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In vitro and in vivo Study of Cross Resistance Between Daunorubicin and Daunorubicin-DNA Complex in Ehrlich Ascites Tumor

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Summary. *The antitumor effect of daunorubicin and of two preparations of daunorubicin-DNA complexes were compared in equitoxic doses against the Ehrlich ascites tumor and in a subline of this tumor resistant to daunorubicin. The therapeutic efficacy of both complexes was significantly better than that of free daunorubicin in the sensitive tumor. The daunorubicin-resistant tumor was found to be cross resistant to both daunorubicin-DNA complexes. In vitro, the steady-state uptake of daunorubicin was considerably lower when the drug was added as a complex compared with addition as free drug. Experiments in which the uptake of daunorubicin and of DNA was determined simultaneously showed no significant uptake of DNA in either cell line. Together, these data indicate that even if daunorubicin is administered as a complex, the membrane transport in Ehrlich ascites tumor cells takes place solely as free drug.*

Introduction

Several studies have confirmed that development of resistance to anthracyclines is accompanied by a reduced cellular drug uptake [4, 5, 7, 8, 10, 15, 16]. Previously, we have shown that the lower uptake of daunorubicin in Ehrlich ascites tumor cells resistant to daunorubicin is mainly a result of a higher specific drug extrusion [7, 15]. As the membrane transport of daunorubicin bound to DNA has been attributed to endocytosis [17], this administration may imply a bypassing of the drug resistance to daunorubicin. To evaluate this possibility, we compared the therapeutic effect of free daunorubicin and daunorubicin-DNA complex in a subline of Ehrlich ascites tumor resistant to daunorubicin. To further elucidate the mechanism of transport, the cellular uptakes of daunorubicin and daunorubicin-DNA complex were compared in vitro.

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Materials and Methods

Drugs. Daunorubicin-DNA complex was kindly supplied in lyophilized form by the Rhône-Poulenc Research Laboratory. Two complexes were tested, in the following referred to as DNR-DNA_1 and DNR-DNA_2; DNA in both complexes originated from salmon eggs and the molar ratio daunorubicin/DNA-nucleotides was in both cases 19. The mean molecular weights were 82,000 (range 14,000–92,000) and 56,000 (range 5,000–300,000) for DNR-DNA_1 and DNR-DNA_2, respectively. Solutions of daunorubicin-DNA complex were always prepared just before use. Daunorubicin as hydrochloride was obtained from Farmitalia Co., Milan, Italy. Nonidet P 40 was obtained from Shell, Carrington, England.

Experimental Animals and Tumors. First-generation hybrids of female random-bred Swiss mice and male inbred DBA mice were used throughout the study. Toxicity experiments were performed as previously described [13]. The sensitive tumor studied was a hypotetraploid strain of Ehrlich ascites tumor. Development of resistance to daunorubicin was performed in vivo by treatment with increasing doses of drug during weekly passages of the tumor [6]. The resistant tumor line was maintained by treatment with doses of daunorubicin equal to LD_{10} during each passage: no drug was given in the last passage before experiments. In therapy experiments, mice received implantation of $15 \cdot 10^6$ cells. In all animal experiments, drug was administered by daily intraperitoneal (IP) injections for four days; in therapy experiments, the first dose was given 24 h after transplantation.

In vitro Experiments. Unless otherwise stated, the preparation of cells, incubation, and sampling procedure were performed as previously described in detail [14]. The standard medium contained 57 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 9 mM NaH_2PO_4, 51 mM Na_2HPO_4, 10 mM glucose, and calf serum constituting 5% by volume (pH adjusted to 7.45). In all experiments (except those shown in Figs. 5 and 6) the cell content in the final suspension was 0.5% by volume.

Lysed cells were prepared by incubation for 5 min in a buffer of the following composition: sucrose 250 mM, $CaCl_2$ 5 mM, Nonidet P 40 0.1% (v/v), and Tris-HCl 25 mM (pH adjusted to 7.45). The cellular content was separated by centrifugation at 6130 *g* for 1 min.

Cellular content of daunorubicin was determined by measuring the total drug fluorescence extracted from the cell pellet with 0.3 N HCl 50% ethanol solution as previously described [2, 14]. The concentration of drug in medium was determined spectrophotometri-

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cally (Unincam SP 1800 spectrophotometer) at 480 nm for daunorubicin and at 505 nm for daunorubicin-DNA complex. DNA in the medium was determined by the diphenylamine reaction after acid hydrolysis to DNA nucleotides [3, 8].

