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Unusual retention of rhodamine 123 by mitochondria in muscle and carcinoma cells

(membrane potential/carcinogenesis/diagnosis/chemotherapy)

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ABSTRACT **Mitochondria in cardiac muscle cells and myoblast-fused myotubes display unusually long (3–5 days) retention times of rhodamine 123, a mitochondria-specific fluorescent probe, in living cells. Among 50 keratin-positive carcinoma or transformed epithelial cell lines tested, mitochondria with prolonged rhodamine 123 retention are detected in most of the transitional cell carcinoma, adenocarcinoma, and chemical carcinogen-transformed epithelial cell lines and in some squamous cell carcinoma lines but not in any oat cell carcinoma lines. The presence of mitochondria having unusual dye retention may be useful for diagnosis and exploitable for chemotherapy of certain human carcinomas.**

The use of rhodamine and cyanine dyes in localizing mitochondria in living cells by epifluorescence microscopy has been described (1–5). The remarkable specificity appears to result from the high membrane potential (negative inside) across mitochondria on the one hand and the net positive charge carried by these dyes at physiological pH on the other. These mitochondrial supravital dyes have been used in a number of studies, including monitoring mitochondrial membrane potential in living cells (2), comparing normal and transformed fibroblasts for dye accumulation (3), assessing the efficacy of chemotherapy (4), detecting mitochondria fragmentation (5), monitoring mitochondrial proliferation during the cell cycle (6) and lymphoblast transformation (7), measuring differences in dye uptake between growing and resting fibroblasts (8) and between young and aged fibroblasts (9), following fusion between karyoplasts and cytoplasts (10), selection of cells having mutations in mitochondria (11), destruction of a single mitochondrion in conjunction with laser beam (12), monitoring the viability of cells (13), and probing the role of mitochondria in erythroleukemia cell differentiation (14).

Here, we report the presence of mitochondria that have unusual rhodamine 123 retention times, much longer than would be predicted from dye reequilibration by diffusion, in cardiac muscle cells, myotubes, and some carcinoma cells.

MATERIALS AND METHODS

Cell Cultures. All cell types and cell lines, unless otherwise specified, were grown in Dulbecco's modified Eagle's medium (GIBCO)/10% calf serum (MA Bioproducts, Walkersville, MD) at 37°C in 5% CO_2/95% air and 100% relative humidity. The following cell lines were obtained from the American Type Culture Collection: CCL 51, CCL 64, CCL 77, CCL 146, CCL 149, Pt K1, Pt K2, CV-1, BS-C-1, Vero, MDCK, SW-480, HeLa, Ca Ski, PaCa-2, PC-3, A549, and SW-13. Human bladder carcinoma lines, mouse bladder epithelial cells, 7,12-dimethylbenz[*a*]anthracene-transformed mouse bladder epithelial cells, rabbit bladder epithelial cells, and benzo[*a*]pyrene-transformed rabbit bladder epithelial cells were as described (15, 16). Butyl(4-hydroxybutyl)nitrosamine-induced mouse bladder carcinoma lines (SH257, SH264, BBN-6) will be described elsewhere. Human breast carcinoma line MCF-7 was from M. Rich (Michigan Cancer Foundation), and lines ZR-75-1, T47-D, BT-20, and HBL-100 were from C. V. Piczak (National Cancer Institute). Human squamous cell carcinoma lines, SCC-4, SCC-9, SCC-12, SCC-13, SCC-15, SCC-25, and SCC-27 (17) and human epidermal cells (18) were from J. G. Rheinwald (Sidney Farber Cancer Institute). Human ovarian carcinoma line OVCA 433 was from H. Lazarus (Sidney Farber Cancer Institute) and line OD562 was from the Dept. of Radiopathology, Institut Curie, Paris. Human lung carcinoma line OH-1 was from S. Baylin (Johns Hopkins Hospital) and lines HUT23, HUT125, U1752, HUT157, LX-1, HUT60, HUT128, HUT231, and HUT64 were from A. Gazdar (National Cancer Institute). Human vulva epidermoid carcinoma line A431 was from J. A. Cooper and T. Hunter (Salk Institute). Human neuroblastoma lines CHP 100, CHP 134, and CHP 212 were from A. Schlesinger (Children's Hospital, Philadelphia). Human leukemia and lymphoma lines HL60, U937, K562, KG1, MOLT-4, LA2-156, LA2-22, and CEM were from H. Lazarus (Sidney Farber Cancer Institute).

