Increased Rhodamine 123 Uptake by Carcinoma Cells

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ABSTRACT

The total cellular content of the fluorescent mitochondrial-specific dye rhodamine 123 (Rh-123) was quantified by butanol extraction as a function of time of exposure and dose for a variety of cell lines. These results were compared with observations made by fluorescence microscopy on dye localization and mitochondrial morphology. There appeared to be two categories of cell types based on Rh-123 uptake: those which progressively accumulate the dye, such as Ehrlich ascites tumor cells, carcinoma-derived lines MCF-7, PaCa-2, EJ, HeLa, and normal fibroblast line CCL 64; and those which appear to equilibrate with the extracellular dye within 1 h of incubation in Rh-123 (1 µg/ml) with a minimal level of uptake, such as the normal epithelial-derived lines CV-1 and MDCK and the transformed fibroblast line 64F3. Within the first category, the absolute value of uptake per cell correlated with the concentration of Rh-123 in the medium and with the period of exposure to the dye up to a point of apparent cellular saturation. The length of time required for apparent saturation depended on the cell type. In the second category equilibrium was very early, and the total uptake was a function of the extracellular concentration of Rh-123. This probably does not represent a saturation level of dye content in the non-accumulating, low uptake cell lines. Fluorescence microscopy revealed that Rh-123 localization was initially mitochondrial-specific for all of the cell lines examined. Over time, alterations in mitochondrial morphology and cytoplasmic fluorescence were observed in the high uptake cell lines but not in the minimal uptake cell lines. Incubation of the high uptake HeLa cell line with the mitochondrial membrane potential inhibitor p-trifluoromethoxyphenylhydrazone substantially decreased Rh-123 uptake. These observations may indicate a transformation-related characteristic of carcinoma cell mitochondria. It may be possible to exploit the mechanism responsible for the progressive accumulation of Rh-123 by carcinoma-derived cell types for chemotherapeutic approaches to certain types of carcinomas.

INTRODUCTION

The permeant cationic fluorochrome Rh-123 has been used in a variety of studies of mitochondria in living cells (reviewed in Refs. 1 and 2). At non-toxic concentrations, Rh-123 provides low background, high resolution fluorescent images of mitochondria in a variety of cell types (3). The preferential accumulation and retention of this dye by mitochondria apparently correlates with the mitochondrial membrane potential (4). Rh-123 has been utilized as an indicator of mitochondrial membrane potential in studies of cells undergoing alterations in metabolic state (5-8). Rh-123 may also demonstrate a qualitative difference between the mitochondria of normal and transformed cells. Johnson et al. (9) observed that mitochondria of feline sarcoma virus-transformed mink fibroblasts retain significantly less Rh-123 after transfer to dye-free medium than do mitochondria of the untransformed parent cells. A more dramatic difference was reported by Summerhayes et al. (10), who found that mitochondria of most carcinoma-derived cells retain Rh-123 for much longer periods than do mitochondria of normal epithelial-derived cells. This phenomenon appears to correlate with selective killing of carcinoma cells in vitro, as well as in vivo (11-13). The mechanism responsible for the difference in interaction of Rh-123 with carcinoma and normal epithelial-derived cells is not understood.

We undertook to examine the first step in this interaction, namely, the uptake of Rh-123 into the cell. Although cellular Rh-123 content can be monitored in terms of relative fluorescence by flow cytometry and microfluorimetry, it is difficult to make quantitative measurements using these techniques. We were especially interested in quantifying the uptake of Rh-123 in cultures of attached cells as a function of dose and exposure time. Here we report a reproducible procedure for measuring total cellular Rh-123 uptake using the butanol extraction method. These experiments included observations by fluorescence microscopy to monitor intracellular Rh-123 localization. The results suggest that Rh-123 uptake among various cell lines can differ both quantitatively and qualitatively and may in fact indicate differing mechanisms of uptake.

