with distilled water) for 10 min. The main population shows increased stainability with propidium iodide and somewhat lowered R123 fluorescence. With time, more and more cells lose stainability with R123 and stain with propidium iodide. *D*, Triton *X*-100 (0.1%)-treated cells. The cells exhibit low R123 fluorescence; their distribution with respect to propidium iodide fluorescence resembles the cell cycle distribution, with a prominent G_1 peak.

staining with trypan blue and viewing the same field alternately in visible and UV light, it was apparent that in preparations in which cells were freshly killed by heat, repeated freezing, alcohol, or hypotonicity, many cells that stained with trypan blue also exhibited strong R123 fluorescence. While some of those cells were characterized by uniform diffuse fluorescence (including nuclei), other cells had a patchy fluorescence pattern resembling swollen mitochondria.

R123 Binding in Relation to Cell Proliferation, Quiescence, and Differentiation. Table 1 summarizes the results of numerous experiments in which the uptake of R123 has correlated with changes in cell metabolism as reflected by altered RNA content. Regardless of the cell type, cells in stationary cultures bound 30 to 45% less R123 than did cells growing exponentially. Cycling lymphocytes from phytohemagglutinin-stimulated cultures accumulated, on average, 7 times more R123 than did nonstimulated cells. Differentiation of FL cells was accompanied by a continuous decrease in R123 uptake. At the time of maximal differentiation (80% benzidine-positive cells), FL cells had 50% less R123 fluorescence than did untreated, exponentially growing cells.

Table 1

Changes in R123 uptake and RNA content of various cell types during their transition from stationary to exponential growth phase or during differentiation

R123 uptake was measured following cell exposure to 5 μg of R123 per ml for 15 min under nonequilibrium. RNA content of all cells in the population, regardless of the cell cycle phase, is given.

Because the settings of the photomultiplier sensitivities were different for different cell types, the results are comparable only within particular cell types.

Cell type	R123	RNA
Lymphocytes		
Quiescent	7.0 ± 2.6[a]	7.4 ± 1.9
Phytohemagglutinin, 3 days	51.1 ± 12.5	25.5 ± 6.8
CHO cells		
Stationary	29.5 ± 12.0	37.5 ± 9.5
Exponential	54.4 ± 18.9	56.6 ± 12.5
L1210 cells		
Stationary	36.2 ± 9.7	39.1 ± 5.7
Exponential	54.2 ± 12.1	52.1 ± 8.3
FL cells		
Stationary	34.7 ± 13.4	33.9 ± 7.9
Exponential	60.4 ± 15.0	54.3 ± 11.6
Differentiated	31.4 ± 11.8	29.8 ± 7.1

[a] Mean ± S.D.

Effects of R123 on Cell Growth and Colony Formation. In the studies reported below we have investigated the effects of R123 and 2 other rhodamine dyes, RB and R110, on cell growth. The latter 2 fluorochromes, in contrast to R123, which is positively charged at physiological pH, are neutral and do not bind to mitochondria (17). As evident from the data in Table 2, RB and R110 at concentrations of 1 to 6 μg/ml have minimal effects on the growth of L1210 cells. In contrast, equivalent concentrations of R123 markedly suppress cell growth, decreasing the number of cells in cultures by 47 to 68%. Although the total number of cells in R123-treated cultures was dimin-

ished, the number of dead cells was minimal and within the range of the control, suggesting that R123 exerts a cytostatic effect on L1210 cells.

Table 2

Cell growth and cell cycle distribution of L1210 cells in control, and in cultures treated with various concentrations of rhodamine dyes for 24 hr

Dye	Concentration (μg/ml)	Cell growth[a]	Cell cycle distribution[b]		
			G_1	S	G_2 + M
None		1.0	47.6	32.4	20.0
R123	1	0.53 ± 0.05[c]	59.8	25.4	14.8
	3	0.37 ± 0.04	72.7	15.6	11.7
	6	0.32 ± 0.03	67.7	18.6	13.8
R110	1	0.96 ± 0.05	46.9	33.7	19.4
	3	0.93 ± 0.05	47.0	32.2	20.8
	6	0.96 ± 0.10	47.7	31.3	21.0
RB	1	1.00 ± 0.03	47.6	31.7	20.7
	3	0.87 ± 0.02	47.1	32.5	20.4
	6	0.88 ± 0.02	47.4	32.9	19.7

[a] Data represent the number of living cells in cultures (in relation to control) after 24 hr of growth with various concentrations of the dyes. There were few dead cells (<2%) in these cultures.
[b] Percentage of cells at the various cell cycle phases was estimated using interactive computer program, after cell staining as shown in Chart 6.
[c] Mean ± S.D.