Primary cardiac muscle cells from neonatal rat were prepared according to Lampidis and Schaiberger (19). Myoblasts/myotubes from chicken embryo and neonatal rat were prepared according to Konigsberg (20) and Yaffe (21), respectively. Chicken embryo fibroblasts were prepared according to Rein and Rubin (22). Human foreskin fibroblasts were prepared by the same procedures. Rat ovary, rat kidney, and mouse kidney epithelial cells were from explant outgrowths prepared by procedures similar to those used for bladder epithelial cells (13). Human endothelial and mast cells and bovine endothelial cells were from B. R. Zetter (Children's Hospital Medical Center, Boston). Mouse macrophages and monocytes were from K. Ho and D. Beller (Harvard Medical School). Human monocytes were from J. Griffin (Sidney Farber Cancer Institute). Human and mouse lymphocytes were from E. L. Reinherz, S. Schlossman, and H. Cantor (Sidney Farber Cancer Institute). Human breast epithelial cells were from M. R. Stampfer (Peralta Cancer Research Institute) and N. S. Yang (Michigan Cancer Foundation). Human embryonic kidney epithelial cells were from J. Ritz (Sidney Farber Cancer Institute). 3T3, SV-3T3, and Py-3T3 were from H. Green (Harvard Medical School). Rat-1 and BHK were from W. Topp (Cold Spring Harbor Laboratory). NIH/3T3-SRD was from G. M. Cooper (Sidney Farber Cancer Institute). 64F3 was from M. Essex (Harvard School of Public Health). THE, T8, and 333-8-9 were from J. K. McDougall

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(Fred Hutchinson Cancer Center). KiSV-3T3 was from E. M. Scolnick (National Cancer Institute). Ann-1 was from D. Baltimore (MIT). C3H/10T1/2 and x-ray-transformed C3H/10T1/2 were from D. Brouty-Boye (Institut de Recherches Scientifiques sur le Cancer, Villejuif Cedex, France). DMBA-BALB/3T3 was from J. A. DiPaolo (National Cancer Institute).

Monitoring Mitochondrial Retention of Rhodamine 123. Living cells grown on 12-mm round coverslips were exposed to rhodamine 123 (10 μg/ml; Eastman Organic Chemicals) for 10 min. Then, the coverslips were extensively washed and mounted on silicon rubber chambers containing dye-free medium as described (1). Cultured medium in the chamber was changed daily for long-term experiments. Before fluorescence measurement, the coverslips were rinsed and mounted in fresh medium. Fluorescence in 100 randomly chosen 625-μm^2 areas that have mitochondria was measured by using a Nanospec-10S