MATERIALS AND METHODS

Cells. Cell lines were obtained as follows: normal mink lung cell line (CCL64) and feline sarcoma virus transformant (64F3) were from M. Essex (Harvard School of Public Health); normal canine kidney epithelial line (MDCK); Ehrlich-Lettre mouse Ascites carcinoma line (CCL77), and human pancreatic carcinoma line (PaCa-2) were from the American Type Culture Collection (Rockville, MD); human bladder carcinoma (EJ) was provided by Dr. Ian Summerhayes (Dana Farber Cancer Institute, Boston, MA); and human breast carcinoma line (MCF-7) was from Dr. M. Rich (Michigan Cancer Foundation). Cells were grown in DME supplemented with either 5 or 10% calf serum (Grand Island Biological Co., Gaithersburg, MD).

Chemicals. Rh-123 was obtained from Eastman Organic Chemicals (Rochester, NY). A 10 mg/ml stock solution in dimethyl sulfoxide was kept in the dark at 4°C and used for the preparation of all solutions and standards used in this study. FCCP was obtained from Sigma Chemical Company (St. Louis, MO) and was stored at 4°C as a stock in dimethyl sulfoxide. Butanol (fluorometric analysis grade 1-butanol) was obtained from Fisher (Medford, MA).

Monitoring of Rhodamine 123 Uptake by Fluorescence Microscopy. Subconfluent cultures of cells on 12-mm round glass coverslips (Rochester Scientific, Rochester, NY) were incubated at 37°C in DME.
with 5% calf serum containing the desired concentration of Rh-123 (usually 1 μg/ml). A coverslip was removed and the cells were observed at hourly intervals. Coverslips were washed by dipping in three successive baths of fresh medium without Rh-123 for approximately 5 s per bath and were mounted on a drop of fresh medium on a live cell observation chamber as described (3). Epifluorescent microscopy and photography were performed essentially as described (3), except that a neutral density filter was used routinely in the excitation beam to reduce bleaching and other photo-induced effects during observation. A photographic record of relative differences in fluorescent intensity was made by manual exposure for 2 and 4 s of several representative fields without a neutral density filter, at hourly time points.

Monitoring of Rh-123 Uptake by Butanol Extraction. Cellular Rh-123 uptake is defined here as the amount of dye, calculated on a per cell basis, which is solubilized from cell cultures by butanol immediately after removal of dye-containing medium and rapid washes with dye-free medium. In a typical 5-h uptake experiment, forty 60-mm tissue culture dishes (Falcon, Cockeysville, MD) were evenly seeded with cells and grown to subconfluence. Prior to the experiment, 18 of the dishes were aspirated free of medium, wrapped in plastic, and placed at −20°C for 12–16 h. On the day of the experiment, these control dishes were allowed to thaw at room temperature for 10 min and then each received 3 ml of freshly prepared Rh-123 (1 μg/ml) in DME with 5% calf serum. At the same time, 18 dishes of live cells were aspirated free of medium and also received dye-containing medium. All dishes were incubated at 37°C. At hourly intervals over a 6-h period, three live-cell and three frozen-cell dishes were removed from the incubator and quickly washed (within 2 min) three times with 3 ml of phosphate-buffered saline (Grand Island Biological Co.), and then received 2.5 ml butanol. After 20 min at room temperature the butanol extracts were transferred to disposable cuvettes (Evergreen Scientific), sealed with Parafilm, and stored in the dark at 4°C. Samples were measured within 48 h against freshly prepared standards of Rh-123 (0–100 ng/ml) in butanol. Control experiments showed less than 1% variation during this storage interval. Measurements were made using a Hitachi Perkin-Elmer model MPF-2A fluorescence spectrophotometer with slit widths set at 10 nm, an excitation wavelength of 512 nm, and an emission wavelength of 532 nm.

The total uptake of Rh-123 per dish of live cells per time point was calculated from the butanol measurements as ng of Rh-123 per dish, and the values from the three live-cell dishes were averaged. Non-specific uptake was calculated for each time point in the same manner from the three frozen-cell butanol measurements. Specific Rh-123 uptake on a "per cell" basis was calculated by subtracting the non-specific uptake from the total uptake and dividing by the average number of cells per dish. Uptake was graphed as a function of time in Rh-123 (1 μg/ml).

The effect of the respiratory uncoupler FCCP on Rh-123 uptake was also examined. An uptake experiment was performed essentially as described above but with 18 additional live-cell dishes incubated in dye-containing medium plus 5 μM FCCP. Parallel coverslip cultures were also treated with FCCP.

