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Reversal of Resistance to Rhodamine 123 in Adriamycin-resistant Friend Leukemia Cells[1]

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ABSTRACT

Pleiotropic resistance to rhodamine 123 (Rho-123) in Adriamycin (ADM)-resistant Friend leukemia cells was circumvented by cotreatment with 10 μM verapamil. Increased cytotoxicity corresponded to higher intracellular Rho-123 levels. The verapamil-induced increase of drug accumulation in resistant cells is accounted for at least in part by the blockage or slowing of Rho-123 efflux from these cells. In contrast, accumulation and consequent cytotoxicity of Rho-123 in sensitive cells are not increased by verapamil. Similar results were obtained when ADM was used in this cell system. These results suggest that the efflux system for Rho-123 and ADM in sensitive cells is either reduced or absent.

Although Rho-123 accumulates specifically in mitochondria and ADM mainly in the nucleus, the loss of these two different classes of compounds from resistant cells appears to occur via a similar or common mechanism. The similarities in drug transport between Rho-123 and ADM may have important implications when applied to an *in vivo* environment.

INTRODUCTION

Pleiotropic drug resistance has been described in cell lines resistant to ADM[3] and DNR (3, 6, 22). We have reported previously that Friend leukemia cell variants resistant to ADM and DNR are cross-resistant to Rho-123 (16, 26) a drug which localizes specifically in mitochondria and is selectively toxic to carcinoma cells *in vitro* (2, 14, 15) and *in vivo* (1, 15). The chemical structure of Rho-123 as well as its cellular localization and biochemical effects (17) differ significantly from the anthracyclines to which it is cross-resistant. However, the similarities of decreased retention in resistant cells between these 2 different classes of compounds provides an opportunity to further explore drug transport as it relates to mechanisms of resistance.

Resistance to ADM or DNR has been shown to be reversed by calcium transport and calmodulin inhibitors (20, 21, 28). The mechanism is thought to involve the inactivation of an active efflux mechanism operating in resistant cells (6, 9, 21, 23). Presumably, by blocking this system, enough anthracycline can accumulate in resistant cells to render them sensitive. We have reported previously that Rho-123 is retained for significantly longer periods in ADM-sensitive as compared to ADM-resistant cell types, which correlates with their respective sensitivities to this agent (16). In the present report, we examine the effects of the calcium transport inhibitor, verapamil, on the cellular accumulation and retention of Rho-123 and ADM as it relates to resistance in these cell variants.

MATERIALS AND METHODS

Cells and Cytotoxicity Assay. FLC were derived from a clone of Friend virus-tranformed 745A cells. Cells were seeded and grown as described previously (8). The cell variant resistant to ADM (ADM-RFLC3) was derived from the above clone by continuous exposure to ADM (8). Resistant cells, grown for more than 150 passages in drug-free medium, maintained their resistance to ADM. To assay for cytotoxicity, exponentially growing cells sensitive and resistant to ADM were treated continuously with increasing concentrations of Rho-123 or ADM in the presence or absence of 10 μM verapamil. At 72 h, cells excluding trypan blue were counted, and survival curves were derived as described previously (8).

Fluorescence Microscopy. Sensitive and resistant cells at 10^6 cells/ml were treated with Rho-123, 10 μg/ml, for 15 min in the presence or absence of 10 μM verapamil. To stop Rho-123 uptake, a 1-ml aliquot of cells was diluted in 10 ml of Rho-123-free medium, either with or without verapamil, and centrifuged at 1000 rpm for 10 min. Pellets were resuspended in 5 ml of the appropriate medium, and droplets of this suspension were placed on slides and mounted with glass coverslips. Epifluorescence was monitored with excitation from a mercury HBO 100-watt bulb using an excitation filter (IF-490), blue/green dichroic mirror, and a 0.515 barrier filter combination on a phase-contrast Olympus BH-2 microscope equipped with a photomicrographic system.

