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Cytofluorescence localization of adriamycin in resistant colon cancer cells*

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Summary. *A simple fluorescent microscopic method demonstrated that adriamycin was distributed in two cellular compartments of living rat colon cancer cells. Adriamycin accumulated slowly in cytoplasmic granules, probably lysosomes, where it persisted long after the drug was removed from the medium. On the other hand, adriamycin accumulated rapidly in the nucleus, but was rapidly cleared in adriamycin-free medium. Drug efflux from the nucleus was blocked by sodium azide in glucose-free medium or by verapamil, a calcium-blocking agent.*

When colon cancer cells were cultivated for 1 day or longer in adriamycin-containing medium no nuclear fluorescence was observed. However, the addition of sodium azide to glucose-free medium or verapamil restores the nuclear fluorescence.

The colon cancer cells had low sensitivity to adriamycin, but the addition of verapamil strongly enhanced adriamycin toxicity. Thus adriamycin is permanently cleared from the nucleus of rat colon cancer cells through an energy-dependent efflux mechanism, which is blocked by verapamil. The efficiency of this efflux mechanism is enhanced by exposure of the cell to adriamycin. This mechanism could be involved in the resistance of colon cancer to adriamycin.

Introduction

Adriamycin (doxorubicin) is a potent antineoplasic agent that is active against a wide variety of human and experimental tumors. Some cancers, however, including human and experimental colon carcinoma [4, 7, 9], are unaffected by adriamycin. The mechanism of this resistance has not yet been clearly established. Adriamycin is a fluorescent anthracycline. Its main site of action is nuclear, the drug intercalating into the DNA double helix [5, 6, 14, 16, 22]; however, extranuclear sites of action have also been suggested [2, 12, 23].

In this work we have used fluorescence microscopy to study adriamycin cellular localization and efflux mechanism of resistance. We demonstrate lack of nuclear fluorescence in resistant colon cancer cells.

Materials and methods

Tumor cell line. A permanent cancer cell line DHD-K12 was established from a transplantable colon carcinoma chemically induced in the syngeneic BDIX rat [13]. DHD-K12 cells were cultivated in Ham's F10 medium supplemented with 4% fetal bovine serum and 8% newborn calf serum; this medium will be referred to below as complete medium. Subcultures between passages 20 and 40 were used for the experiments. Absence of contamination by mycoplasma was checked according to Chen's method [3].

Chemicals. Adriamycin (doxorubicin, mol. wt. 580) was obtained from Roger Bellon Laboratories (Neuilly, France), verapamil (mol. wt. 454) from Biosedra (Malakoff, France), and sodium azide from Merck (Darmstadt, Germany). They were freshly dissolved or diluted before each experiment. Ham's F10 medium, glucose-free or supplemented with glucose (1.1 mg/ml), was obtained from Gibco (Grand Island, NY, USA).

Cytofluorescence of adriamycin. The intracellular localization of adriamycin was studied by ultraviolet illumination, which induced an orange fluorescence at the sites of adriamycin accumulation [8]. DHD-K12 cells were cultivated in complete medium on sterile glass cover slips in petri dishes. After exposure to adriamycin, living cells were examined in a fluorescence microscope after the wet cover slip had been turned over and set on the edges of two other cover slips (0.15 mm thick) placed 10 mm apart on the slide. This allowed us to observe the living cells for a few minutes without their drying and to use an oil immersion objective without contact between oil and cells. A Dialux 20 fluorescence microscope (Leitz, Wetzlar, Germany) equipped with a high-pressure mercury lamp, an N-2 filter set, and a Leitz 40× or 100× oil immersion objective was used. Living cells can be observed for a few minutes before UV illumination induces fluorescence fading and cell structure alteration. Microphotographs were taken on Kodak Tri-X panfilm (400 ASA) with an exposure time of about 1 min or Agfa Vario XL (1600 ASA) with an exposure time of 10–15 s.