Rh-123 uptake as a function of Rh-123 concentration was examined by incubating three live-cell and three frozen-cell dishes in the desired concentration of Rh-123 for 2 h at 37°C. Specific Rh-123 uptake per dish was plotted as a function of Rh-123 concentration.

For calculations of uptake of the dye on a per cell basis, the average cell number per dish was determined. Within 3 h of the start of each experiment, the cells in the four remaining untreated live-cell dishes were removed from the dish with trypsin-EDTA (Grand Island Biological Co.), and suspended in medium. Two samples were taken from each suspension, and two hemocytometer fields were counted for each sample (usually 50–100 cells per field). The four counts from each suspension were averaged, and these in turn were averaged for the four dishes, to give the average number of cells per dish.

RESULTS

Butanol Extraction of Cellular Rh-123. Fluorescent dyes are known to be sensitive to their surroundings. Fluorescence intensity can be affected by metal ions or dye concentration and by the polarity of the environment. pH levels may alter the chemical form of the dye (ionization, dimerization, etc.), and salts can cause spectral shifts. A red shift in the fluorescence emission of Rh-123 within the mitochondria of living cells has been reported (6). Rather than measure Rh-123 fluorescence within the cellular environment, butanol extraction was used to effect the rapid and efficient solubilization of Rh-123 from all cellular compartments. With this method, more than 95% of the dye is extracted from the culture dish. Prolonging the incubation time with butanol by 50% did not significantly increase the amount of dye extracted (data not shown). Solubilizing Rh-123 in butanol, which is a uniform, nonpolar solvent, negates possible microenvironmental effects on the excitation and emission spectra and fluorescence intensity. The fluorescence intensity of Rh-123 in butanol was linear with its concentration over the range measured in these experiments (0–150 ng/ml). The excitation and emission maxima were 512 and 532 nm, respectively; these values did not shift significantly over this concentration range.

Rh-123 uptake as measured by butanol extraction has two components, one nonspecific and one specific. Rh-123 seems to bind nonspecifically to some degree to both substratum and cell and this nonspecific binding is substantial for virgin tissue culture dishes or dishes which have contained complete medium but no cells. We therefore estimated the level of nonspecific binding in each butanol extraction experiment by using control dishes of cells which had been exposed to −20°C overnight. Epifluorescence microscopy of such frozen cells after staining with Rh-123 revealed very little fluorescence, except for occasional small bright dot-like structures. The binding of Rh-123 (1 μg/ml in culture medium) to these frozen cell control cultures was examined by butanol extraction; the results are shown by the dashed line in Chart 1. The amount of dye nonspecifically bound per dish reached a plateau within the first hour of continuous incubation, irrespective of cell line. In some cases, the washing procedure removed remnants of frozen cells from the control dishes, but the amount of Rh-123 extracted from these dishes did not differ significantly from control dishes in which cell loss did not occur. Variations in cell number per dish also had...
In contrast to nonspecific binding, the total uptake in experimental dishes is dependent on the particular cell line and the number of cells per dish. Chart 1 (solid lines) shows the total Rh-123 uptake by cultures of MCF-7 (human breast carcinoma) and 64F3 (feline sarcoma virus-transformed mink fibroblast) cells. The uptake kinetics of these two cell lines are quite dissimilar. The total uptake by MCF-7 initially shows a substantial increase and begins to plateau after about 5 h, whereas total uptake by 64F3 is minimal and parallels the kinetics of nonspecific binding. A comparison of the nonspecific uptake and total uptake illustrates the contribution of nonspecific binding to uncorrected total uptake measurements. Chart 1 shows that for incubation in Rh-123 (1 μg/ml), nonspecific binding reaches a plateau at approximately 25–35 ng per 60-mm dish. For the total uptake of MCF-7, which in the experiment of Chart 1 reaches 325 ng/dish, the nonspecific contribution is on the order of 10%. In contrast, the nonspecific level of dye uptake for 64F3 cells is about 50% of the total uptake. It is apparent that nonspecific uptake can make a large contribution to butanol extraction measurements of Rh-123 uptake. Charts 2 and 3 show data after this contribution was analyzed and deleted from total uptake to yield specific uptake values.