Laser-excited Flow Cytometry. Procedures already described for laser excitation and quantitation of interacellular anthracycline fluorescence were used for both Rho-123 and ADM studies (12, 13). Two ml of 10^6 cells/ml were treated with Rho-123, 5 μg/ml, in the presence or absence of 10 μM verapamil for 10 min and resuspended in 10 ml of Rho-123-free medium, with and without verapamil, respectively. The same procedure was followed for ADM assays except that drug, 2 μg/ml, was applied for 2 h. Cells were centrifuged at 1000 rpm for 5 min, and pellets were resuspended in 2 ml of the appropriate Rho-123 or ADM-free medium and analyzed in a Coulter Electronics Epics V cell sorter using 500 megawatts power output at 488 nm of an argon ion laser either immediately or after 20 min of incubation for Rho-123 and 2 h for ADM. Data on the 90°-light scatter and fluorescence (above 530 nm) were collected in a Coulter electronics MDADS system. Details of our instrumentation and procedures have been described earlier (11).

Chemicals. Rho-123 was obtained from Eastman Kodak Co. (Rochester, NY), ADM was from Adria Laboratories, Inc. (Columbus, OH), and verapamil was from Biosedra Laboratories (Malakoff, France).

RESULTS

Reversal of Resistance to Rho-123 by Verapamil. Sensitive (FLC) and resistant (ADM-RFLC3) cells were treated continuously with Rho-123 in the presence or absence of 10 μM of verapamil. Cells normally resistant to Rho-123, 30 μg/ml, become sensitive to Rho-123, 1.5 μg/ml (ID_{50}) in the presence of verapamil. In contrast, the cytotoxicity of Rho-123 in cells normally

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[3] The abbreviations used are: ADM, Adriamycin; Rho-123, rhodamine 123; DNR, daunorubicin; FLC, Friend leukemia cells; ADM-RFLC3, Friend leukemia cells resistant to Adriamycin; ID_{50}, dose causing 50% growth inhibition.

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sensitive to the dye, 0.1 μg/ml (ID_{50}), is not enhanced by verapamil (Chart 1). Although verapamil increases the sensitivity of resistant cells to Rho-123, they still remain 10-fold more resistant than sensitive FLC. At the concentrations used in these experiments, verapamil alone had no effect on cell growth in either cell type.

Verapamil-induced Increase in Rho-123 Accumulation and Retention. Differences in Rho-123 retention between sensitive and resistant cells were observed using fluorescence microscopy (Fig. 1). Each cell type was incubated with Rho-123 (10 μg/ml) for 15 min, centrifuged, and resuspended (as above) in drug-free medium. Rho-123 mitochondrial fluorescence was retained in sensitive (Fig. 1*B*) but was quickly lost from resistant cells (Fig. 1*D*) when incubated for 15 min in drug-free medium. However, when Rho-123-treated cells were resuspended in drug-free medium containing 10 μM verapamil, resistant cells retained drug in their mitochondria for as long as they were exposed to verapamil (4 h) (Fig. 1*F*). In sensitive cells, there was no apparent effect of verapamil on drug retention. When cotreated with Rho-123 and verapamil, resistant cells appeared brighter than when treated with Rho-123 alone. When these cells were cotreated with verapamil but resuspended in medium without verapamil, Rho-123 fluorescence was quickly lost. This loss was not detected in sensitive cells similarly treated.

Although these differences were observed by fluorescence microscopy, we used multiparameter laser flow cytometry to measure the accumulation and retention of Rho-123 fluorescence in these 2 cell types. Chart 2*A* demonstrates Rho-123 cellular fluorescence accumulation in sensitive cells after 10 min of incubation in drug-containing medium. In Chart 2*B*, a similar aliquot of cells after washing and reincubating for 20 min in drug-free medium is shown. The mean peak fluorescence in these histograms is 8 and 6, respectively. In contrast, when drug-resistant cells are incubated for 10 min with Rho-123, cellular fluorescence is barely detectable (Chart 2*C*), and on reincubation of cells in drug-free medium, this amount is further reduced (Chart 2*D*).