Inhibition of [^{3}H]thymidine incorporation by DHD-K12 cells. To evaluate the cytostatic effect of adriamycin and/or verapamil, 3×10^4 DHD-K12 cells in 200 µl complete culture medium were seeded in the wells of Microtest 3040 tissue culture plates (Falcon Plastics, Oxnard, Calif., USA) and incubated at 37° C in a controlled atmosphere (95% air, 5% CO_2) for 48 h. The medium was then aspirated and replaced by 200 µl complete medium supplemented with adriamycin and/or verapamil and ^{3}H-thymidine, specific activity 25 Ci/m*M*

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(CEA, Gif-sur-Yvette, France) at 10 μCi/ml. After incubation at 37° C for 24 h the plates were washed four times with phosphate-buffered saline, then dried. Residual cells were lysed with 500 μl *N* sodium hydroxide. The well contents were quantitatively transferred into counting microvials containing 4 ml Picofluor (Packard Instruments, Rungis, France) and their radioactivity was measured in a Tricarb Prias liquid scintillation counter (Packard). The percentage inhibition of [^{3}H]-thymidine incorporation was calculated using the following formula:

$$\% \text{ effect} = (1 - a/b) \times 100,$$

where a represents the mean radioactivity of eight wells containing adriamycin and/or verapamil and b is the mean radioactivity of eight wells containing complete culture medium without drug. The toxic effects of adriamycin, verapamil, and both were compared by the nonparametric Mann-Whitney U-test.

Results

Cellular localization of adriamycin after a first short incubation

Before contact with adriamycin, DHD-K12 cells were not fluorescent except for a pale greenish autofluorescence in some cytoplasmic granules. When DHD-K12 cells were incubated in 1 μg/ml adriamycin for 1 h at 37° C fluorescence was observed at two distinct cellular sites (Fig. 1):

A dark yellow fluorescence in the nuclei of 100% of cells. Chromatin was brighter than nucleoli. Chromosomes of mitotic cells and any dead cells were intensively fluorescent. Nuclear fluorescence was stable under UV illumination.

Bright yellow fluorescence in scattered cytoplasmic granules of various sizes (0.1 to 0.4 μ*M*).

Neither cytoplasmic membrane nor other cell components were fluorescent. The fluorescence of cytoplasmic granules faded rapidly under UV illumination (1 min), but it did not disappear completely. Both cytoplasmic and nuclear fluorescence intensity varied from cell to cell.

When DHD-K12 were incubated for 1 h with adriamycin at a concentration lower than 0.05 μg/ml only autofluorescence was seen. Above 0.05 μg/ml and up to 10 μg/ml for 1 h, fluorescence was augmented in both the nucleus and the cytoplasm in parallel as the extracellular drug concentration rose.

Efflux experiments from adriamycin-preloaded cells

DHD-K12 cells were incubated for 1 h in complete medium containing 1 μg/ml adriamycin. The presence of fluorescence in both the nucleus and the cytoplasm was verified. Then cells

