formed is not clear. We believe that the use of a carotenoid dispersion with proxanol holds better promise, for this polymer has been long used in the food and medical industry for the stabilization of perfluorocarbons as components of artificial blood, and the method of preparing the agent on the basis of proxanol is simple and easily reproducible.

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Intravitam Quantitative Assessment of the Intracellular Distribution of Doxorubicin in Tumor Cells

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 120, № 11, pp. 518-521, November, 1995
Original article submitted April 12, 1995

Intravitam quantitative assessment of the intracellular distribution of doxorubicin was carried out in anthracycline-sensitive tumor cells of CaOv human ovarian carcinoma grown in a monolayer culture *in vitro* and in P388 leukemia cells transplanted to mice *in vivo*. The intracellular content of antibiotic unbound to DNA was assessed from the total fluorescence of cells at the end of incubation. The DNA-bound active fraction of intracellular doxorubicin makes up the bulk of intracellular doxorubicin in both cell lines, whereas only less than 30% of anthracycline accumulated in a cell is not bound to DNA. The results confirm that the predominant accumulation of doxorubicin in cell nuclei may characterize their sensitivity to anthracyclines.

Key Words: *doxorubicin; tumor cells; intracellular distribution*

The intracellular content of antitumor drugs is an important parameter determining their biological activity. This is indeed true for the anthracycline antibiotics, which for many years have been widely used in the chemotherapy of malignant tumors. Nevertheless, the mechanism of action of anthracyclines, and, specifically, the factors governing the development of resistance, are still not clear. For example, some authorities claim that anthracycline resistance correlates with a decrease of their content in the cell [6,12]. But such a relationship is not always observed [3,5], and the intracellular distribution of the antibiotics appears to be a more important factor, specifically, the amount interacting with DNA and accumulated in the nucleus [4,9], or the ratio of the nuclear to cytoplasmic fractions of the drug [8,11].

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0007-4888/95/0011-1140$12.50

Nuclear fluorescent microscopy and extraction biochemical methods routinely used for this purpose preclude investigations with live cells, require sophisticated equipment, and are time-consuming. With this in mind, we developed a new methodological approach to the intravitam assessment of the share of the active nuclear fraction of anthracycline bound to DNA in the total amount of the cytostatic accumulated in the cell. It is based on the known fact that interaction with DNA, but not with other cellular macromolecules, leads to an appreciable reduction of antibiotic fluorescence. Hence, by comparing the extinction of anthracycline fluorescence in the course of a short-term incubation with cells with the total fluorescence of cells it is possible to determine the share of the active fraction of anthracycline in the total amount of intracellular accumulated drug. In this study such an approach was used to investigate the intracellular distribution of doxorubicin (DOX) in sensitive cells of a monolayer culture of CaOv human ovarian carcinoma and *in vivo* transplanted mouse leukemia P388.

MATERIALS AND METHODS

Anthracycline-sensitive cells of a monolayer culture of CaOv human ovarian carcinoma and of P388 leukemia transplanted in BDF_1 mice were used. CaOv cells were cultured *in vitro* as described previously [2]. All experiments were carried out on cells in the stationary growth phase. Leukemia P388 was transplanted intraperitoneally with ascitic fluid diluted in Hanks' solution (10^6 cells per mouse). The final concentration of cells of both types in the suspension was 2×10^6 cells/ml. The cells were counted under the microscope in Goryaev's chamber. DOX (Farmitalia) in a concentration of 1×10^{-6} M was used in the study.

The interaction between anthracycline and DNA was assessed by a spectrofluorometric method developed previously by us [1] and simultaneously by other scientists [13]. This method permits a quantitative assessment of anthracycline binding to DNA from the extinction of its fluorescence during a brief incubation with live cells. The method is based on the phenomenon of an appreciable reduction of anthracycline fluorescence during its reaction with DNA, but not with other cellular macromolecules.

Since there is a direct relationship between the antibiotic concentration and fluorescence in the studied range of DOX concentrations, the amount of DOX bound to DNA (nuclear fraction of DOX - DOX_N) was calculated after the formula: $DOX_N = DOX_{IN}[(DOX_{FL.IN}-DO_{FL.REM})/DOX_{FL.IN}]$, where

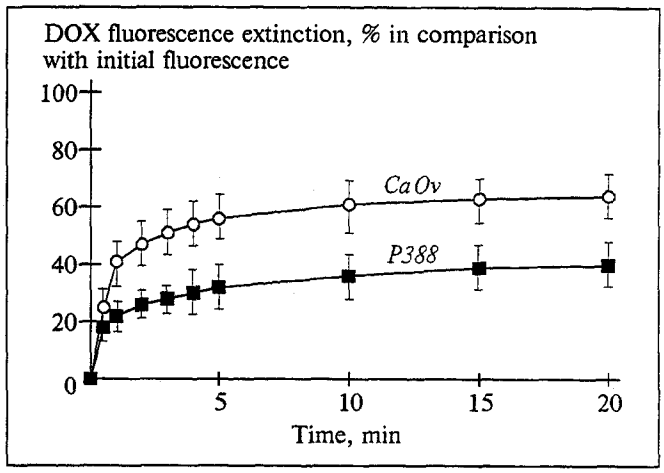

Fig. 1. Extinction of DOX fluorescence during incubation with CaOv human ovarian carcinoma and P388 murine leukemia cells. Here and in Fig. 2: the concentration of cells in the suspension was 2×10^6 cells/ml; the initial concentration of DOX was 1×10^{-6} M.