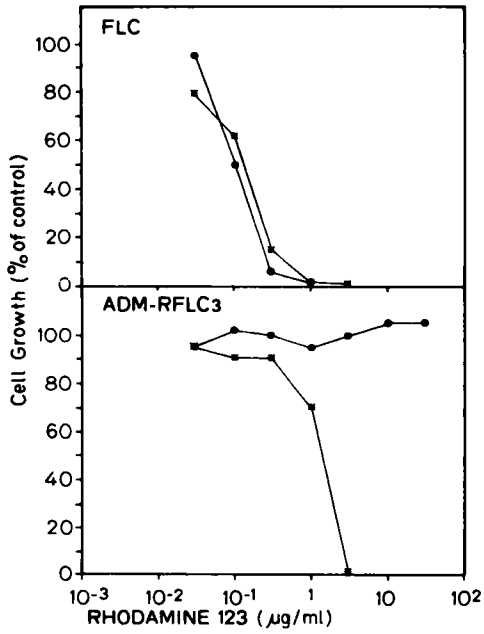

Chart 1. Effect of verapamil on the cytotoxic activity of Rho-123 in ADM-sensitive and -resistant FLC. Exponentially growing sensitive (*FLC*) and resistant (ADM-RFLC3) cells were treated continuously with increasing concentrations of Rho-123 in the presence (*) or absence (●) of 10 μM verapamil. At 72 h, trypan blue-excluding cells were assayed. Each *point* is the mean of 3 determinations with coefficients of variation less than 10%.

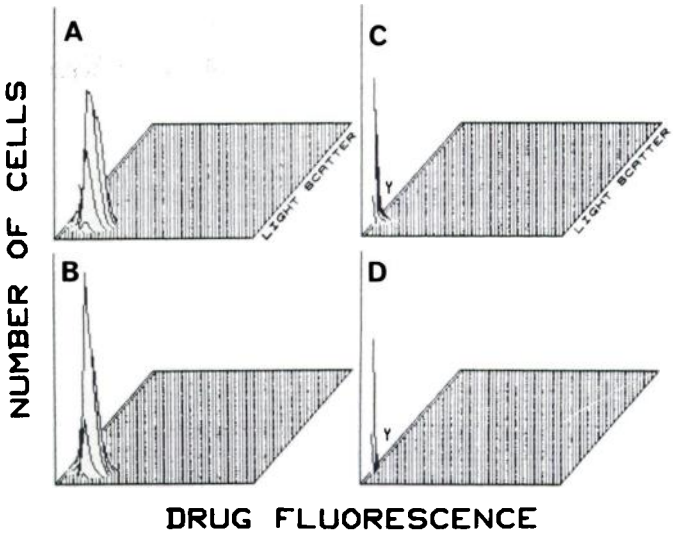

Chart 2. Multiparameter analysis of Rho-123 accumulation and retention in ADM-sensitive and -resistant FLC. Uptake of Rho-123 (5 μg/ml for 10 min) in exponentially growing sensitive (*A*) and resistant (*C*) cells. Following uptake, sensitive (*B*) and resistant (*D*) cells were washed, resuspended, and incubated in drug-free medium (20 min). For each histogram, 10,000 cells were analyzed. Peak height records the number of cells. On the *x* axis, intracellular drug fluorescence is recorded on a 2 decade log scale. On the *y* axis, laser scatter (approximately proportional to cell size) is recorded.