Analysis of the cell cycle distribution provided direct evidence of the cytostatic effect of R123. As is evident in Table 2, this dye arrests cells in the G_1 phase of the cell cycle; RB and R110 are without effect. The data in Chart 6 indicate that as a result of R123 treatment, cells become preferentially blocked in the G_{1A} compartment of the G_1 phase. Cells in this compartment have RNA values below the minimum RNA content of S-phase cells (5, 7, 8, 13). As shown before (7) and will be discussed further, the G_{1A} compartment represents a functionally distinct subphase of the cell cycle.

The RNA content of R123-treated cells is similar to that of cells growing exponentially. An interesting detail related to the RNA values of the S-phase cells is, however, apparent (Chart 6). Namely, the rate of RNA increase during S phase is lowered in the cell population treated with R123, as evidenced by the more vertical inclination of the S cluster. Assuming that R123 does not accelerate the rate of DNA synthesis, the data indicate that in the presence of this dye the rate of RNA accumulation slows down during cell progression through S phase. There is also a higher intercellular variation in RNA content among S cells in the R123-treated cultures, as reflected by the widening of the S-phase cluster. The coefficient of variation of the RNA values of the S-phase cells (over similar range of DNA values)

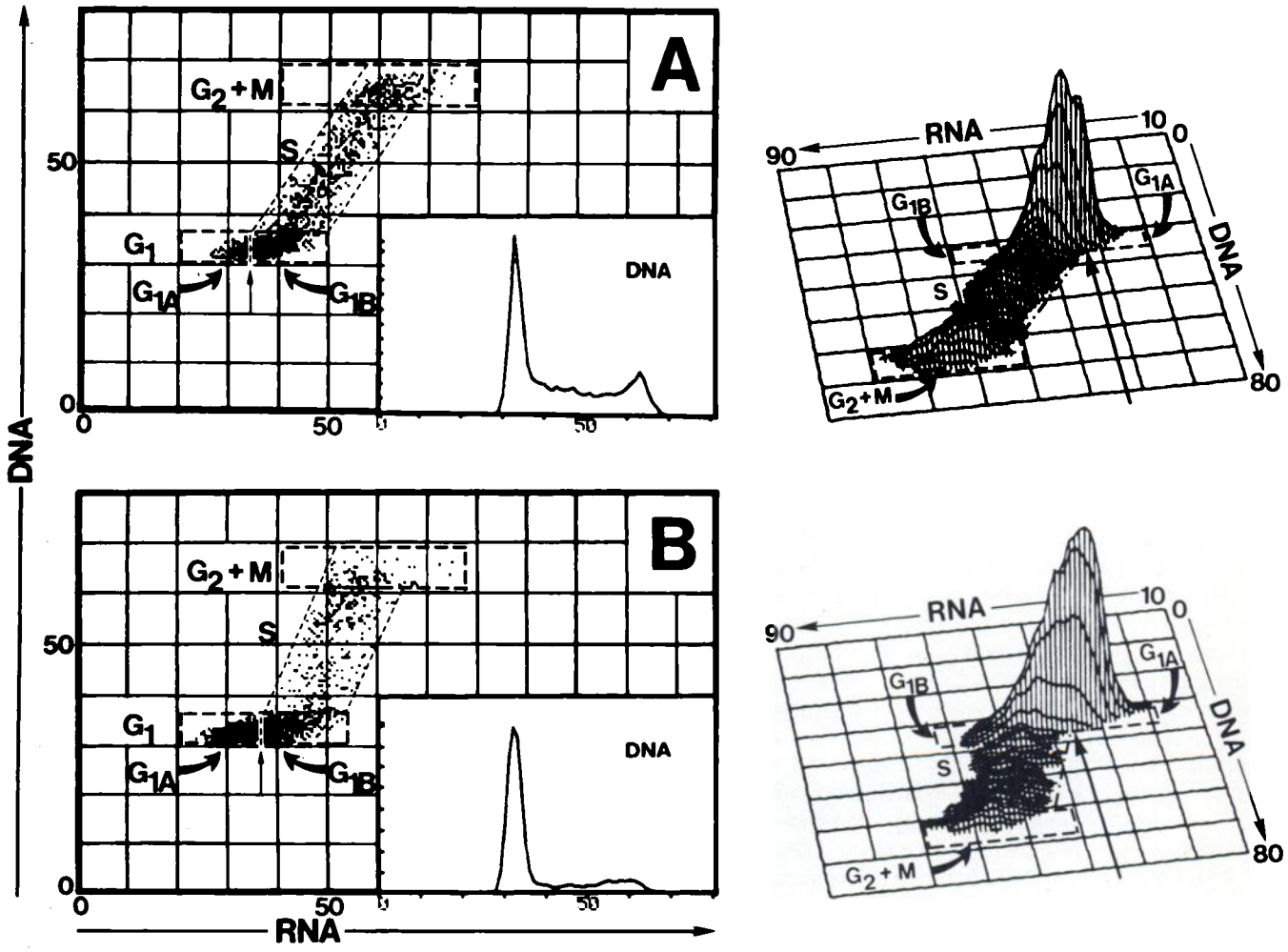

Chart 6. Arrest of L1210 cells in the G_{1A} compartment of the G_1 phase as a result of their growth with 6 μg of R123 per ml for 24 hr. Scattergrams and histograms showing DNA and RNA values of the exponentially growing, control L1210 cells (*A*) and cells grown in the presence of R123 (*B*). As a result of R123 treatment, cells accumulate preferentially in the low RNA compartment of G_1 phase, with RNA values below the critical threshold (*arrows*). Such cells cannot enter the S phase.

is about twice higher in R123-treated cultures than in control.