**Uptake of Rh-123 by Several Cell Types.** Chart 2 illustrates the dye uptake per cell over a 6-h period of continuous incubation with Rh-123 (1 μg/ml) for a number of cell lines. To account for the contribution of nonspecific binding, uptake values for the frozen-cell dishes were subtracted before the data were plotted. The cell lines that exhibited minimal uptake were normal epithelial- and mink fibroblast lines CV-1 (African green monkey kidney) and MDCK (canine kidney) and feline sarcoma virus-transduced mink fibroblast line 64F3. Even after 7 h of continuous incubation with Rh-123 (1 μg/ml), the average uptake for these cell lines reached a plateau within the first hour and did not exceed 2 × 10⁻⁵ ng per cell.

In contrast to CV-1, MDCK, and 64F3, some cell lines exhibited a substantial progressive accumulation of Rh-123. The cell line with the highest uptake was the Ehrlich-Lettre ascites tumor line (Chart 2). This cell line was unique in that the uptake of Rh-123 was initially very rapid, approaching a high plateau after only 3 h. The average value of 5 × 10⁻⁴ ng per cell at plateau is more than 20 times that of the CV-1, MDCK, or 64F3 lines. Two other lines which exhibited high levels of uptake were human bladder carcinoma-derived line EJ, and human pancreatic carcinoma-derived line PaCa-2. Moderate but significant Rh-123 accumulation was seen in normal mink fibroblast line CCL64, human breast carcinoma line MCF-7, and cervical carcinoma line HeLa. Despite variation in the absolute amount of uptake per cell in these cell lines, they share the common feature of continual dye uptake over time until a “saturation” cellular dye content is approached. This differentiates them from the minimal uptake CV-1, MDCK, and 64F3 lines, which appear to equilibrate rapidly with the dye and do not exhibit progressive dye uptake.

**Uptake of Rh-123 as a Function of Dye Concentration.** We investigated further whether the plateau levels of dye uptake exhibited by some cell types (for example, Ehrlich ascites tumor cells, Chart 2) reflect an absolute saturation amount of Rh-123 per cell. Chart 3 shows uptake by 64F3 and Ehrlich ascites tumor cell lines at various external Rh-123 concentrations. The nonspecific uptake per dish was directly proportional to the dye concentration (data not shown) and was subtracted from the total dye uptake before these data were plotted. The amount of dye accumulated in 2 h by 64F3 cells was very low but showed a slight dose-dependent increase over the entire range of 1–50 μg/ml of external Rh-123. 64F3 uptake appeared to be directly proportional to dose over this concentration range, suggesting that the early (within 1 h) plateau level of uptake shown in Chart 2 does not reflect a saturation level. In contrast, the accumulation of dye by Ehrlich ascites tumor cells increased substantially in proportion to concentration until about 10 μg/ml, where it remained level. Higher extracellular dye concentrations did not increase uptake in these cells, which suggested that a saturation dye content had been attained.

**Intracellular Rh-123 Localization.** Since the uptake results seemed to suggest that there is a qualitative as well as quantitative difference in the interaction of Rh-123 with low uptake and high uptake cell lines, cellular dye localization was examined by...
fluorescence microscopy at different incubation times. Fig. 1, a and b, shows fluorescence micrographs of MDCK (non-accumulating, low uptake) cells that had been continuously exposed to Rh-123 for 1 and 6 h, respectively. Representative fields were chosen, and identical exposure times were used. Both micrographs show a characteristic heterogeneity in the intensity of the fluorescence of the MDCK cells. Continuous exposure to Rh-123 (1 μg/ml) for 1 or 6 h did not result in a noticeable increase in fluorescence intensity or alteration in mitochondrial morphology (Fig. 1, a and b). Similar observations were made on the 64F3 and the CV-1 lines. Even after 32 h of continuous incubation in Rh-123 (1 μg/ml), no cytoplasmic fluorescence, alteration in mitochondrial morphology, or obvious increase in the number of more strongly stained cells was detected in MDCK cell cultures (not shown).

