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Subcellular Localization of Daunorubicin in Sensitive and Resistant Ehrlich Ascites Tumor Cells

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Abstract—*Cellular uptake and subcellular localization of daunorubicin were studied in Ehrlich ascites tumor cells, either* in vivo *after inoculation of the drug to mice or* in vitro *after incubation of cultured Ehrlich ascites tumor cells with the drug. These cells were either sensitive (wild-type) or resistant to daunorubicin. The uptake of daunorubicin was 2–4 times less in resistant cells. About 90% of the drug which had penetrated in the cells was recovered in the nuclei. By cell fractionation, it has been established that the extranuclear daunorubicin was localized in the lysosomes. The lower uptake of the drug in resistant cells and the similar subcellular localization in both types of cells were in favour of an increased active outward transport of the drug in resistant cells.*

INTRODUCTION

THE DEVELOPMENT of resistance against the action of daunorubicin (DNR), a very potent anti-leukemic agent [1], is an important cause of treatment failure. It has been established that, in resistant Ehrlich ascites tumor (EAT) cells, there is a reduced uptake of the drug which is related to a reduced transport through the cell membrane and to an active transport of the drug out of the cells [2–4]. This has been confirmed in P 388 leukemia cells, the resistance of which is primarily due to a diminished capability of retaining the anthracycline drugs DNR and doxorubicin (adriamycin) [5]. At the molecular level, until now it has not been possible to observe differences in the affinity of the drug for DNAs or chromatins extracted from sensitive and resistant cells or in the inhibition by DNR of RNA polymerases A and B prepared from the two types of cells [6, 7]. In order to determine if the drug resistance may be related to modifications in the subcellular distribution of DNR, we have measured the amount of DNR present in cell fractions obtained from wild type or resistant cells according to the technique of de Duve *et al.* [8].

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MATERIALS AND METHODS

Daunorubicin

DNR and (14-^{14}C) DNR (spec. act. = 50 mCi/mM) were obtained from Rhône-Poulenc (Paris, France). Solutions in saline were prepared extemporaneoulsy (2 mg/ml).

Ehrlich ascites tumor cells

In vivo *experiments.* CD_1 female mice (Charles River, France) 6–8 weeks-old (20 g) were inoculated i.p. with 10^6 cells/mouse and resistance was developed by treatment with subtherapeutic doses of DNR (0.25 mg/kg i.p. for 5 days). After 8 days for sensitive cells and 15 days for resistant cells, 25–50 mg/kg of DNR or 0.62 to 1.25 mg/kg of (14-^{14}C) DNR were given i.p. After 1 hr, mice were sacrificed and ascites cells were collected. Due to the short time interval following treatment the death rate of the tumor cells, as determined by the trypan blue exclusion test, was similar in all cases and never higher than 10%.

In vitro *experiments.* EAT cells were collected 8 or 15 days after inoculation, washed 2–4 times with saline phosphate buffer (0.15 M sodium chloride, 10.7 mM disodium phosphate, 2.6 mM monopotassium phosphate, pH 7.2) and then transferred in a spinner flask containing RPMI 1640 medium, supplemented with 20% of foetal calf serum, 10% of tryptose phosphate broth, 2% of glutamine, 1% of a solution of amino acids (L-alanine, L-proline, L-serine, L-glycine, L-asparagine, L-aspartic acid and L-glutamic acid, 0.01 M, pH 7.4), 1% of sodium bicarbonate and antibiotics (4.10^4 U.I of penicillin, 2.5 mg of aureomycin, 20 mg of streptomycin and 5000 U.I. of mycostatin %). 10^6 cells/ml were incubated in the spinner flask at 37°C for 18 hr. Five μg/ml of DNR were added to the cell culture and incubation was stopped at various times (1–24 hr).

Cell fractionation. Homogenization of the cells and fractionation by differential centrifugation were described earlier [9]. Cells were fractionated to yield the following four fractions: a nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and a final supernatant S.