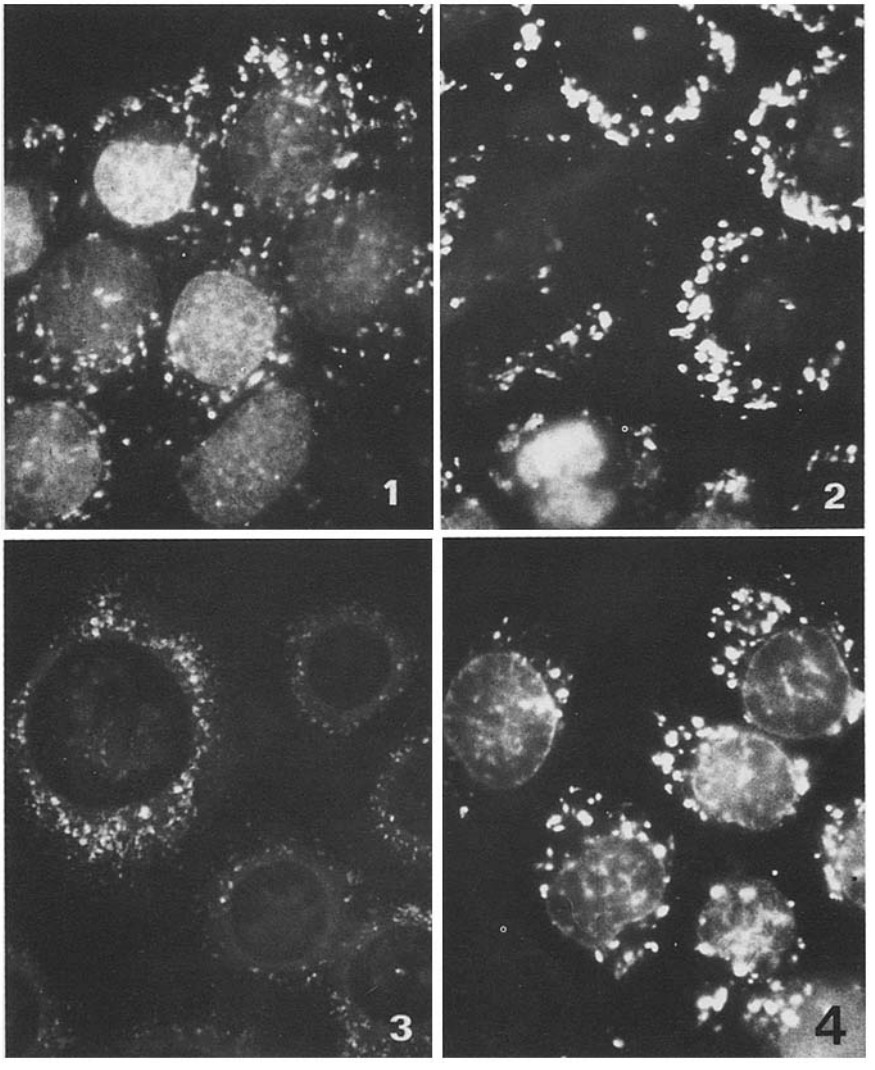

Fig. 1. DHD-K12 cells incubated for 1 h in adriamycin (1 μg/ml). Fluorescence is localized in nuclei and in cytoplasmic granules probably lysosomes (oil immersion × 1,200)

Fig. 2. Cells are preloaded 1 h in adriamycin-containing medium (1 μg/ml), then incubated for 1 h in drug-free medium. Only cytoplasmic fluorescence persist (oil immersion × 1,200)

Fig. 3. Culture of DHD K12 cells in adriamycin-containing medium (1 μg/ml) for past 72 h. Only cytoplasmic fluorescence is observed (oil immersion × 1,200)

Fig. 4. DHD-K12 cells accustomed to adriamycin for 72 h then incubated for 1 h in medium containing adriamycin (1 μg/ml) + verapamil (5 μg/ml). Nuclear fluorescence is restored (oil immersion × 1,200)

Table 1. Efflux experiments from adriamycin-preloaded cells[a]

Washing medium[b]	Nuclear fluorescence	Cytoplasmic fluorescence
C. M.[c]	0	+
Ham's F 10	0	+
Glucose-free Ham's F 10	0	+
Glucose-free Ham's F 10 + sodium azide (10 m*M*)	+	+
Ham's F 10 + sodium azide (10 m*M*)	0	+
C. M. + verapamil (5 μg/ml)	+	+

[a] Cells are preloaded 1 h in complete medium with 1 μg/ml adriamycin. Nuclei and cytoplasm are both fluorescent (+)
[b] Washing time: 1 h at 37° C in a controlled atmosphere
[c] Complete medium

Table 2. Kinetics of adriamycin cellular localization[a]