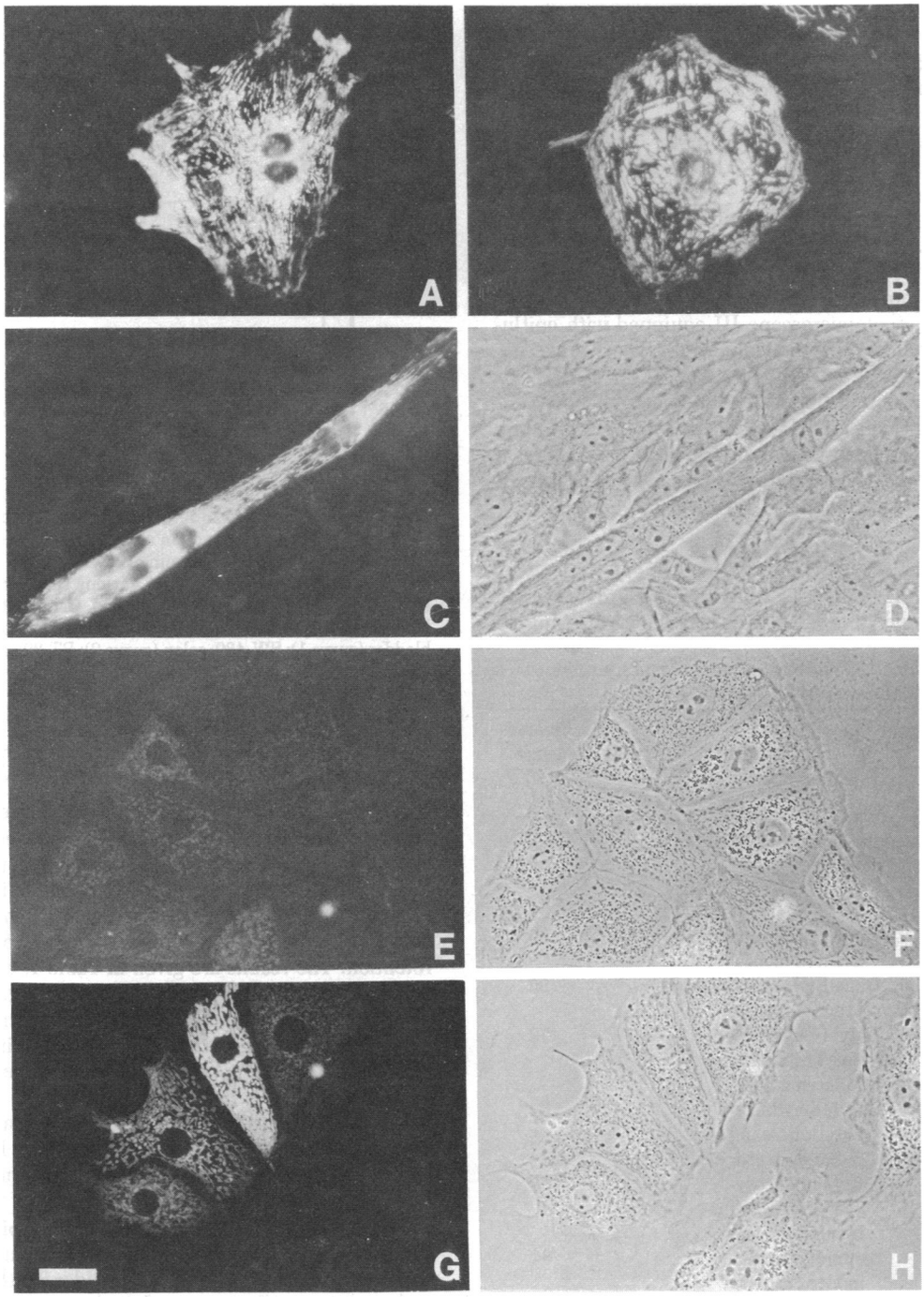

FIG. 1. Fluorescence (exposure time, 2 sec) and phase-contrast micrographs showing rhodamine 123 retention by various cell types. (*A*) Rat cardiac muscle cell stained with rhodamine 123 (10 μg/ml) for 10 min and visualized immediately after washing. (*B*) Rat cardiac muscle cell stained with rhodamine 123 and kept in dye-free medium for 3 days before visualization. (*C*) Rat myoblasts and a myotube stained with rhodamine 123 and kept in dye-free medium for 1 day before visualization. (Only the myotube retained rhodamine 123.) Fluorescence intensity in the myotube was approximately the same after 3 days. (*E*) Rat adult ovary epithelial cells stained with rhodamine 123 and kept in dye-free medium for 16 hr before visualization. (*G*) Primary human embryonic kidney epithelial cells treated the same as *E*. (*D*, *F*, and *H*) Phase-contrast images corresponding to *C*, *E*, and *G*, respectively. Bar = 50 μm.