Analysis by centrifugation in a sucrose gradient was performed in a SW 50 rotor, using a Beckman ultracentrifuge L5 75. After cell homogenization and separation of the nuclei, the cytoplasmic fraction containing extranuclear DNR was first centrifuged 30 min at 40,000 rev/min and the supernatant discarded. The pellet, corresponding to fractions MLP was resuspended in sucrose 0.5 M, EDTA 0.5 mM, Tris-HCl 1 mM, pH 7.6. Of this suspension, 0.2 ml, corresponding to 1–1.5 mg of proteins, were layered on top of a sucrose gradient (1–1.8 M in EDTA 0.5 mM, Tris-HCl 1 mM, pH 7.6) After 7 hr of centrifugation at 4°C and 40,000 rev/min, fractions of 15 drops were collected.

Biochemical assays. Assay conditions for marker enzymes, DNA, RNA and protein are described in [9]. The DNR concentration in the subcellular fractions was determined by spectrofluorimetry (λ excitation 485 nm, emission 570 nm) according to Noël *et al.* [10].

RESULTS

In vitro *cellular uptake*

The cellular uptake of DNR inside sensitive and resistant cells after various incubation times is given in Fig. 1. The amount of drug recovered in the cells is 2–4 times smaller in resistant cells than in sensitive ones.

Subcellular localization

After various incubation times of DNR with sensitive or resistant EAT cells *in vitro*, cellular fractionation was performed. The distribution of drug, marker enzymes, protein and nucleic acids after 17 hr of incubation is given in Fig 2. A similar pattern was observed at all incubation times between 1 and 24 hr. DNR is mainly recovered in fractions containing DNA (N and M fractions). As shown in Table 1, the percentage of DNR recovered in the N and M fractions is comparable for cells incubated with the drug *in vitro* and for cells exposed to DNR *in vivo.*

The amount of DNR recovered in the M, L, P fractions raises the problem of the homogeneity of the fractions. To determine more precisely the localization of the extranuclear DNR, a cytoplasmic fraction corresponding to

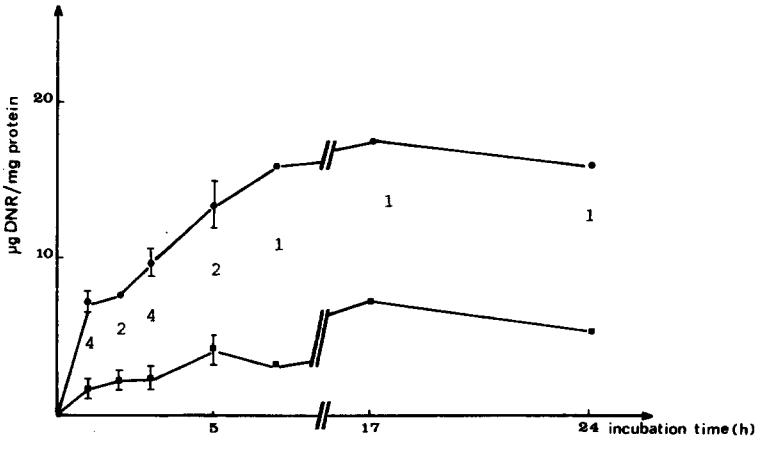

Fig. 1. *Accumulation kinetics of daunorubicin in sensitive* (●) *and resistant cells* (■). *Cells are incubated with* 5μg *of DNR per* ml *of incubation medium. Numbers give the number of experiments.*