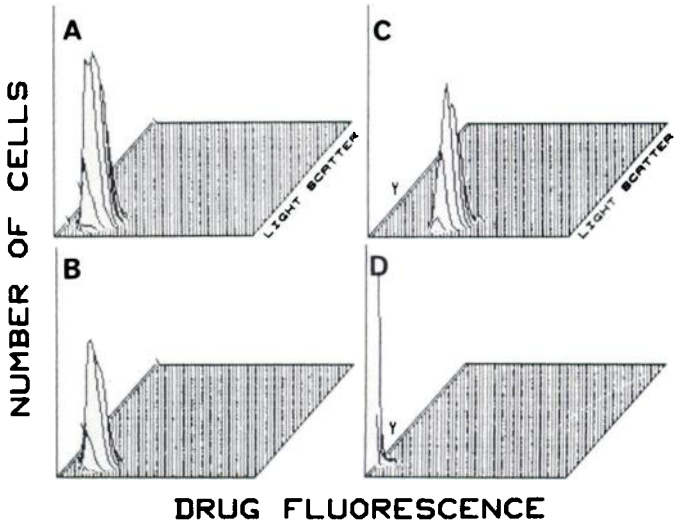

Chart 3. Multiparameter analysis of the effect of verapamil on Rho-123 accumulation and retention in sensitive and resistant FLC. Cells were incubated with Rho-123 (5 μg/ml for 10 min) in the presence of verapamil (10 μM), washed, resuspended, and incubated (20 min) in either the presence [sensitive (*A*), resistant (*B*)] or absence [sensitive (*C*), resistant (*D*)] of verapamil (10 μM). For each histogram, 10,000 cells were analyzed. Peak height records the number of cells. On the *x* axis, intracellular drug fluorescence is recorded on a 2 decade log scale. On the *y* axis, laser scatter (approximately proportional to cell size) is recorded.

Chart 3 illustrates the differences between sensitive and resistant cells in drug accumulation and efflux when they are coincubated with Rho-123 and verapamil for 10 min, followed by washing and reincubation for 20 min in Rho-123-free medium in the presence or absence of verapamil. Sensitive cells are found

to accumulate the same amounts of Rho-123 fluorescence whether or not they are coincubated with verapamil (Charts 2*A* and 3*A*). Similarly, incubation with (Chart 3*A*) or without verapamil (Chart 3*B*) in Rho-123-free medium has no significant effect on the loss of the accumulated drug fluorescence. However, when resistant cells are coincubated with verapamil, they accumulate significantly higher amounts of Rho-123 fluorescence than do cells treated with Rho-123 alone. Reincubation of these cells for 20 min in Rho-123-free medium containing verapamil results in maintenance of the enhanced Rho-123 fluorescence (Chart 2*C*). However, when resistant cells are washed and reincubated in verapamil-free medium, all of the accumulated Rho-123 fluorescence is lost, as shown in Chart 3*D*.

Reversal of Resistance to ADM by Verapamil. When ADM instead of Rho-123 was used in these cell types, similar results were obtained. The ID_{50} for ADM in resistant cells treated continuously with the drug was between 1 and 3 μg/ml when cells were cotreated with 10 μM verapamil. In sensitive cells, no significant differences in cytotoxicity were observed between cells treated with ADM alone or cotreated with verapamil. Both treatments yielded ID_{50}s between 1 and 3×10^{-3} μg/ml.

Verapamil-induced Increase in ADM Accumulation and Retention. Laser flow cytometry was used to assess the differences between the 2 cell types in ADM uptake and efflux as well as the effects of verapamil on these processes (Charts 4 and 5). Sensitive FLC (Chart 4*C*) exposed to ADM, 2 μg/ml, for 2 h accumulated significantly greater amounts of drug than did resistant cells (Chart 4*D*). When cells were cotreated with verapamil, a significant increase in ADM accumulation was observed in the resistant cell type (Chart 4*B*), whereas drug accumulation in the sensitive cells was relatively unaffected (Chart 4*A*). When resistant cells cotreated with verapamil were incubated in ADM-free medium for 2 h in the presence of verapamil, most of the accumulated drug was retained (Chart 5*D*). In contrast, when resistant cells were placed in drug-free medium in the absence of verapamil, loss of the drug was observed (Chart 5*B*). Under the same treatment conditions, sensitive cells retained most of the accumulated drug with (Chart 5*C*) or without (Chart 5*A*) verapamil in the ADM-free incubation medium.