Exposure of CHO cells to up to 10 μg of R123 per ml for 24 hr does not markedly affect cell viability as measured by cell clonogenicity. Thus, while control CHO cultures had 72.0 ± 8.6 (S.D.) colonies/plate, cells incubated with 3 or 10 μg of R123 per ml showed 73.3 ± 8.3 or 65.0 ± 5.7 colonies, respectively.

Effect of R123 on the Cell Cycle Kinetics of L1210 Cells. To analyze the effect of R123 on the cell cycle progression in more detail, a stathmokinetic experiment was performed in which the effect of the dye on cell progression at various points of the cell cycle could be studied. In this experiment, exponentially growing, asynchronous cultures are treated with the stathmokinetic agent (vinblastine), in the presence or absence of the drug, and sampled at various times to obtain the percentage of cells in G_{1A}, G_{1B}, various portions of S, G_2, and M (13). All those phases are identified, based on a distinctive distribution of native *versus* denatured DNA on 2-parameter histograms after partial denaturation of DNA *in situ*, as described (13). This approach (13) allows one to measure the effect of the drugs on the G_{1A} to G_{1B} transition, cell exit from G_1, progression through S, duration of G_2, cell transit from G_2 to M, and to detect the terminal point of its action.

Chart 7 shows the rate of accumulation of cells in G_2 + M and M phases in the presence and absence of R123 added either at the same time or 16 hr prior to vinblastine. In the control, the G_2 + M and M slopes are linear (M slope become linear after a 1-hr delay), thus indicating that cells are growing exponentially. The duration of the cell cycle estimated from these slopes (13) is 10.2 hr. The duration of the G_2 phase, estimated from the delay of the M slope (after correction for the initial lag period) as compared with G_2 + M slope is 2.1 hr.

Cells treated with R123 at the time of addition of vinblastine are not affected during the first 3 hr; then the rate of cell entry into M and G_2 + M slows down. The extent of the slowdown is similar for both the M and G_2 + M compartments, indicating that the first effects of R123 manifest as a decrease in cell progression through late S and G_2 phases. In cultures pretreated with R123 for 16 hr prior to vinblastine, the rate of entry into M or G_2 + M is minimal.

