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Uptake of the Daunorubicin-DNA Complex in Cultured Fibroblasts

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Summary. *The uptake and fate of the daunorubicin-DNA complex have been studied in cultured rat embryo fibroblasts. ^{125}I-DNA was digested by the cells and appeared as low molecular weight fragments in the incubation medium. Subcellular fractionation of fibroblasts, previously incubated with the complex, showed that daunorubicin was localized to nuclei and lysosomes. At a high incubation concentration (17.5 μM), the accumulation of the free drug exceeded that of the complex. However, at a lower concentration (1.75 μM), the accumulation of the complex was as high as that of the free drug.*

The results are consistent with an uptake of the complex into cultured rat embryo fibroblasts by endocytosis. However, it cannot be excluded that the complex partly dissociates in the incubation medium, and that daunorubicin and DNA thereafter enter the cells separately.

Introduction

Daunorubicin (DNR) is widely used to induce remission in patients with hematologic malignancies (Bernard et al., 1969; Weil et al., 1973). Its antitumoral activity is most probably due to intercalation of drug molecules between adjacent base pairs in the DNA double helix, thereby inhibiting gene duplication and transcription (Calendi et al., 1965; Pigram et al., 1972). The use of DNR for maintainance therapy is limited by a poorly understood, deleterious effect on the cardiac muscle, which is dose dependent (von Hoff et al., 1977).

In 1972, a new principle was introduced into cancer chemotherapy when evidence was presented that the selectivity of DNR could be enhanced by administering the drug linked to DNA (Trouet et al., 1972). Thus, the therapeutic activity of DNR in animals with experimental tumors could be enhanced, and toxicity reduced, by giving the drug as a DNA complex.

According to this concept, the cellular uptake of DNR-DNA occurs by endocytosis. Since lysosomes contain DNA-digesting enzymes (de Duve et al., 1955), DNR molecules can then be liberated and diffuse into other cellular compartments, e.g., the nucleus. The cytotoxic effects of DNR-DNA on different cell types will therefore depend on the endocytotic properties of the cells. Evidence has been presented that at least some malignant cells have a higher endocytotic activity than their normal counterparts (Easty, 1964; Gey, 1956; Ghose et al., 1962).

The DNR-DNA complex can easily be formed by taking advantage of the high affinity of DNR for its natural target. The binding constant to DNA in vitro is about 10^6 M^{-1} (Zunino et al., 1972). However, since the binding is noncovalent in nature, free DNR molecules are also present.

To study the mechanisms involved in the cellular transport and processing of DNR-DNA, we have used cultured rat embryo fibroblasts as a model system. A preliminary report of some of these results has been presented earlier (Noël and Trouet, 1977).

Materials and Methods

Cell Culture. Rat embryo fibroblasts were obtained and cultivated as described by Tulkens et al. (1974). For incubation with drugs, cells from the first or second subculture were used as soon as they reached confluency. All incubations were performed at 37° C.

After the incubation, the cells were washed three times with phosphate buffered saline (PBS) (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 8 mM; and KH_2PO_4, 1.5 mM; pH 7.4), harvested in this medium by scraping with a rubber 'policeman', and finally disrupted by ultrasonication (15 s at 75 W, 20 KHz).

Cell Fractionation Procedure. After incubation with the drug, the cells were washed, harvested, homogenized, and fractionated as described earlier (Tulkens et al., 1974; Noël et al., 1978). Cell fraction-

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ation involves the preparation of a nuclei-free cytoplasmic extract by differential centrifugation and the centrifugation of the various components of this extract to their equilibrium densities in a sucrose gradient.

Drugs. Daunorubicin hydrochloride was kindly supplied by Rhône-Poulenc, S. A., Paris, France: DNA (herring sperm, type VII) was purchased from Sigma Chemical Co., St. Louis, USA. It was dissolved in 0.15 M NaCl, heated to 95° C, and filtered through a Millipore filter (0.8 μ). Before use, the solution was autoclaved at 120° C for 15 min. After slow cooling, DNR was added to a final molar ratio of 1 : 20 (drug/nucleotides). Concentrations of DNR-DNA given in the text refer to DNR.

DNA (autoclaved) was iodinated according to the method of Orosz and Wetmur (1974). $Na^{125}I$ was obtained from Amersham Searle, Inc., England. More that 95% of the radioactivity of the iodinated DNA solution could be precipitated by TCA (27%).

Assays. DNR in the sonicated cell suspensions and in the subcellular fractions was assayed by fluorometry (excitation and emission wavelengths: 485 and 580 nm, respectively) after precipitation with TCA (27%) followed by centrifugation. This treatment releases DNR from its association with cellular constituents. The drug concentration in each sample was calculated by comparison with identically treated standard solutions.