Table 1. Rhodamine 123 retention of various normal cell types after 24 hr in dye-free medium

Cell type	Source	Fluorescence
Cardiac muscle	Neonatal rat	100
Myotube	Neonatal rat	100
Myotube	Chicken embryo	100
Fibroblast	Human foreskin	23
Fibroblast	Neonatal rat	10
Fibroblast	Chicken embryo	5
Endothelial	Human aorta	4
Endothelial	Bovine aorta	4
Lymphocyte (T and B)	Human	0
Lymphocyte (T and B)	Mouse	0
Monocyte	Human	0
Monocyte	Mouse	0
Macrophage	Mouse	10
Mast	Human	2
Epithelial	Rat ovary (adult)	5
Epithelial	Rat intestine (adult)	10

microspectrophotometer (Nanometric, Sunnyvale, CA) attached on a Zeiss photomicroscope III equipped with epifluorescent optics (Planapo objective lens, ×63). The average fluorescence of rat cardiac muscle cells after 24 hr in dye-free medium was taken as 100. Butanol extraction of mitochondria-associated dye and fluorescence measurement by Perkin–Elmer MPF-4 spectrophotometer were as described (3). Fluorescence micrographs were made according to Johnson *et al.* (1) except that a constant exposure time of 2 sec was used.

RESULTS

Mitochondrial Rhodamine 123 Retention by Cardiac Muscle Cells, Myotubes, and Other Cell Types. When cardiac muscle cells stained with rhodamine 123 (10 μg/ml for 10 min) were kept in dye-free medium for 3 days, a significant level of mitochondrial fluorescence was still detected (Fig. 1 *A* and *B*). Myotubes generated by fusion of myogenic cells from neonatal rat skeletal muscle in cultures retain rhodamine 123 at a level comparable with that of cardiac muscle cells (Fig. 1 *C* and *D*). We have thus far screened fibroblasts, epithelial cells, endothelial cells, epidermal cells, macrophages, lymphocytes, monocytes, and mast cells from various sources for rhodamine 123 retention. The results are given in Table 1, and an example of rat adult ovary epithelial cells is shown in Fig. 1 *E* and *F*. After 16 hr in dye-free medium, human, mouse, and rat embryonic kidney cells show a great variation in rhodamine 123 retention. Although many of the cells lose fluorescence in the course of 16 hr, some retain a high level of rhodamine 123, as shown in Fig. 1 *G* and *H*. Bladder epithelial cells from adult mice, which have the lowest capacity to retain rhodamine 123, were chosen for studying the effect of oncogenic transformation on rhodamine 123 retention in epithelial cells.

Table 2. Rhodamine 123 retention of normal and transformed bladder epithelial cells after 24 hr in dye-free medium

Cell type or line	Comment	Fluorescence
Primary bladder epithelial		
Mouse		0
Rabbit		0
Carcinogen transformed		
MB48	Dimethylbenzanthracene-induced mouse	90
MB49	Dimethylbenzanthracene-induced mouse	90
BBN-6	Butyl-(4-hydroxybutyl)nitrosamine-induced mouse	70
SH264	Butyl-(4-hydroxybutyl)nitrosamine-induced mouse	62
SH257	Butyl-(4-hydroxybutyl)nitrosamine-induced mouse	0
RBC-1	Benzo[*a*]pyrene-induced rabbit	84
Human bladder tumor		
EJ	Transitional cell carcinoma	90
J82	Transitional cell carcinoma	15
RT4	Transitional cell carcinoma	70
RT112	Transitional cell carcinoma	75
HT-1376	Transitional cell carcinoma	93