In contrast to cell lines with minimal Rh-123 uptake, cell lines which exhibited progressive Rh-123 accumulation generally did exhibit alterations after prolonged continuous exposure to the dye. After 1 h of continuous incubation in Rh-123 (1 μg/ml), EJ mitochondria were brightly stained and typically filamentous (Fig. 1c). Five h later fluorescence was detected in the cytoplasm, as well as in the mitochondria, and short “globular” mitochondria could be seen in many cells (Fig. 1d, arrowheads and arrows, respectively). Similar observations were made on all of the cell lines which exhibited progressive dye uptake.

Effect of FCCP on Rh-123 Uptake. Johnson et al. (4) observed, using fluorescence microscopy, that the accumulation of Rh-123 in living cells is affected by mitochondrial membrane potential. Therefore, we examined the uptake of Rh-123 in the presence and absence of the respiratory uncoupler FCCP, which is known to dissipate the mitochondrial membrane potential (14). As illustrated by Chart 4, in the absence of FCCP, HeLa cells exhibited a nearly linear uptake of the dye over a 6-h time period. However, addition of 5 μM FCCP to the Rh-123 medium greatly reduced the accumulation of the dye by these cells. At any given point, FCCP reduced uptake by a factor of 6–8. This observation is consistent with the hypothesis that mitochondria play an important role in Rh-123 uptake in living cells.

To investigate whether or not FCCP has a corresponding effect on mitochondrial fluorescence and morphology, fluorescence observations were made during the uptake experiments. After 1 h in medium containing Rh-123 (1 μg/ml) alone, HeLa cell mitochondria were bright, filamentous structures (Fig. 2a). After 6 h the mitochondria were slightly swollen and highly fluorescent, and low-level cytoplasmic staining had appeared (Fig. 2b, arrows). In contrast, HeLa cells exposed to both Rh-123 (1 μg/ml) and 5 μM FCCP for 1 h typically had dimly fluorescent, fragmented mitochondria (Fig. 2c), which after 6 h were only slightly brighter, with some cytoplasmic fluorescence (Fig. 2d). These fluorescence observations demonstrate an effect of FCCP on mitochondria and correlate with the observed reduction in dye uptake.

DISCUSSION

Previous attempts to quantify Rh-123 uptake have included visual estimation of dye binding by fluorescence microscopy and the more quantitative method of flow cytometry (5–8). Fluorescence microscopy is an invaluable tool for comparing individual cells within a culture in terms of relative fluorescence and dye localization. However, quantification of dye binding by fluorescence microscopy requires supplementation with microspectrofluorometry or densitometry on carefully obtained photographic images. Flow cytometry is useful for the quantification of dye binding per cell for large populations of cells but requires that the cultures be in suspension. Attached cells cannot be studied without introducing possible artifacts due to their removal from the substratum. Fluorescence microscopy and flow cytometry measure the fluorescence of the dye within different cellular microenvironments, in which the fluorescence intensity and spectra of a fluorochrome can be affected by complex formation, pH, lipid solubility, etc. These factors can make precise quantitation of dye binding by these methods difficult. The butanol extraction method circumvents these factors by solubilizing the dye before quantification by fluorometry. Butanol extractions can be performed on attached cultures, suspension cultures, and whole tissues.

Butanol extraction of attached cell cultures solubilizes the Rh-123 bound nonspecifically to the substratum in addition to that taken up by live cells. The amount of nonspecific binding is minor compared with the total Rh-123 accumulation by carcinoma cell lines such as Ehrlich ascites, EJ, PaCa-2, MCF-7, and HeLa. However, for cell lines with low Rh-123 uptake, such as CV-1, MDCK, or 64F3, the proportion of nonspecific binding becomes significant in comparison to the total extractable dye (Chart 1). It is essential therefore to develop a reliable method to estimate the level of nonspecific binding. The freezing of attached cells on culture dishes at −20°C for 12–16 h gave highly reproducible results for nonspecific binding.