After incubation with ^{125}I-DNA, the radioactivity was measured (Autogamma scintillation spectrometer Model 5160, Packard Instruments) in the harvested cell suspensions, as well as in the supernatants and precipitates of the incubation media after treatment with TCA (27%).

Marker enzymes and protein were assayed as described by Tulkens et al. (1974).

Results

During incubation of fibroblasts with ^{125}I-DNA (117 μg/ml), the radioactivity associated with the cells reached a plateau within 5 min (Fig. 1). On the other hand, there was a continuous increase in the level of TCA-soluble radioactivity in the incubation medium.

Time courses for the accumulation of DNR in fibroblasts incubated with free or DNA-bound DNR are shown in Fig. 2. At a drug concentration of 17.5 μM, the accumulation of free DNR markedly exceeded that of the complexed drug. However, at a concentration of 1.75 μM, the accumulation of DNA-bound DNR was as large as that of the free drug.

Figure 3 shows the distributions of DNR, protein, and marker enzymes in the cytoplasmic extract of fibroblasts previously incubated for 10 h with DNR-DNA (17.5 μM). The results have been plotted in the form of standardized histograms (Leighton et al., 1968). The drug distribution corresponded very well to the distribution of the lysosomal marker enzymes N-acetyl-β-glucosaminidase and cathepsin B. On the other hand, the distribution of DNR did not correspond to the distribution of cytochrome oxidase (marker enzyme for mitochondria), NADPH: cytochrome-C reductase (endoplasmic reticulum) or 5′-nucleotidase (plasma membrane).

The nuclear concentration of DNR cannot be determined directly from the drug concentration in the nuclear fraction, since this is heavily contaminated with lysosomes. However, taking into account the relative amount of lysosomal enzymes in the nuclear fraction, it is possible to calculate the true distribution of DNR between nuclei and lysosomes (Noël et al., 1978). Figure 4 shows that the lysosomal concentration of DNR markedly exceeded the nuclear concentration after incubation of fibroblasts for 10 h with DNR-DNA (17.5 μM).

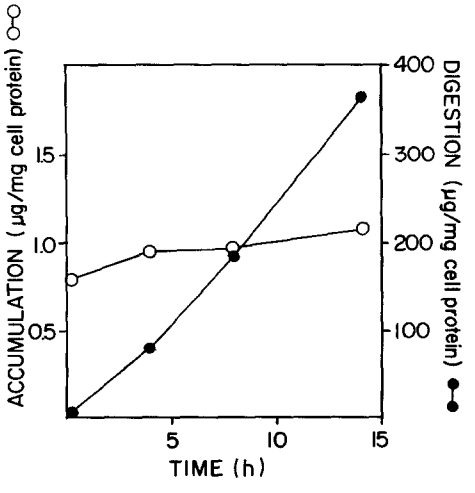

Fig. 1. Accumulation (*open circles*) and digestion (*filled circles*) of ^{125}I-DNA in cultured fibroblasts incubated for different periods of time at a concentration of 117 μg/ml

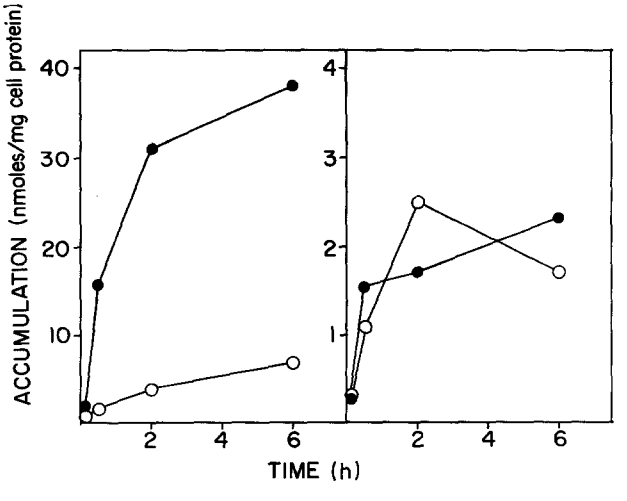

Fig. 2. Accumulation of DNR in cultured fibroblasts incubated for different periods of time with DNR (*filled circles*) or DNR-DNA complex (*open circles*) at a concentration of 17.5 μM (*left*) or 1.75 μM (*right*)