The data in Chart 8 illustrate the effect of R123 on cell exit from G_1 and G_{1A}. When cells are blocked in mitosis, the rates of emptying of G_1 and G_{1A} may be used to estimate cell transit times through those compartments, assuming that the stathmokinetic agent does not affect progression through G_1. As shown before (5, 13), the curve representing emptying of the G_1 compartment is biphasic; the first phase is linear (a shoulder on the log plot), while the second is exponential (a straight line on a log plot). These phases reflect the presence of 2 compartments in the G_1 phase, one which has transit times of rather constant duration and the other having exponentially distributed residence times (5, 7, 13). The exponentially declining slope of the G_{1A} exit curve (Chart 8) indicates that cell residence times in G_{1A} are exponentially distributed; the half-time of cell residence in G_{1A} (5) estimated from the slope is 55 min.

The effect of R123 on G_1 cells is already apparent 1.5 to 2 hr after addition of the dye and manifests as a decrease in the G_{1A} exit rate. The half-time of cell residence in G_{1A} is increased to 85 min in the presence of 3 μg of R123 per ml. The diminished exit rate in the presence of R123 does not change up to the sixth hr, at which time nearly all cells have left G_{1A}.

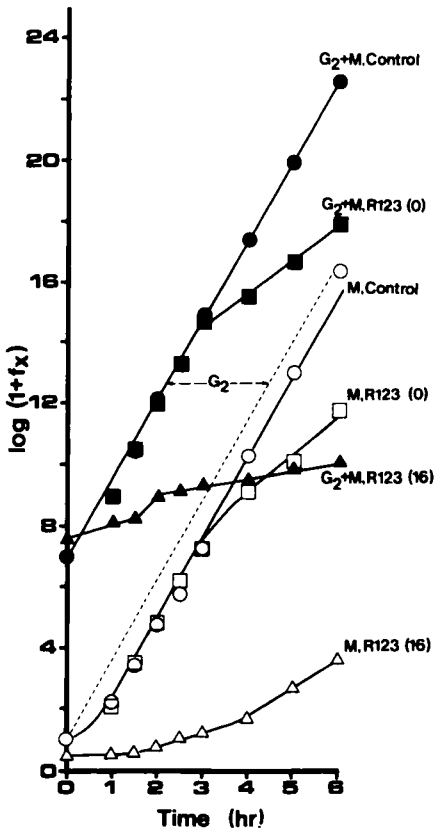

Chart 7. Kinetics of entrance of L1210 cells into mitosis (*open symbols*) and into G_2 + M (*closed symbols*) in the presence and absence of R123 in culture. At 0 time, vinblastine was added to all cultures; some cultures (□) were treated with 3 μg of R123 per ml added simultaneously with vinblastine. Other cultures (△) were pretreated with 3 μg of R123 per ml for 16 hr prior to addition of vinblastine. All cultures were then sampled at 0.5- or 1-hr intervals, and cell numbers of M and G_2 + M peaks were estimated after cell staining as described (13). Following a short lag, accumulation of control cells in M is linear, indicating that cells were growing exponentially and that their arrest in M is not leaky. The first effects of R123 are already seen after 4 hr and manifest as a decreased rate of entrance into M and G_2 + M.

The rate of cell exit from G_1 (*i.e.*, G_1 to S transition) decreases 4 hr after addition of R123. Of the 2 phases of emptying of the G_1 compartment, only the latter one (represented by an exponential slope), is influenced by the dye. Few cells leave the G_1 or G_{1A} compartments in cultures treated for 16 hr with R123.

By applying appropriate thresholds during data analysis, it is also possible to measure cell progression through selected window(s) in S phase (13). In the present experiments, progression of cells through the early S window was also analyzed (data not shown). A slowdown in the rate of cell traverse through the S phase was evident 4 hr after addition of R123.

DISCUSSION

R123 appears to be a useful mitochondrial probe that may be applied to studying living cells for discrimination of their different metabolic states, *i.e.*, as related to proliferation or differentiation. The excitation and emission wavelengths of the free dye, and dye taken up by cells, are within a range of 450 to 560 nm, making it suitable for study with commercially available flow cytometers using argon ion laser illumination.