DISCUSSION

We have shown previously that ADM- and DNR-resistant FLC are cross-resistant to Rho-123 (16, 26). We found that reduced cytotoxicity in resistant as compared to sensitive cell variants correlated with decreased levels of intracellular Rho-123 accumulation. Tsuruo *et al.* (28, 29) have reported that cells resistant to either ADM or vincristine could be made sensitive when cotreated with verapamil or other calcium transport and calmodulin inhibitors. In this report, we have demonstrated that verapamil significantly increases the sensitivity of ADM-resistant cells to Rho-123 or ADM. This increased sensitivity correlates with greater cellular Rho-123 or ADM accumulation and retention in the presence as compared to the absence of verapamil in the incubation medium. The question arose as to whether the increase in Rho-123 or ADM accumulation induced by verapamil was due to increased drug uptake or decreased efflux. Our experiments demonstrate that blockage or slowing of efflux of both Rho-123 and ADM from resistant cells accounts, at least in part, for the increase in drug accumulation. However, additional effects on influx are not excluded by these results. Similar data have been reported for increased accumulation and retention of ADM in ADM-resistant P388 cells (28).

Thus, a corollary can be made between the effect of verapamil on the accumulation and retention of ADM and Rho-123 in cells resistant to both agents. Although these agents localize in different compartments of the cell (ADM mainly in the nucleus and Rho-123 in mitochondria), they appear to be utilizing a similar or