Time	% of cells with fluorescent nuclei	Intensity of nuclear fluorescence[b]	Intensity of cytoplasmic fluorescence[c]
0	0	0	±
7 min	6	+	±
15 min	85	+	±
30 min	93	++	+
1 h	100	++	++
2 h	100	++	++
5 h	72	+	+++
8 h	17	+	+++
24 h	0	0	+++
1 week	0	0	+++
2 week	0	0	+++
3 week	0	0	+++

[a] Cells are incubated in complete medium supplemented with 1 μg/ml adriamycin. With incubations longer than 1 h, new medium with 1 μg/ml adriamycin is added 1 h before the assays
[b] Subjective nuclear intensity: + nuclei are seen but limits are vague. No detail is visible; ++ nuclei are clearly seen. Fluorescence is bright, clumps of chromatin and nucleoli are clearly visible
[c] Subjective cytoplasmic intensity: ± autofluorescence pale, greenish, granular and different from orange-yellow adriamycin fluorescence; + faint granular fluorescence; ++ bright fluorescence in numerous granulations; +++ full-intensity fluorescence in numerous and large granulations

Table 3. Inhibition of efflux on adriamycin-accustomed cells[a]

Incubation medium[b] + 1 μg/ml adriamycin	Nuclear fluorescence	Cytoplasmic fluorescence
C. M.[c]	0	+
Ham'2 F 10	0	+
Glucose-free Ham's F 10	0	+
Glucose-free Ham's F 10 + sodium azide (10 m*M*)	+	+
Ham's F 10 + sodium azide (10 m*M*)	0	+
C. M. + verapamil (5 μg/ml)	+	+

[a] Cells treated with 1 μg/ml adriamycin for at least 72 h prior to experiment have no nuclear fluorescence (0), but bright cytoplasmic fluorescence (+)
[b] Incubation: 1 h at 37° C in a controlled atmosphere, adriamycin 1 μg/ml
[c] Complete medium

were put in different adriamycin-free media for 1 h at 37° C (Table 1).

In complete medium, nuclear fluorescence disappeared whereas cytoplasmic fluorescence persisted (Fig. 2). At 72 h 40% of the cells still had a cytoplasmic fluorescence. Nuclear fluorescence persisted if the energetic metabolism was inhibited both in the absence of glucose and with sodum azide. Drug efflux out of the nuclei was also blocked by verapamil. Cytoplasmic granular fluorescence is not much influenced by metabolic inhibitors or by verapamil.

Effect of exposure time on cellular adriamycin localization

DHD-K12 cells were incubated in 1 μg/ml adriamycin-containing medium at 37° C for the appropriate time.

If necessary the culture medium was changed three times weekly. With incubation for longer than 1 h new medium with 1 μg/ml was added before the microscopic observations. Results are presented in Table 2.

Nuclear fluorescence reached its full intensity at 1 h in 100% of the cells, but afterwards decreased and disappeared. Cytoplasmic fluorescence reached its full intensity slowly but persisted subsequently.

Resistant cells survived for up to 3 weeks in 1 μg/ml adriamycin-containing medium and were able to incorporate at ^{3}H-thymidine. Lack of nuclear fluorescence in resistant cells was confirmed many times (Fig. 3). Nevertheless, their proliferation was slowed down and the morphology altered, with an increased size of both the cytoplasm and the nucleus. If adriamycin was removed from the medium proliferation and morphology returned to normal.

Restoration of nuclear fluorescence and toxicity on resistant cancer cells

DHD-K12 cells were accustomed to adriamycin for at least 72 h. Lack of nuclear fluorescence was verified. Then cells were incubated for 1 h at 37° C in different media with 1 μg/ml adriamycin. Results of the observations presented in Table 3 demonstrated that sodium azide in glucose-free medium or verapamil restored an intense nuclear fluorescence (Fig. 4).

Prolonged inhibition of cellular metabolism was followed by cell death, but a long incubation in 5 μg/ml verapamil was easy to perform with low toxicity for a long time. DHD-K12 cells were able to survive for 3 weeks or longer in 1 mg/ml adriamycin-containing medium. The addition of both 5 μg/ml verapamil and 1 μg/ml adriamycin resulted in 100% cell death after 24 h.