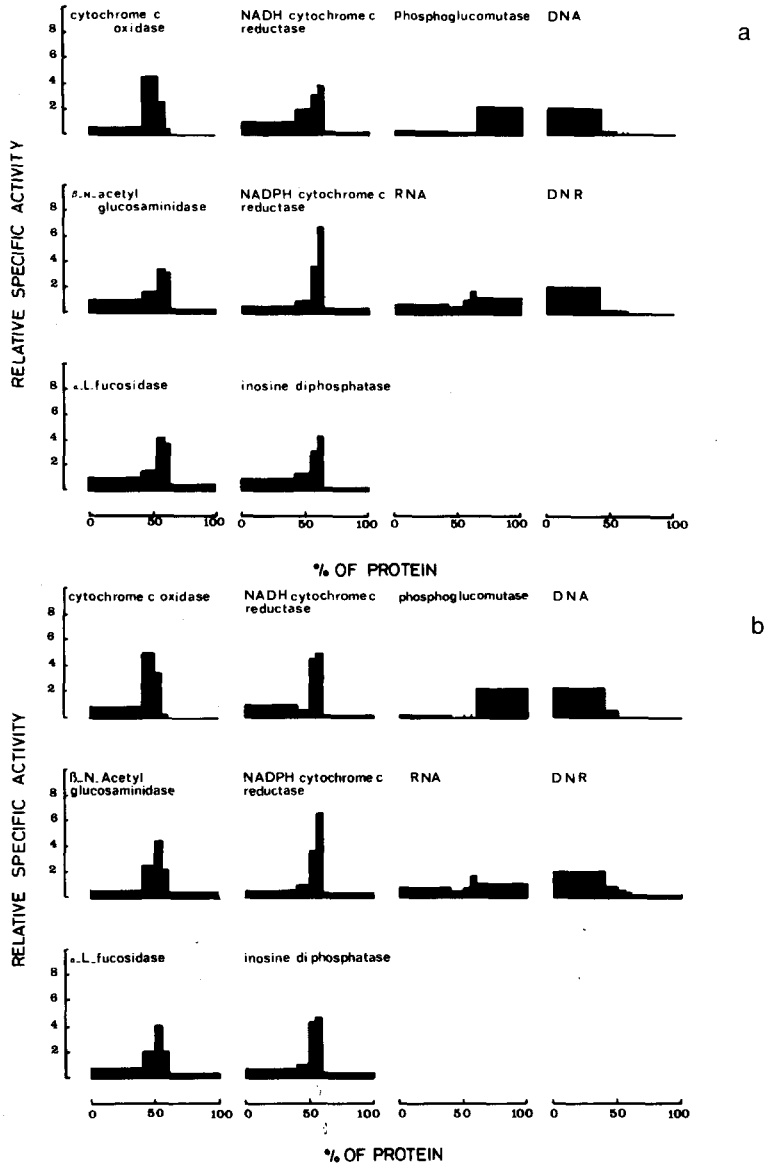

Fig. 2. Distribution of DNR in EAT cells as determined by subcellular fractionation. Sensitive (part a) and resistant (part b) EAT cells were incubated in vitro *with 5 μg of DNR per* ml *of incubation medium for* 17 hr. *N, M, L, P and S fractions are represented in blocks ordered in the same sequence along the abscissa where the length is proportional to the protein content. The ordinates give the relative specific activity (or the amount of DNR, DNA or RNA) which is the percentage of activity (or amount) recovered in each fraction over the percentage of protein in the same fraction.*

Table 1. *Percentage of daunorubicin recovered in DNA containing fractions (N and M fractions) of sensitive and resistant cells*

Incubation time (hr)	Sensitive cells	Resistant cells
	*In vitro**	
1	91.98	82.49
2	93.49 ± 1.00	86.24 ± 1.60
17	94.87	88.60
24	93.61	88.18
	In vivo†	
0.5	95.36	—
1	94.65 ± 0.92	84.67 ± 7.11

*Cells were incubated with 5 μg of DNR/ml in the incubation medium at 37°C in spinner flasks during various incubation times.

†Mice were inoculated i.p. with 0.5 mg of DNR/mouse and sacrificed after 30 or 60 min.