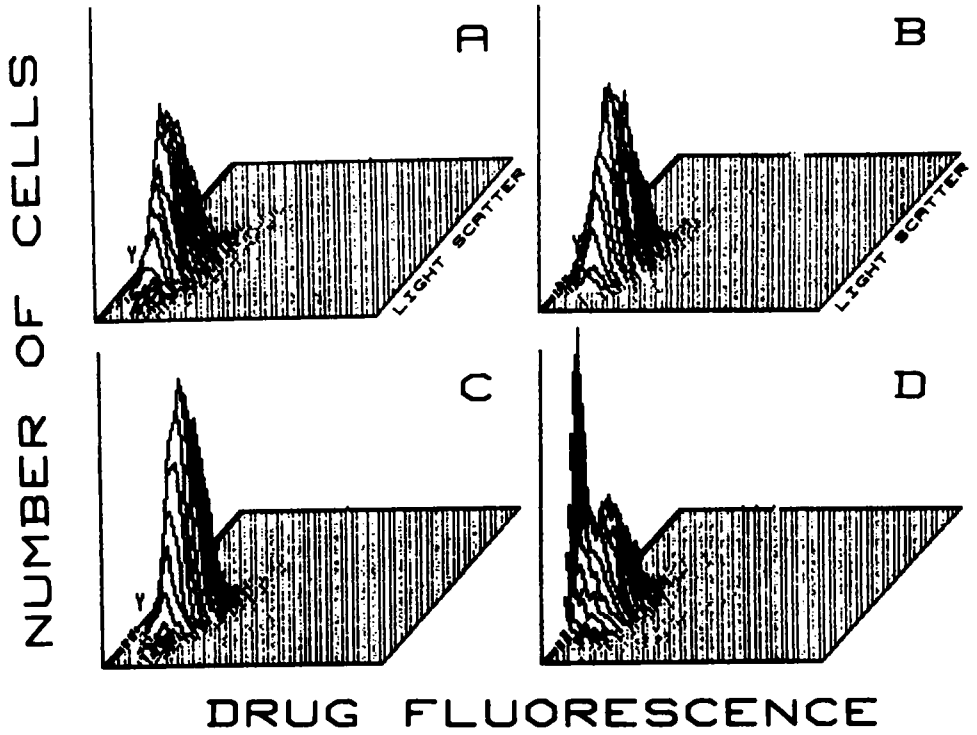

Chart 4. Multiparameter analysis of ADM accumulation and effects of verapamil in sensitive and resistant FLC. Contrasted are the uptake of ADM (2 μg/ml for 2 h) in sensitive (*C*) and resistant (*D*) cells and the cotreatment of sensitive (*A*) and resistant (*B*) cells with 10 μM verapamil. For each histogram, 10,000 cells were analyzed. Peak height records the number of cells. On the *x* axis, intracellular drug fluorescence is recorded on a 2 decade log scale. On the *y* axis, laser scatter (approximately proportional to cell size) is recorded.

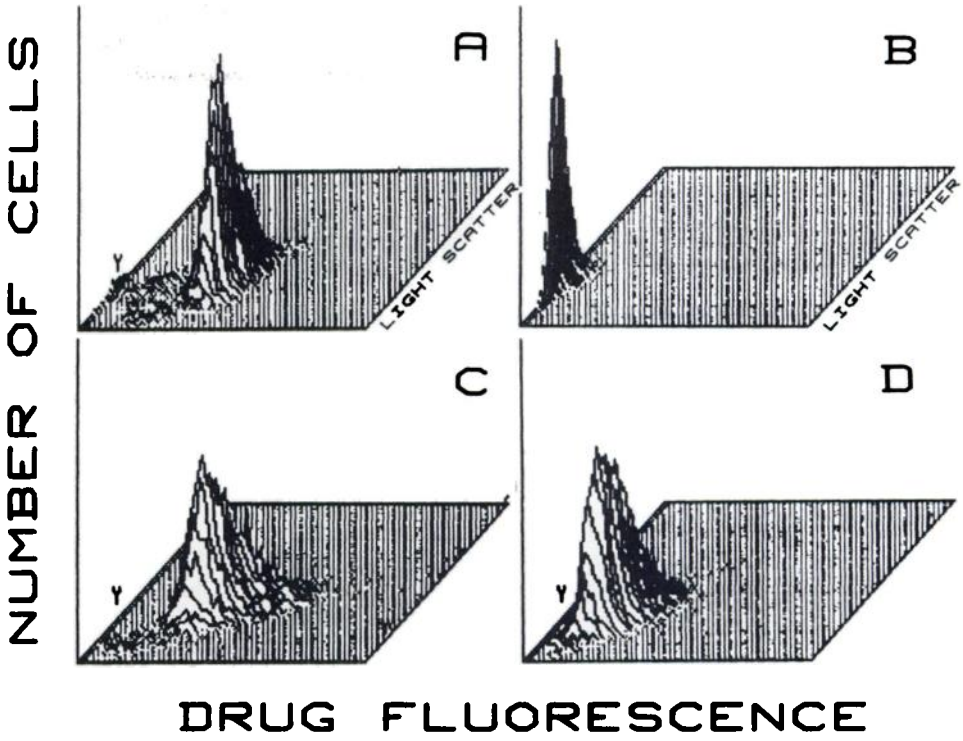

Chart 5. Multiparameter analysis of ADM efflux and effects of verapamil in sensitive and resistant FLC. Cells were incubated with ADM (2 μg/ml for 2 h) in the presence of 10 μM verapamil, washed, resuspended, and incubated (2 h) in either the presence or absence of verapamil (10 μM). Sensitive cells were incubated for 2 h with (*C*) and without (*A*) verapamil. Resistant cells were incubated similarly with (*D*) and without (*B*) verapamil. For each histogram, 10,000 cells were analyzed. Peak height records the number of cells. On the *x* axis, intracellular drug fluorescence is recorded on a 2 decade log scale. On the *y* axis, laser scatter (approximately proportional to cell size) is recorded.

common efflux mechanism. It is important to note, however, that ADM has also been shown to have effects on NADPH dehydrogenase in isolated mitochondrial preparations (7, 27).