Results

To compare the therapeutic activity of free drug and complex in vivo, toxicity experiments were performed for the three compounds. Figure 1 shows a log probit plot of the results. As shown in other studies [12, 17] complex-bound daunorubicin was significantly less toxic than the free compound. In our study, LD_{10} of daunorubicin was found to be 1.6 mg/kg (5% confidence limits 1.45–1.77) as compared with 2.6 mg/kg (5% confidence limits 2.25–3.17) for DNR-DNA_1 and 3.2 mg/kg (5% confidence limits 2.72–3.80) for DNR-DNA_2. For both complexes, doses are calculated as daunorubicin.

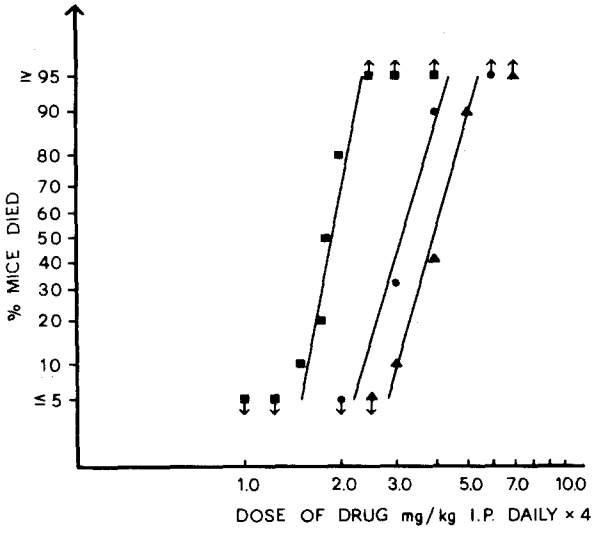

Fig. 1. Log probit plot of toxicity of daunorubicin and daunorubicin-DNA complexes. Mice in groups of ten were treated intraperitoneally with the drugs for four consecutive days. The toxicity of each dose was measured by the percentage of mice dying within one month. DNR (■); DNR-DNA_1 (●); DNR-DNA_2 (▲)

Table 1 shows the response of wild-type Ehrlich ascites tumor and a subline resistant to daunorubicin subjected to therapy with equitoxic doses of the three compounds. Cells were transplanted intraperitoneally, and the mice were treated intraperitoneally for four days. It appears that the therapeutic effect of both complexes was significantly better than that of free daunorubicin. On the other hand, neither daunorubicin nor complex-bound daunorubicin showed a significant therapeutic effect on the subline resistant to daunorubicin, i.e., complete cross resistance was demonstrated between daunorubicin and complex-bound daunorubicin.

Figure 2 shows the results of dialysis experiments with daunorubicin and daunorubicin-DNA complexes. It appears that the binding of daunorubicin to DNA reduces the rate of dialysis considerably. Thus, the half-life of daunorubicin was calculated as 80 min as compared with 630 min for both complexes.

Figure 3 shows the cellular drug uptake in vitro in the sensitive tumor and in the subline resistant to daunorubicin at equimolar concentrations with respect to daunorubicin. In all cases, a steady state is reached within 2 h. In both tumor lines the cellular uptake of daunorubicin was considerably lower when the drug was added as complex compared with free drug. However, the ratio between uptake at steady state in sensitive and resistant cells was equal for the three compounds, suggesting a common mechanism of transport for free drug and complex-bound drug in resistant cells.

To further investigate the mechanism of uptake of the daunorubicin-DNA complexes, the daunorubicin uptakes in whole cells and in cells lysed by the detergent Nonidet P 40 were compared. Exposure to this deter-

Table 1. Comparison of the effect of daunorubicin and daunorubicin-DNA complex on the life-span of mice bearing sensitive or resistant cells of Ehrlich ascites tumor

Compound	Dose mg/kg daily × 4	Sensitive tumor[a]			Daunorubicin-resistant tumor[a]		
		Survival time (range) days	% increase in life-span	No. of long-term survivors[b]	Survival time (range) days	% increase in life-span	No. of long-term[b] survivors
Control	–	11 (10–18)	–	–	15 (5–17)	–	–
DNR	1.5	18 (12–23)	64	0	16 (7–21)	7	0
DNR-DNA_1	2.5	21 (17–26)	91	1	17 (15–20)	13	0
DNR–DNA_2	3.0	23 (18–32)	109	2	15 (10–22)	0	0

Each group is composed of 18–20 mice
[a] 15×10^6 cells were inoculated IP on day 0
[b] Numbers of mice that survived 60 days after receiving the drug

gent converted the cells to isolated nuclei [14]; on the other hand, no significant effect on dialysis of daunorubicin-DNA complex could be demonstrated (data not shown). Figure 4 shows that the daunorubicin uptake in the lysed cells was considerably higher than that of whole cells, and no difference was observed between nuclei of sensitive cells and resistant cells. The high binding of daunorubicin to the lysed cells, which are unable to carry out endocytosis, and subsequent dissociation of the complex, suggests that a significant part of daunorubicin in the complex may be released fairly easily when a binding substance, such as nuclear DNA, is present and competes for the free drug.