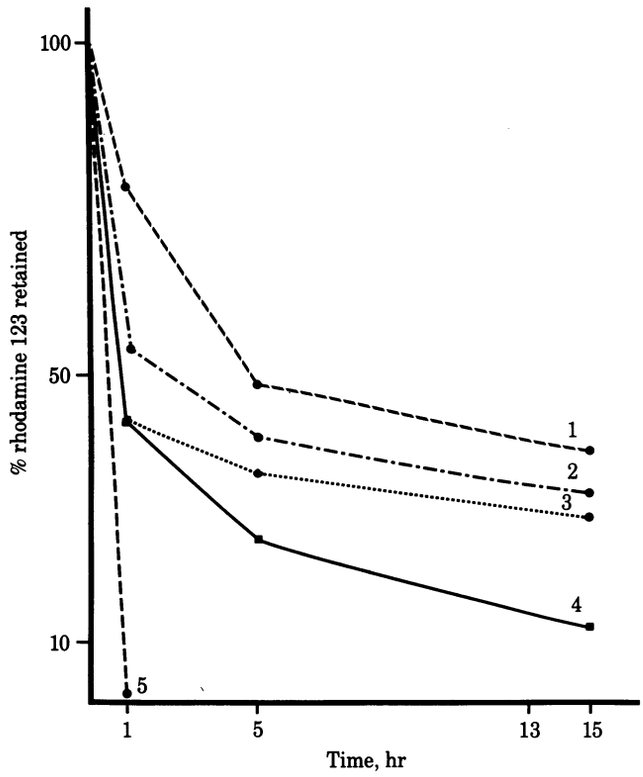

FIG. 2. Rhodamine 123 retention (percentage of initial uptake) as measured by butanol extraction and fluorescence spectrophotometry. Cells were stained with rhodamine 123 (10 μg/ml) for 10 min, washed, and kept in dye-free medium. At various times, rhodamine 123 was extracted with butanol and quantitated. Cells tested included mouse bladder epithelial cells (curve 5) and human carcinoma lines [HT-1376, bladder (curve 1); SW-480, colon (curve 2); BT-20, breast (curve 3); and A549, lung (curve 4)].

Rhodamine 123 Retention by Bladder Epithelial Cells and Transformed Derivatives. We have previously shown that a confluent monolayer of mouse bladder epithelial cells accumulates very small amounts of rhodamine 123 (2). Cells at the periphery of bladder epithelial outgrowths and along the edge of a wound within an epithelial sheet accumulate rhodamine 123 more than cells in other regions (2). However, even those brightly stained cells release rhodamine 123 within 2 hr. In contrast, several dimethylbenzanthracene-transformed mouse bladder epithelial cells, benzo[*a*]pyrene-transformed rabbit

Table 3. Rhodamine 123 retention of untransformed and transformed established cell lines after 24 hr in dye-free medium

Cell line	Source	Fluorescence
Untransformed epithelial		
Pt K1	Kangaroo rat	0
Pt K2	Kangaroo rat	0
CV-1	Monkey kidney	0
BS-C-1	Monkey kidney	0
Vero	Monkey kidney	0
MDCK	Canine kidney	5
Untransformed fibroblast		
3T3	Mouse embryo	18
BALB/3T3	Mouse embryo	5
NIH/3T3	Mouse embryo	10
BHK	Hamster kidney	15
Rat-1	Rat embryo	15
CCL 149	Rat lung	0
CCL 64	Mink embryo	40
C3H/10T1/2	Mouse embryo	12
Transformed epithelial		
THE	Simian virus 40-transformed hamster	80
Ehrlich ascites	Mouse	60
CCL 51	Mouse (mammary)	60
Lewis lung	Mouse	0
Transformed fibroblast		
SV-3T3	Simian virus 40-transformed 3T3	10
Py-3T3	Polyoma-transformed 3T3	10
NIH/3T3-SRD	Rous sarcoma virus-transformed 3T3	3
AnAn	Rous sarcoma virus-transformed rat	5
64F3	Feline sarcoma virus-transformed CCL 64	0
T8	Adenovirus serotype 2-transformed rat	0
KiSV-3T3	Kirsten sarcoma virus-transformed 3T3	10
333-8-9	Herpes–hamster	15
Ann-1	Abelson murine leukemia virus-transformed 3T3	10
DMBA-BALB/3T3	Dimethylbenzanthracene-transformed	12
X-ray-C3H/10T1/2	X-ray-transformed	18
CCL 146	Gerbil fibroma	0
SV-BHK	Simian virus 40-transformed hamster	15
Osteosarcoma		
N377/H135	Human lung metastasis	10
Neuroblastoma	Human	
CHP 100		0
CHP 134		0
CHP 212		0
Leukemia and lymphoma	Human	
HL60		0
U937		0
K562		0
KG1		0
MOLT-4		0
LA2-221		0
CEM		0
LA2-156		0

bladder epithelial cells, and butyl-(4-hydroxybutyl)nitrosamine-induced mouse bladder transitional cell carcinoma retained a significant level of rhodamine 123 in dye-free medium even after 24 hr. Some of these tumorigenic cell lines (e.g., MB48, MB49, and RBC-1) still retain brightly stained mitochondria 4 days after incubation in dye-free medium, similar to the mitochondria of muscle cells. Most tumorigenic bladder epithelial cell lines tested retained mitochondrial fluorescence in dye-free medium for a prolonged period of time as assessed by fluorescence microscopy (Table 2). One butyl-(4-hydroxybutyl)nitrosamine-induced mouse bladder carcinoma line, SH257, is a notable exception in having a short retention time.