Striking dissimilarities in the amounts of uptake over time were seen among different cell lines after incubation in Rh-123 (1 μg/ml) (Chart 2). Carcinoma-derived cells (MCF-7, PaCa-2, EJ, HeLa) took up substantial amounts of this dye, whereas normal epithelial-derived cells (CV-1, MDCK) took up minimal amounts. Dye uptake by normal fibroblasts (CCL 64) was intermediate but was significantly higher than dye uptake by transformed fibroblasts (64F3). The mouse Ehrlich-Lettre ascites tumor line (CCL 77) exhibited the highest uptake. All of these lines showed progressive dye accumulation with time. In contrast, several lines (normal epithelial-derived CV-1 and MDCK lines; transformed fibroblast line 64F3) took up very little dye initially and then appeared to
reach a plateau despite prolonged exposure to Rh-123. These results suggest that cell types can be divided into two main categories, those which progressively accumulate Rh-123 ("high uptake") and those which do not ("low uptake").

Chart 2 also shows that several high uptake lines (e.g., carcinoma-derived lines EJ, PaCa-2) exhibited a fairly linear increase in uptake over the first 6 h of incubation, whereas others (especially Ehrlich ascites tumor cells but also carcinoma-derived MCF-7 and HeLa and fibroblast line CCL 64) appeared to approach plateau values of uptake after 3 h or more. These data suggest that a saturation value for each cell type exists beyond which the mechanism responsible for progressive dye uptake does not operate. The experiments illustrated in Chart 3 examine this possibility for Ehrlich ascites tumor cells. For 2-h incubation periods, uptake by Ehrlich ascites tumor cells is proportional to extracellular dye concentrations up to extracellular Rh-123 concentrations of 10 µg/ml. Higher concentrations do not increase dye accumulation. The low uptake cell lines 64F3, CV-1, and MDCK also reach plateau values of uptake (Chart 2), but the minimal levels of the plateau values suggest that they do not represent saturation. Additionally, Chart 3 shows that 64F3 cells, unlike Ehrlich ascites tumor cells, exhibit a linear dose-dependent increase in uptake. These data suggest that the plateau values for the low and high uptake cell lines (Chart 2) may be determined by alternate mechanisms.

Fluorescence observations showed another dissimilarity between the high and the low uptake lines. All of the cell lines that exhibited progressive Rh-123 uptake also eventually developed altered mitochondrial morphology and cytoplasmic fluorescence, usually within 6 h (Figs. 1d and 2b). The low uptake lines did not exhibit these alterations, even with 32 h continuous exposure to Rh-123 (MDCK cells; data not shown). This is a probable consequence of the high intracellular concentrations of Rh-123 attained by high uptake cell lines. These changes may indicate initial cellular alterations which result in Rh-123 toxicity for carcinoma-derived cells but not for normal epithelial-derived cells (12).

Increased uptake of Rh-123 by carcinoma cells may partially explain the observation that carcinoma cells generally show significantly longer retention of Rh-123 than do normal epithelial cells (10). The correlation, however, is not absolute for all cell types. For example, the high uptake lines Ehrlich ascites, EJ, PaCa-2, MCF-7, CCL 64, and HeLa (Chart 2) exhibit Rh-123 retention of 60, 90, 90, 95, 40, and 70%, respectively, after 24 h in dye-free medium (10). In addition, the normal rat lung fibroblast line CCL 149 exhibits Rh-123 uptake comparable to that of EJ (data not shown) yet shows 0% retention after 24 h (10). Uptake is not therefore the single factor involved in retention, at least for cells of nonepithelial origin.

The results presented here document major differences in Rh-123 uptake among different cell lines. The mechanism responsible for uptake remains to be determined. However, the data presented here that show that the respiratory uncoupler FCCP decreases uptake (Chart 4) indicate a significant role of the mitochondrial membrane potential. The differences in uptake between carcinoma-derived cells and normal epithelial-derived cells may be related to differences in their mitochondrial membrane potentials.

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REFERENCES

Fig. 1. Fluorescence microscopy of a low uptake cell line (MDCK; a and b) and a high uptake cell line (EJ; c and d) after 1 h (a and c) and 6 h (b and d) of incubation in Rh-123 (1 µg/ml) in culture medium. Arrowheads indicate cells with cytoplasmic fluorescence; arrows indicate mitochondria with altered morphology. Bar, 20 µm.
Fig. 2. Fluorescence micrographs of HeLa cells incubated in Rh-123 (1 µg/ml) in the absence (a and b) or presence (c and d) of 5 µM FCCP for 1 h (a and c) or 6 h (b and d). Arrows indicate mitochondria with altered morphology. Bar, 20 µm.
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