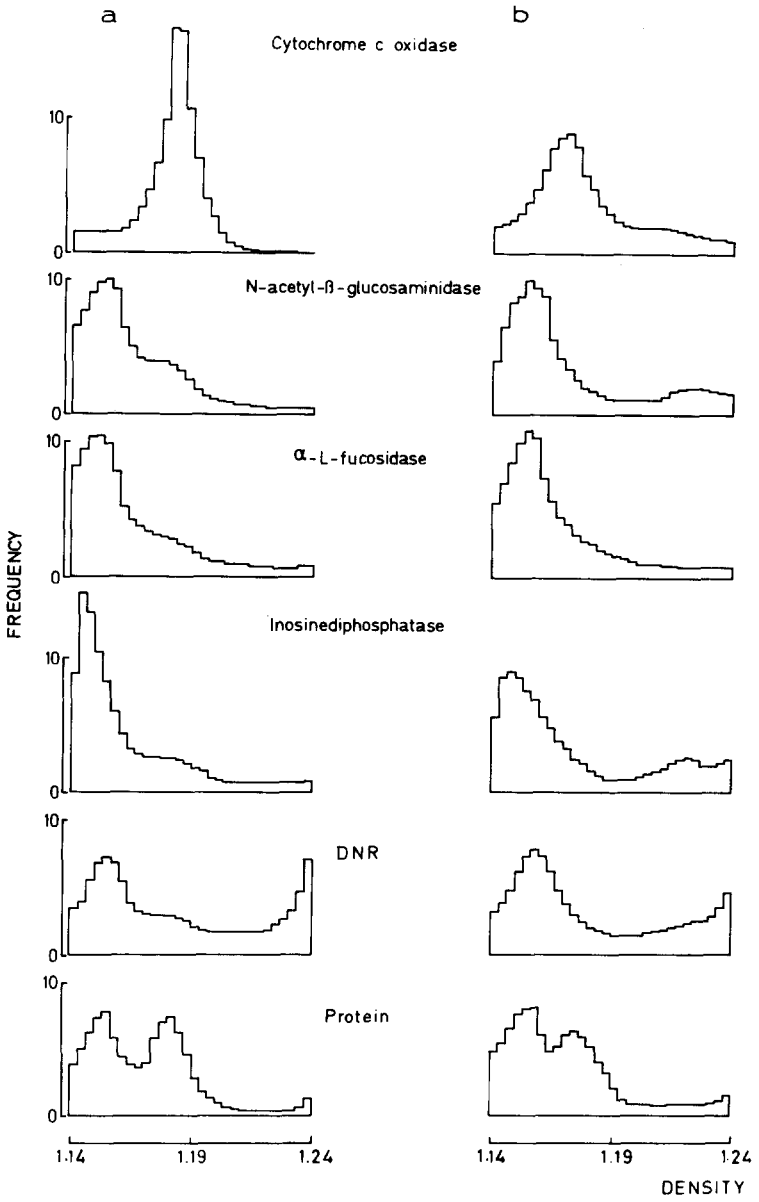

Fig. 3. Distribution of DNR, proteins and marker enzymes of the MLP fraction after centrifugation in a sucrose gradient. Mice bearing sensitive (part a) or resistant (part b) Ehrlich ascite tumor were injected i.p. with 0.62 mg/kg *of* (14−^{14}C) *DNR. One* hr *after the drug injection mice were sacrificed and EAT cells were collected. The ordinate indicates the percentage of total enzymatic activity or the amount of DNR or protein. The abscissa indicates sucrose density.*

MLP fraction was analyzed by centrifugation in a sucrose gradient after incubation of cells *in vivo* with (14-^{14}C) DNR at 0.62 mg/kg for 1 hr.

As shown in Fig. 3, the distribution pattern of DNR is clearly bimodal and distinct from that of cytochrome c oxidase (mitochondrial enzyme) and that of inosine diphosphatase (microsomal fraction enzyme). Part of DNR is recovered with the heavy fraction which could correspond to the nuclei or nuclear fragments which contaminate the MLP fraction (Fig. 2). Another major part of DNR follows the distribution of β-*N*-acetylglucosaminidase and α-L-fucosidase, marker enzymes for the lysosomes.

Analogous distributions were observed after incubation of cells *in vivo* with DNR at 25–50 mg/kg (12.5–25 times the LD_{50}). One can eliminate the possibility of a redistribution of the drug during the homogenization of the cells since control experiments in which DNR was added to the cell homogenates showed a distribution of the drug along the gradient, without specific localization.