Although 90% of ADM taken up by cells can be recovered from the nucleus (25), the possibility that ADM localizes and effects mitochondria *in situ* cannot be overlooked. Thus, verapamil could be probing a similarity in drug transport between these 2 agents at a mitochondrial level. On the other hand, Chauffert *et al*. (4) have recently reported inhibitory effects of verapamil on ADM transport from the nucleus of resistant cells. This suggests either that verapamil is acting directly at the nuclear membrane level or that its effects at the plasma membrane are associated with subsequent internal transport to and from other organelles.

Tsuruo *et al*. (29) and later Skovsgaard and Friche (21) and more recently Rogan *et al*. (20) have demonstrated an increase in life span of animals implanted with vincristine-, DNR- or ADM-resistant tumor cells when cotreated with verapamil and the appropriate antitumor agent. The implications of what reversal of resistance to these agents may mean in the treatment of cancer patients have been proposed by these investigators, and trials have begun to test the efficacy of using modifiers of resistance in cancer patients (20). Since Rho-123 has been shown previously to be selective for a wide variety of carcinoma cells *in vitro* (15) and has shown antitumor activity *in vivo* (1, 15), the effect of verapamil on resistance *in vitro* may also have relevance to the clinical use of this drug.

Prolonged mitochondrial retention of Rho-123 in carcinoma cells as compared to relatively rapid loss of drug from mitochondria in normal epithelial cells (24) has been proposed as a reason for the selective toxicity of this agent in these cell types (2, 5). It was suggested that Rho-123 is an indicator of mitochondrial potential (10). Cells that did not stain brightly, or those that lost their mitochondrial fluoresence staining quickly, were presumed to have a lowered mitochondrial potential than those that stained brightly and retained the dye (5, 10). Our data suggest that cells which lose their mitochondrial Rho-123 fluorescence quickly and are correspondingly resistant to Rho-123 have an efflux mechanism that reduces the amount of drug that can accumulate or be retained in this cell type. At present, it is unclear where verapamil is exerting its major effects on drug transport. The possibilities remain that verapamil may either be blocking this process at the level of the plasma membrane, cytoplasm, or nucleus or may be affecting transport directly at the mitochondria. In regard to the latter possibility, there are several reports indicating effects of verapamil in isolated heart mitochondria (30, 31) and demonstrating that verapamil enters and concentrates in the intact cell (18, 19). We cannot exclude the possibility that verapamil affects mitochondrial membrane potential in such a way as to increase the accumulation and retention of Rho-123. However, since sensitive cells treated with verapamil did not show an increase in their Rho-123 fluoresence and consequent sensitivity to the drug, this possibility seems less likely.

We have also been studying the effects of verapamil on the retention and cytotoxicity of Rho-123 in carcinoma and nontumorigenic epithelial cells and have found a similar increase in accumulation and reversal of resistance in normally resistant epithelial cells (this data will be presented in a following report). It appears therefore that the active efflux mechanism for Rho-123 in cells which have an acquired resistance to ADM may be a common mechanism utilized by cells normally resistant to the drug.