A close relationship between cell fluorescence and dye concentration suggests that the fluorescence intensity of individual

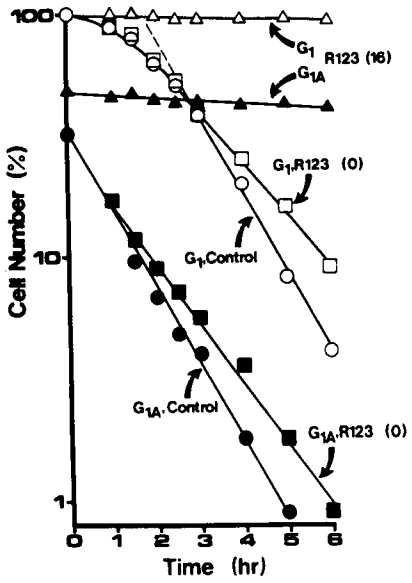

Chart 8. Kinetics of exit of L1210 cells from the G_1 (*open symbols*) and G_{1A} compartments (*closed symbols*) in the presence and absence of R123 in cultures. Experimental scheme is described in legend to Chart 7. Following additions of vinblastine at 0 time, the number of cells remaining in the G_1 and G_{1A} compartments, distinguished as described before (13), was estimated at various time intervals.

cells is closely related to the quantity of dye taken up. Because the intensity of R123 in solutions is proportional to dye concentration (when correction for the inner filter effect is made), it appears that R123 does not form aggregates as some cyanine dyes do (3, 25), in which fluorescence quenching takes place. The dye taken up remains in the cell for a considerable period of time, and during division is distributed into progeny cells. After 2 cell generations the daughter cells have about one-fifth of the fluorescence of the original, labeled cells. Because the fluorescence of R123-labeled cells is strong, the dye may be detected even 48 hr after labeling, which makes it useful for supravital cell labeling. It should be stressed, however, that since the dye leaks from the cells, albeit slowly, cocultivation of labeled and unlabeled cells results in progressive dye uptake by unlabeled cells. This restricts use of R123 as a cell tracer in situations where labeled and unlabeled cells remain in contact with each either directly, or via culture medium for an extended period of time.

The dye may be retained not only in living but also in dead cells. Freshly killed cells often retain the ability to bind R123 for up to 8 hr. The dye is then distributed either homogeneously in the cells or in organelles resembling swollen mitochondria. The fluorescence of such cells is greater than the fluorescence of living cells, although they could be counterstained with trypan blue or propidium. These data suggest caution in interpreting binding of R123 as evidence of cell viability. The increased binding of R123 during cell death may reflect a transient change in membrane polarization, *e.g.*, as related to swelling of the mitochondria.

Binding of R123 during cell proliferation, quiescence, and differentiation correlated with the metabolic state of the cells as reflected by their RNA content. Thus, cells in stationary cultures bound 30 to 45% less R123 than did cells growing exponentially. Cells in deep quiescence (noncycling lymphocytes) bound several times less R123 than their cycling counterparts. Erythroid differentiation of FL cells was accompanied by a 50% decrease in R123 binding. Simultaneous cell staining with R123 and ethidium or propidium thus provides a rapid assay of cell populations with respect to cell viability and their metabolic state; the latter may reflect their proliferative or motility potential (see "Addendum").

In the situations discussed above, changes in R123 accumulation may reflect either an altered number of mitochondria per cell, altered transmembrane potential, or both. Unless another mitochondrial probe unrelated to the membrane potential is found for comparison, a distinction between those alternatives cannot be made. Cohen *et al.* (3), Johnson *et al.* (16), and Shapiro *et al.* (20) reported rapid changes in binding of R123 or cyanine dyes following cell stimulation induced by "wounding" of monolayer cultures or PHA treatment of lymphocytes. Changes occurring in a time much shorter than that required for the synthesis and assembly of new mitochondria most probably reflect altered transmembrane potential. According to the work of Johnson *et al.* (16), stimulation of cells in these experiments primarily triggered an increase in their motility, which in turn was correlated with change in the membrane potential. At present, we did not attempt to investigate the early changes in R123 binding in response to cell stimuli (which would be primarily an indication of their hypo- or hyperpolarization). Instead, efforts have been made to compare R123 binding by various cell types in different functional states under standard culturing conditions.