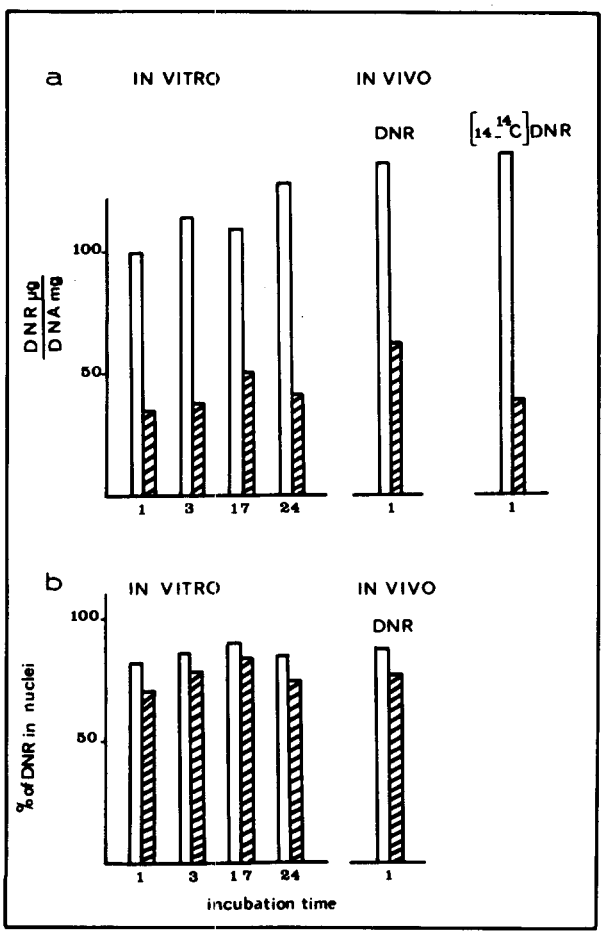

Fig. 4. DNR/DNA ratio and nuclear distribution of DNR in sensitive (open bars) and resistant (hatched bars) EAT cells. In vitro *experiments: EAT cells were incubated in a spinner flask with* 5 µg *of DNR per* ml *of the incubation medium.* In vivo *experiments: mice bearing Ehrlich ascites tumor were injected i.p. with* 25 mg/kg *of cold DNR or with* 0.62 mg/kg *of* (14^{-14}C) *DNR. Part a: Ratio DNR* (µg/ml)/*DNA* (*mg/ml*) *at different incubation times. Part b: Percentage of intracellular DNR recovered in the nuclei at different incubation times.*

DISCUSSION

We have compared the cellular uptake of DNR in sensitive and resistant cells and determined the subcellular localization of DNR after treatment with DNR of mice bearing an Ehrlich ascite tumor or after addition of DNR to Ehrlich cells *in vitro*.

The cellular uptake of the drug is 2–4 times lower in resistant cells. Cellular fractionation experiments show that if one takes into account the contamination of the nuclear fraction by lysosomes [10], it is possible to estimate the true nucleo-cytoplasmic repartition of the drug in sensitive and resistant cells. At all incubation times, the largest part of DNR which has penetrated in the cells is recovered in nuclei and the percentage of DNR associated to the nuclei is slightly lower in resistant cells (Fig. 4b). On the base of these results and given the 2–4 times decreased uptake of DNR in resistant cells, it is obvious that 2–4 times less DNR is found associated with nuclear DNA in resistant cells (Fig. 4a).

We can assume that extranuclear DNR is localized in the lysosomes since its distribution follows that of the lysosomal hydrolases. The repartition between nuclei and lysosomes of intracellular DNR is analogous to that described by Noël *et al.* [10] for cultured rat fibroblasts. The fact that ±90% of DNR is found in nuclei of EAT cells as compared to 20% in the nuclei of fibroblasts can partly be attributed to the higher DNA content and the tetraploid character of EAT cells.

The data of the literature [3, 6, 7, 10, 11] and the results presented here are consistent with a mechanism of cell resistance to DNR relying on an increased outward transport mechanism of DNR. DNR seems indeed to get access to the cell by permeation across the plasma membrane and to be trapped intracellularly firstly in the nuclei by interaction with DNA and secondly in lysosomes by protonation. The lower overall uptake of DNR in resistant cells combined with an unchanged nucleo-lysosomal distribution of the drug allows us to establish that the mechanism of cell resistance is not the result of a modified intracellular distribution of DNR and supports the hypothesis that it may be attributed to an increased outward transport localized on the plasma membrane.

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