In the mechanism of action of daunorubicin-DNA complex, the route of translocation of the drug across the membrane is an important factor. To study this process the uptake of daunorubicin and DNA was followed by simultaneous determination of the loss of each component separately from the medium during the incubation. Figures 5 and 6 show the concentrations of daunorubicin and DNA-nucleotides in the medium of cell suspension after incubation with the two complexes. Figure 5 shows that when sensitive cells were incubated with these complexes, the daunorubicin concentration in the medium decreased by more than 50% within 2 h, reflecting the cellular drug uptake. On the other hand, the DNA concentration in the medium was unchanged or slightly increased. The increment observed may reflect a release of cellular nucleotides induced by the complex or it may be due to DNA in cellular fragments.

Figure 6 shows the results obtained for resistant cells. As for sensitive cells, a significant disappearance of daunorubicin from the medium was noted without a

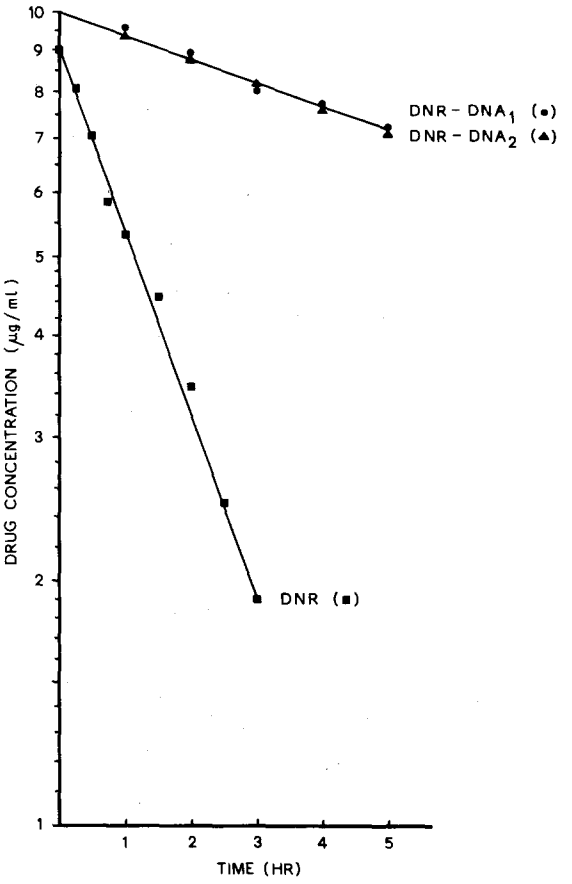

Fig. 2. Dialysis of daunorubicin and daunorubicin-DNA complexes. Dialysis bags of 10 ml containing 10 μg/ml drug were incubated in a large volume of isotonic Ringer solution, pH 7.4, at 37° C. The dialysis medium was stirred and removed at intervals of 15–30 min. At the times indicated, the concentrations of daunorubicin or complex in the bags were determined by spectrophotometry