Rhodamine 123 Retention by Human Bladder Carcinoma Cells. To examine whether extraordinary rhodamine 123 retention by mitochondria is also a characteristic of human bladder carcinoma cells, five cell lines, EJ, J82, RT4, RT112, and HT-1376, were analyzed for dye retention. Four of them, all but J82, retained mitochondria-associated rhodamine 123 for a prolonged period, similar to that observed with carcinogen-transformed mouse bladder epithelial cells (Fig. 2 and Table 2). Unfortunately, no cell line or primary culture derived from normal human bladder is available for comparison.

Rhodamine 123 Retention by Other Carcinoma Cells and Nontumorigenic Epithelial Cell Lines. In view of the difference in rhodamine 123 retention between normal mouse bladder epithelial cells and their tumorigenic counterparts, a variety of epithelial cell lines derived from kidney, ovary, pancreas, lung, adrenal cortex, skin, tongue, breast, prostate, cervix, vulva, and colon were examined for rhodamine 123 retention. All of these cell lines, verified to be of epithelial origin by keratin staining,

Table 4. Rhodamine 123 retention of human carcinoma lines after 24 hr in dye-free medium

Carcinoma	Type	Fluorescence
MCF-7	Breast (adeno-)	95
T47-D	Breast (adeno-)	95
ZR-75-1	Breast (adeno-)	80
BT-20	Breast (adeno-)	80
HBL-100	Breast (adeno-)	80
SW-480	Colon (adeno-)	80
HeLa	Cervix (adeno-)	70
Ca Ski	Cervix (epidermoid)	90
A431	Vulva (epidermoid)	85
PaCa-2	Pancreas	90
PC-3	Prostate	90
SW-13	Adrenal cortex (adeno-)	90
OD562	Ovary	80
OVCA 433	Ovary	70
SCC-4	Tongue (squamous)	60
SCC-9	Tongue (squamous)	65
SCC-12	Skin (squamous)	60
SCC-13	Skin (squamous)	50
SCC-15	Tongue (squamous)	40
SCC-25	Tongue (squamous)	20
SCC-27	Skin (squamous)	50
HUT23	Lung (adeno-)	75
HUT125	Lung (adeno-)	60
A549	Lung (adeno-)	60
U1752	Lung (squamous)	60
HUT157	Lung (large cell)	0
LX-1	Lung (poorly differentiated)	0
OH-1	Lung (oat cell)	0
HUT60	Lung (oat cell)	0
HUT128	Lung (oat cell)	0
HUT231	Lung (oat cell)	0
HUT64	Lung (oat cell)	0