The results in Table 4 demonstrate that incorporation of ^{3}H-thymidine by DHD-K12 cells was severely inhibited when verapamil and adriamycin were presented together in the incubation medium.

Discussion

Cytofluorescence localization of adriamycin is not a new technique [8], but we improved it by observation of whole living cells in their own incubation medium and by use of an immersion oil objective with better magnification and resolution. One risk with the cytofluorescence technique is that the nuclear concentration could be misjudged, since the binding of adriamycin to DNA could quench durg fluorescence. However, Egorin [8] demonstrated that quenching was moderate when DNA or whole nuclei were added to adriamycin. Thus, a

Table 4. Effect of adriamycin and verapamil on ^{3}H-thymidine incorporation of DHD-K12 cells

Complete medium supplemented with	Residual dpm[b] ($\times 10^{-2}$)	% Inhibition[c]	Enhancement[d] adriamycin cytostasis by verapamil
0	1,096 (± 124)	–	–
Verapamil (5 μg/ml)	616 (± 145)	43 ($P < 0.001$)	–
Adriamycin (0.1 μg/ml)	986 (± 154)	9 (NS)	–
Adriamycin (1 μg/ml)	466 (± 55)	57 ($P < 0.001$)	–
Adriamycin (0.1 μg/ml), verapamil (5 μg/ml)	123 (± 63)	89 ($P < 0.001$)	$P < 0.01$
Adriamycin (1 μg/ml), verapamil (5 μg/ml)	10 (± 3)	99 ($P < 0.001$)	$P < 0.01$

[a] Cells are seeded and incubated for 48 h, then ^{3}H-thymidine and drugs are added in complete medium for 24 h before the end of the assay

[b] Disintegrations per minute; mean of eight wells ± SD

[c] Percentage of inhibition (Student's *t*-test); NS, not significant

[d] Significance of enhancement effect of verapamil on adriamycin-induced cytostasis (Mann and Whitney's U-test)

complete lack of fluorescence in the nucleus cannot be an artifact, but may really reflect a low concentration.

We were able to show both a nuclear and a cytoplasmic localization. Granular cytoplasmic fluorescence is probably due to adriamycin capture by lysosomes. Using cell fractionation techniques, Noel and al. [15] observed that 60% of adriamycin was located in the nuclei of rat embryo fibroblasts, whereas 40% had accumulated in the lysosomes. They suggested that adriamycin was trapped in the lysosomes in a protonated form, due to the locally lower H, and preventing back-diffusion. Our observations confirm that intralysosomial adriamycin is not very mobile, since cytoplasmic fluorescence persisted beyond 72 h after removal of the drug. In contrast, after a first short incubation adriamycin is rapidly taken up in the nucleus but quickly eliminated when the drug is removed. Efflux of movable adriamycin from the nucleus is dependent on energetic metabolism, as Skovsgaard and other authors have demonstrated [10, 17–20].

Sodium azide is required to block oxidative phosphorylation and glucose-free medium is required to block glycolysis. Inhibition of both these two metabolic pathways is necessary to inhibit adriamycin efflux. We have also confirmed Tsuruo's observations [24] with reference to the possibility that verapamil, a calcium inhibitor, can inhibit adriamycin efflux and is able to restore cytotoxicity in resistant cells. A major finding in this work is the demonstration that inherently resistant cancer cells are able to remove adriamycin from the nucleus permanently after a short period of adaptation. Adriamycin cannot remain in the nucleus, its main site of action. By this permanent efflux mechanism cells are able to survive and to grow in a culture medium containing 1 μg/ml adriamycin. In man, pharmacologic studies [1] show that such a concentration is only reached in the first few minutes after a bolus injection. Moreover, this efflux mechanism of resistance is shared with other anthracyclines and some nonanthracycline agents [11, 21, 24]. We can therefore understand the usual resistance of colon cancer to chemotherapy.

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