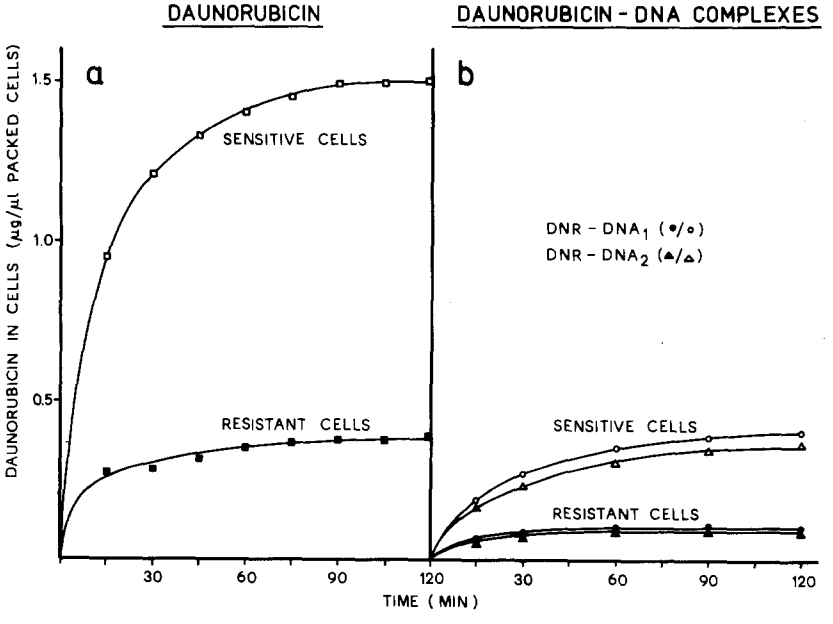

Fig. 3. Time course of the uptake of daunorubicin in sensitive cells from Ehrlich ascites tumor and in cells from the subline resistant to daunorubicin. Suspensions of cells (5-μl packed cells/ml) were incubated in a phosphate-buffered medium, pH 7.45, at 37° C. Daunorubicin (**a**) and complexes (**b**) were added at 10 μg/ml at zero time. At time indicated, the cellular content of daunorubicin after washings was determined by measuring the total drug fluorescence extracted from the cells

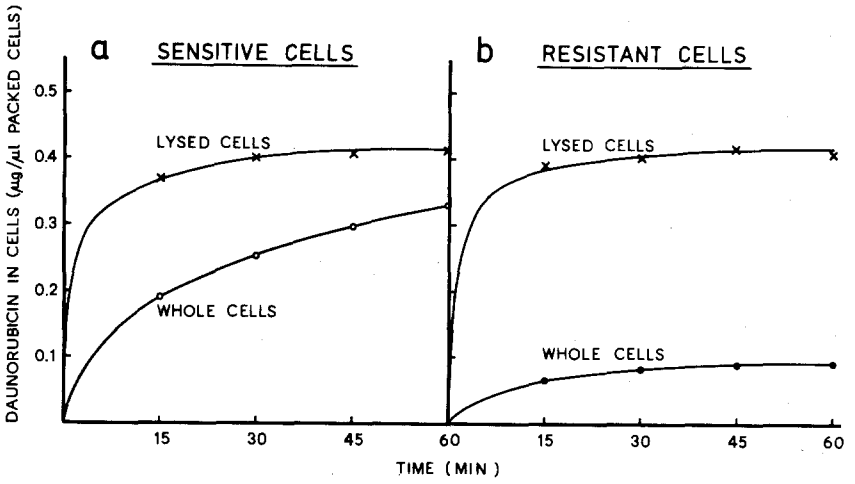

Fig. 4. Comparison of daunorubicin content in whole cells and lysed cells from sensitive cells (**a**) and resistant cells (**b**) in suspensions corresponding to 5-μl packed cells/ml. Daunorubicin-DNA complexes were added at 10 μg/ml with respect to daunorubicin at zero time. Method of drug uptake as described in Fig. 3

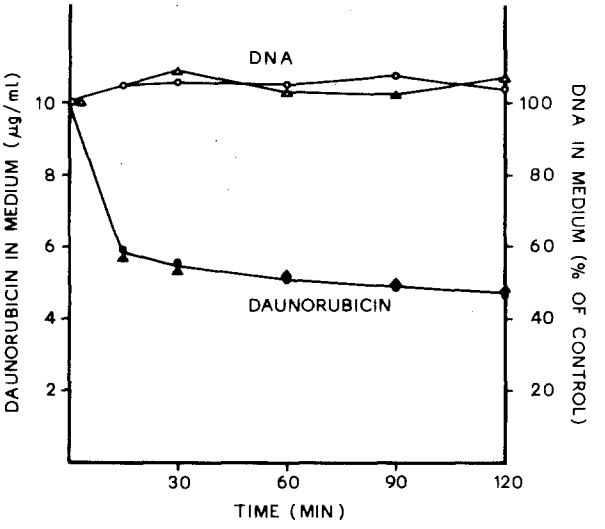

Fig. 5. Sensitive cells. Concentrations of daunorubicin and DNA nucleotides in medium of a suspension of sensitive cells corresponding to 50-μl packed cells/ml. Daunorubicin-DNA complexes were added at 10 μg/ml with respect to daunorubicin at zero time. The concentration of daunorubicin in the medium was determined by spectrophotometry. DNA in the medium was determined by the diphenylamine reaction after acid hydrolysis to DNA nucleotides. The determination of DNA is expressed as the percentage of a control which is a medium with corresponding drug content without cells. DNR-DNA$_1$ (●/○); DNR-DNA$_2$ (▲/△)