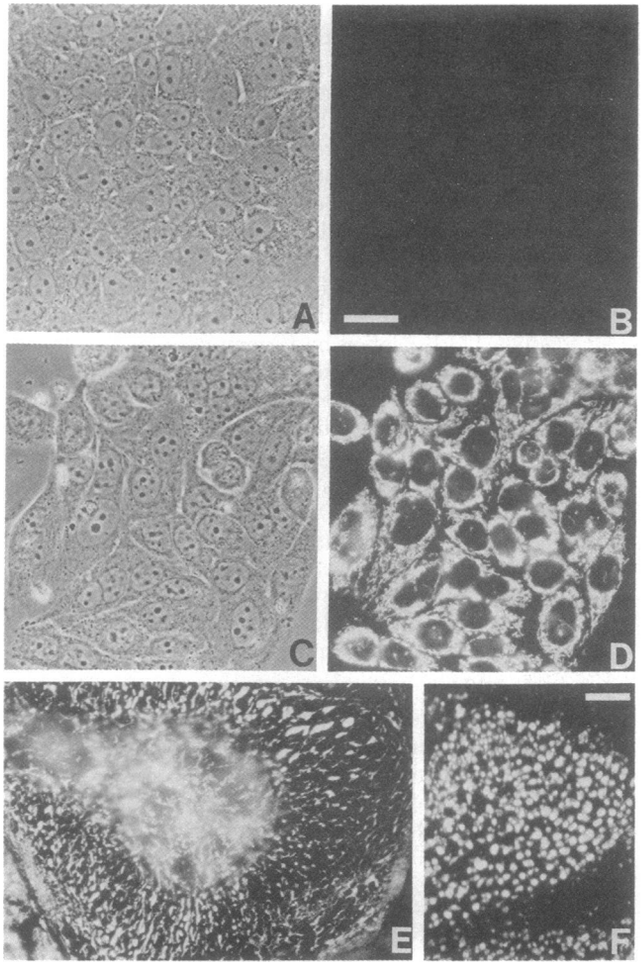

FIG. 3. Fluorescence (exposure time, 2 sec) and phase-contrast micrographs showing rhodamine 123 retention by normal and carcinoma cells. Cells were stained with rhodamine 123 (10 μg/ml) for 10 min, washed, and kept in dye-free medium for 2 days. (*B*) Tertiary human breast epithelial cells. (*D*) Human breast carcinoma, MCF-7. (*E*) Human ovarian carcinoma, OVCA 433. (*F*) Human cervical carcinoma, Ca Ski. (*A* and *C*) Phase-contrast images corresponding to *B* and *D*, respectively. (*B* and *D*) Bars = 80 μm. (*E* and *F*) Bars = 30 μm.

were assayed for rhodamine 123 retention by fluorescence microscopy (Tables 3 and 4), and a few of them were further analyzed for dye retention by butanol extraction (Fig. 2). With several exceptions, the overall results show a remarkable pattern of short rhodamine 123 retention times in nontumorigenic epithelial cell lines and cell lines established from normal epithelium, while many carcinoma-derived cells had characteristically long rhodamine 123 retention times. The most significant exceptions are oat cell (five human lines and one mouse line, Lewis lung) and large cell carcinomas, which display short rhodamine 123 retention times.

Many cells having long retention times—for example, human breast (MCF-7), cervical (Ca Ski), and ovarian (OVCA 433) carcinoma cell lines—display unusual mitochondrial morphology (Fig. 3), some of it reminiscent of the cardiac muscle cell mitochondria reported previously (2) and those shown in Fig. 1.

Unusual dye retention shown by mitochondria of muscle and some carcinoma cells has thus far rarely been detected in established fibroblastic lines, transformed fibroblasts, sarcomas, leukemias, lymphomas, neuroblastomas, and osteosarcomas (Tables 3 and 4). The notable exception has been the mink fibroblastic line, CCL 64 (Table 3).

DISCUSSION

The molecular basis for the extended retention of rhodamine 123 in muscle and carcinoma mitochondria remains to be determined. Irrespective of the mechanism responsible for extended rhodamine 123 retention, it is of interest that normal mouse bladder epithelial cells differed significantly from their carcinogen-transformed derivatives in retention time. That three different carcinogens—dimethylbenzanthracene, butyl-(4-hydroxybutyl)nitrosamine, and benzo[*a*]pyrene all lead to a similar alteration suggests that the long retention of rhodamine 123 may result from a high probability event accompanying oncogenic transformation in bladder epithelial cells. However, the identification of one tumorigenic butyl-(4-hydroxybutyl)-nitrosamine-induced mouse bladder carcinoma line without longer retention of rhodamine 123 suggests that the acquisition of such a phenotype is neither necessary nor sufficient for oncogenic transformation of bladder epithelial cells. Thus, we wish to emphasize that we are not reporting a phenotype that is essential for neoplasia.

That rhodamine 123 is retained much longer by many carcinoma cells provides an opportunity for diagnosis as well as chemotherapy. It would be of interest if carcinoma cells could be eradicated selectively by a mitochondrial poison that is selectively retained by carcinoma mitochondria, like rhodamine 123.

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