The present data indicate that R123 may be used as a supravital cell probe for long-term experiments. Pulse labeling of CHO cells at up to a concentration of 10 μg/ml for up to 24 hr does not affect their cloning efficiency by more than 10%. Lower concentrations, still adequate for good cell labeling, are without effect on cell viability. Thus, as long as R123 is used for pulse labeling and cells are then allowed to grow in dye-free medium, their growth is not perturbed despite the presence of the dye in the mitochondria. These data may be interpreted as indicating that the R123 binding sites, once saturated by the dye, can later recover during cell growth in dye-free medium with restoration of mitochondrial function. Since the dye still remains in the cell, the recovery may indicate the appearance of new R123 binding sites, perhaps as a result of their *de novo* synthesis.

Continuous cell growth in the presence of R123, however, induces their arrest in the cell cycle with preferential cell accumulation in the G_{1A} compartment. The first signs of perturbation of the cell cycle are observed 2 hr following addition of R123. At that time, a slowdown of cell exit from the G_{1A} compartment is seen. Slightly later (4 hr), the rate of progression through other phases decreases. Cell exit from G_{1A}, however, is more affected than is the transit through other phases, and as a consequence, more and more cells reside in G_{1A}. After 16 to 24 hr, the cells are predominantly blocked in G_{1A}; the few cells that still remain in G_{1B}, S, or G_2, progress through those phases at a very slow rate. The RNA content of the R123-treated cells is not much different from the control values of cells in their respective phases of the cell cycle; there is higher heterogeneity, however, of the S cells and a minor RNA deficit in late-S-phase cells. The rhodamine analogs that do not bind to mitochondria and do not interrupt energy metabolism (*i.e.*, RB and R110) (14, 16, 17) have no effect either on the cell cycle or cell survival.

The G_{1A} compartment is the most sensitive to R123 action and is the cell cycle point through which the treated cells cannot progress. As shown before (5, 7, 8, 13), this compartment, representing early G_1 cells, is a functionally distinct subphase of the cell cycle. Cells in G_{1A} have lowered RNA content and more condensed chromatin as compared with the remaining cells of G_1 (G_{1B}). The cell residence times in G_{1A}, in contrast to other phases, are distributed exponentially (5, 7, 13). It is probable that the presence of R123 results in inhibition of the synthesis of essential macromolecules (*i.e.*, rRNA, proteins), which precludes G_{1A} cells from accumulating threshold values of the constituents necessary to leave G_{1A} and enter the prereplicative (G_{1B}) phase. Since progression of individual cells through other phases is slowed down rather than terminated by R123, with time, more and more cells arrest in G_{1A}.

The mechanism by which R123 induces a cytostatic effect may be inferred from the specificity of its binding. This specific mitochondrial probe, upon binding, may neutralize the electronegative sites of the mitochondrial membrane and thus lower the transmembrane potential. Thus, the dye may act as an antimetabolite disrupting energy metabolism at the mitochondrial level. It was shown recently by Conover and Schneider (4) that cationic dyes of the cyanine series specifically inhibit NAD^+-linked respiration in rat liver mitochondria. Positively charged rhodamines 6G and 3B were also inhibitory (although at higher concentrations), while B, neutral at physiological pH, was without effect. The data of Gear (14) indicate that rhodamine 6G strongly inhibits oxidative phosphorylation. Zigman and Gilman (26) reported recently that cyanine dyes with reduction potential (E_R) more negative than -1.0 V inhibited division of fertilized sea urchin eggs, most likely by disrupting the energy metabolism of those cells. The authors postulate that these cationic dyes bind to the mitochondrial membrane and a dye with an E_R more negative than that of respiratory chain reactions could occupy electron acceptor sites, turning off the transport of electrons and cellular respiration. Thus, depending on their E_R, the dyes would form electron barriers interupting the flow of electrons in key transport systems (26). Disruption of the energy metabolism will result in suppression of the synthesis of macromolecules essential for cell progression through the cell cycle.

ADDENDUM

Recently, simultaneous staining with R123 and ethidium of sperm cells provided a means to assay rapidly sperm motility and viability by flow cytometry (Evenson, D. P., Darzynkiewicz, Z., and Melamed, M. R. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J. Histochem. Cytochem. *30:* in press, 1982.)

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