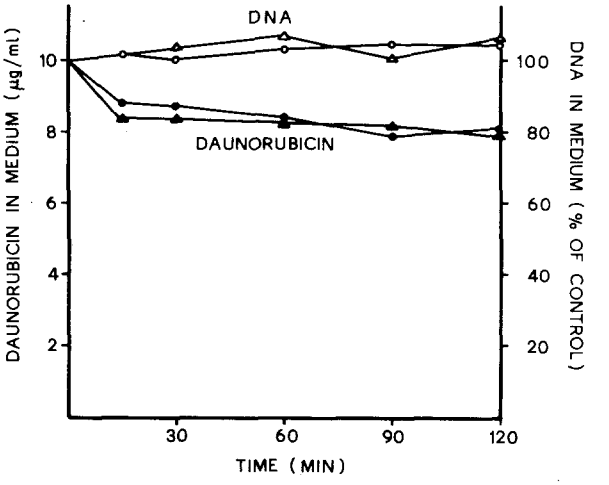

Fig. 6. Resistant cells. Concentrations of daunorubicin and DNA nucleotides in medium of a suspension of resistant cells corresponding to 50-μl packed cells/ml. The experimental conditions as described in Fig. 5. DNR-DNA$_1$ (●/○); DNR-DNA$_2$ (▲/△)

corresponding disappearance of DNA. The drop in the daunorubicin concentration was significantly lower in these cells than in sensitive cells, owing to the lower cellular uptake previously demonstrated for these cells. The steady-state level of DNA in the medium, despite the significant uptake of daunorubicin, may be an expression of an exchange of the extracellular macromolecular DNA with nucleotides originating from DNA taken up by endocytosis and subsequently released from lysosomes. However, in separate experiments in which dialysis of the medium was performed for 24 h prior to the determination of DNA, the dialyzable fraction of total DNA in the medium was less than 8% for both cell lines after incubation for 120 min.

Discussion

The present data demonstrate that daunorubicin-DNA complex has a lower toxicity and – in equitoxic doses – a higher therapeutic activity compared with free drug when injected into the same compartment as the inoculated tumor cells. These findings agree well with data obtained in other studies for daunorubicin-DNA [12, 17] or adriamycin-DNA [9, 12, 18]. According to the

concept of Trouet et al. [17], these results may be attributed to entry of the drug-carrier molecule by endocytosis preferentially in tumor cells and subsequent release of the free drug in the lysosomes. The results in this study do not support this hypothesis. Thus, no significant loss of DNA from the medium was observed in the suspension of sensitive cells even if more than 50% of the total daunorubicin content was taken up by the cells. In controls, no significant fraction of the extracellular DNA was dialyzable, indicating that the DNA measured does not represent nucleotides originating from DNA digested in lysosomes. This finding provides evidence that DNA does not serve as a carrier in the transport of DNR into Ehrlich ascites tumor cells.

The lower steady-state level of daunorubicin in both cells lines when added as complex compared with those obtained for the free drug further supports this concept. A lower level of uptake at steady state cannot be explained by endocytosis, as this mode of transport may only influence the rate of uptake. At equilibrium, the cell/medium ratio is expected to be equal for free drug and complex as the drug is released from the carrier intracellularly. On the other hand, the high affinity of daunorubicin to DNA indicates that DNA may serves as an extracellular vehicle for daunorubicin. If this function of DNA is maintained in a closed compartment, the cellular steady-state level of daunorubicin is expected to be reduced.

Comparing the cellular effect of free drug and complexes of daunorubicin and adriamycin on Novikoff hepatoma cells, human mammary carcinoma cells, and human acute leukemia blast cells, Seeber et al. [11] also reach the conclusion that DNA complex dissociates at the outer cell membrane. The demonstration in this study of cross resistance between daunorubicin and daunorubicin-DNA also agrees well with this concept. However, the slower release of anthracyclines from DNA may result in significant influence on the pharmacokinetics of the drugs. Thus, Attasi et al. [1] showed that free adriamycin was cleared from plasma of mice about nine times faster than that of adriamycin-DNA complex prepared according to the method of Trouet el al. [17]. This difference may explain the superior antitumor effect of the complexes. Another explanation could be a synergism in the antitumor action between DNA and the drug as suggested by Marks et al. [9].

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