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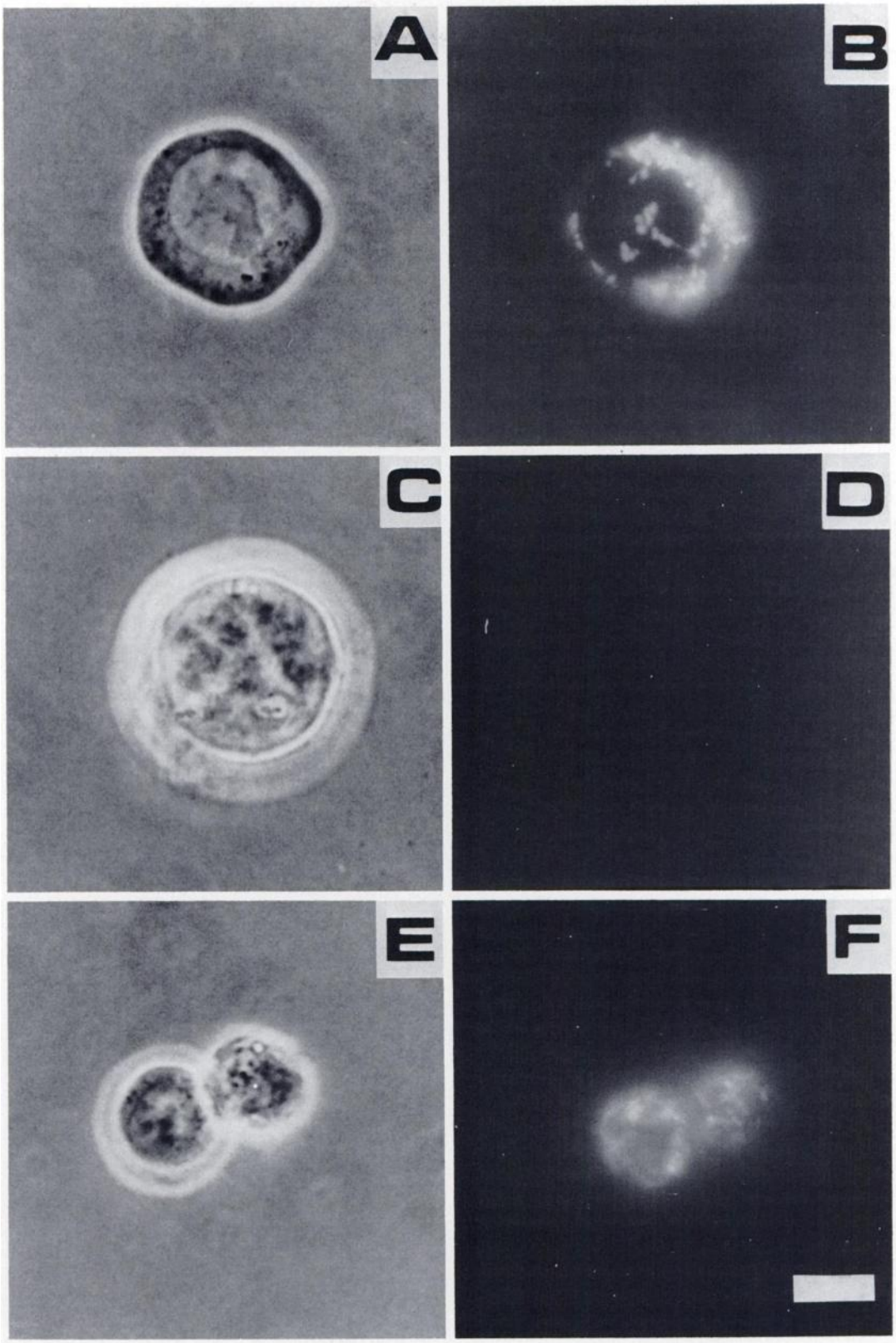

Fig. 1. Retention of Rho-123, analysis by phase-contrast and fluorescence microscopy. Exponentially growing ADM-sensitive (*A* and *B*) and resistant (*C* and *D*) FLC were treated for 10 h in drug-free medium and visualized under phase-contrast (*A*) and fluorescence microscopy (*B*). Resistant cells were washed, incubated either for 15 min in drug-free medium [visualized under phase-contrast (*C*) and fluorescence (*D*) microscopy] or 4 h in drug-free medium containing 10 μM of verapamil [phase-contrast (*E*) and fluorescence (*F*) microscopy]. *Bar*, 30 μm.