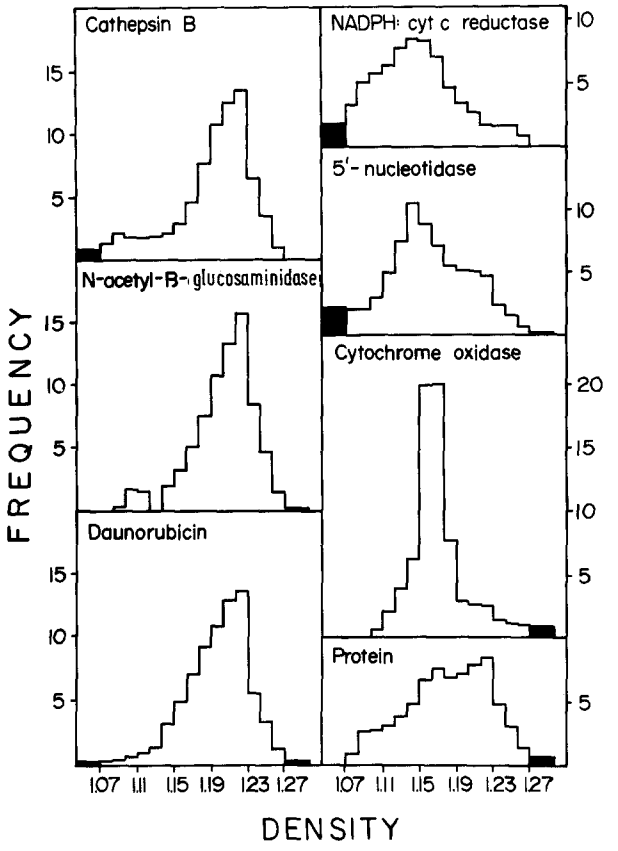

Fig. 3. Distribution of DNR, protein, and marker enzymes in the cytoplasmic extract of fibroblasts incubated with DNR-DNA (17.5 μM) for 10 h. The frequency (*ordinate*) is $\Delta Q/(Q \times \Delta p)$, where ΔQ is the amount of constituent in the section, Q the total amount of the constituent in all sections, and Δp the density increment for each section (0.0133). *Solid areas* represent the amount of constituent recovered at densities below 1.07 and above 1.27

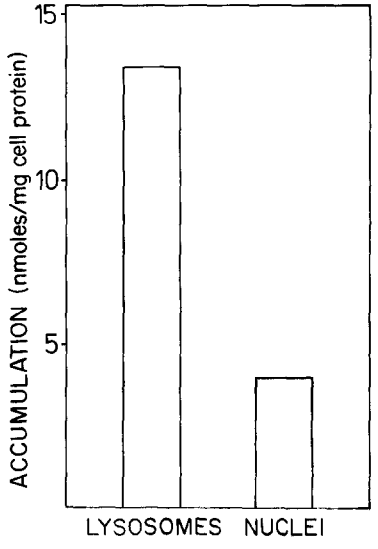

Fig. 4. Distribution of DNR between nuclei and lysosomes in fibroblasts incubated for 10 h with the DNR-DNA complex (17.5 μM)

Discussion

After incubating fibroblasts with ^{125}I-DNA, very little radioactivity was found associated with the cells. Quantitatively, the appearance of TCA-soluble radioactivity in the incubation medium was much more important. After incubation for 14 h, the digestion of ^{125}I-DNA exceeded the amount associated with the cells by a factor of 400. This cannot be explained by he DNAse activity of the culture medium (containing 10% calf serum), since the TCA-soluble radioactivity in incubations without cells has been deducted from the values presented. Neither can it be explained by secretion of hydrolytic enzymes from the cells, since incubation of ^{125}I-DNA in a medium previously incubated with fibroblasts for 4 days did not increase the TCA-soluble radioactivity as compared with incubation in a fresh medium. The most likely explanation for the appearance of TCA-soluble radioactivity in the culture medium is that after being taken up into the fibroblasts by endocytosis the ^{125}I-DNA is digested in the lysosomes and thereafter the low molecular fragments diffuse out from the cells.

At high DNR concentration (17.5 μM), the accumulation of complexed DNR was considerably lower than that of the free drug. On the other hand, at 1.75 μM, which is closer to the plasma concentrations found in patients during treatment with the complex (Eksborg et al., 1978), the accumulation of complexed DNR was as high as that of the free drug. This is in agreement with observations on human leukemia cells (Paul et al., 1979). Since endocytosis is an active process, it should be saturable. However, at least part of the difference observed at high and low DNR concentrations could be explained in another way. Considering the equilibrium reaction DNR + DNA $\rightleftarrows$ DNR-DNA, it can be calculated that the *fraction* of free drug decreases with increasing concentrations of DNR and DNA (added in a constant molar ratio).

After incubation of the fibroblasts with DNR-DNA, the drug was found almost exclusively in the lysosomes (76%) and in the nuclei (24%). However, the presence of DNR in the lysosomes cannot be taken as evidence that the drug enters the cells as the DNA complex by endocytosis, since free DNR also accumulates in the lysosomes of cultured fibroblasts (Noël et al., 1978).

From the present data, it can be concluded that DNR added as a DNA complex to fibroblasts in culture reaches the nuclei as well as the lysosomes. However, the experiments do not permit a quantitative evaluation of the possible uptake mechanisms, i.e., endocytosis versus diffusion.

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