DOX_{IN} is the initial concentration of DOX in M; $DOX_{FL.IN}$ is the initial fluorescence of DOX; and $DOX_{FL.REM}$ is the fluorescence of the DOX remaining after a 20-min incubation with cells.

Similar calculations were performed when assessing the amount of DOX not bound to DNA (cytoplasmic fraction of DOX - DOX_{CYT}):

$$DOX_{CYT}=DOX_{IN}(DOX_{FL.C}/DOX_{FL.IN}),$$

where DOX_{IN} is the initial concentration of DOX in M; $DOX_{FL.IN}$ is the initial fluorescence of DOX; and $DOX_{FL.C}$ is the fluorescence of DOX in the cell seduiment after a 20-min incubation.

The intensity of anthracycline fluorescence was measured with a Hitachi F2000 spectrofluorometer. The experiments were carried out with constant stirring of the suspension. Hanks' solution, pH 7.0, was the incubation medium. The measurements were carried out at an escitation wavelength of 470 nm and a fluorescence wavelength of 590 nm, and an optical slit of 10 nm. A ZhS-18 yellow filter was used to cut off the esciting light scattered by the cells during work with the cell suspension. Quartz cuvettes with a 1-cm optical path were used in which the absorption of DOX in Hanks' solution was no more than 5%.

The measurements were carried out in 3 or 4 samples of each experimental group, and each experiment was repeated three times. The results were statistically processed after Fisher-Student. The mean values and standard deviations are presented.

RESULTS

The sequence of the procedures was as follows. First, the amount of DOX binding to intracellular

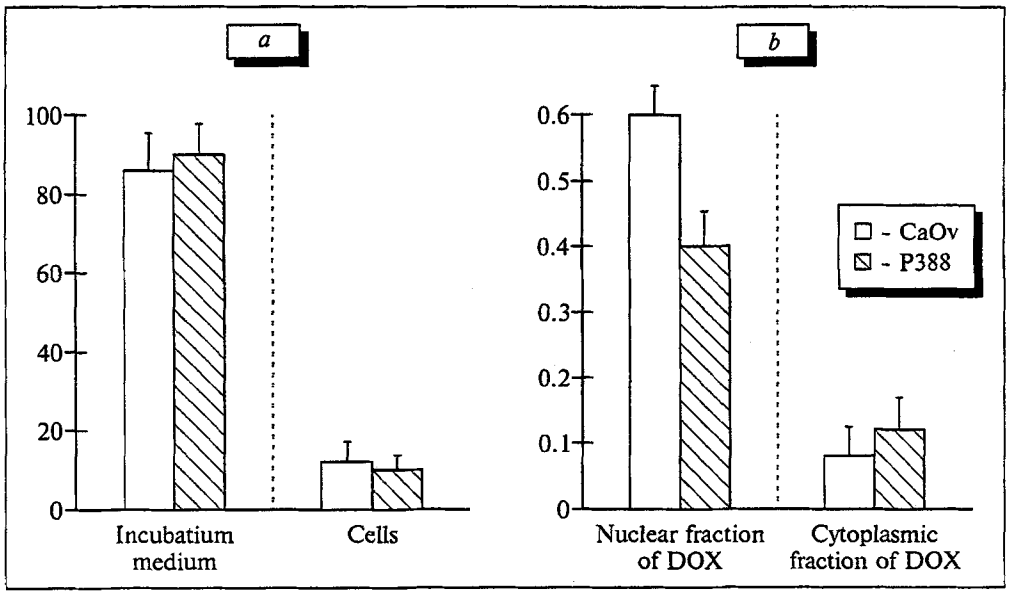

Fig. 2. Distribution of DOX in CaOv human ovarian carcinoma and P388 murine leukemia cell suspensions. Ordinate: *a*) DOX fluorescence, in % of residual fluorescence of DOX in cell suspension after 20-min incubation; *b*) amount of DOX ($\times 10^{-6}$ M).

DNA during the 20-min incubation with the cell suspension was assessed. Previously it had been shown that it was during binding with DNA but not with other cellular macromolecules that an appreciable reduction of anthracycline fluorescence occurred. This gave grounds for developing a method for quantitative assessment of the active intracellular DNA-binding fraction of anthracyclines. The magnitude of antibiotic fluorescence extinction during its brief incubation with live cells is an indicator of the active fraction [1,13]. Figure 1 shows that the extinction of DOX fluorescence was more expressed in the CaOv cell suspension than in the P388 leukemia cells and at the end of incubation was 60 and 40%, respectively, in comparison with the initial fluorescence of DOX added to the cells. This means that 60 and 40% of the initial amount of DOX added to cells interact with intracellular DNA in suspensions of CaOv and P388 cells, respectively.

Second, after the incubation was over, the residual fluorescence of DOX was measured separately in the cells and incubation medium, for which the cell suspension was centrifuged for 5 min at 1000 rpm. The results are presented in Fig. 2. The fluorescence of DOX in the supernatant in comparison with the residual fluorescence of the antibiotic in the cell suspension after incubation was approximately the same in both cell lines: 87% for CaOv and 90% for P388. The ratio of DOX fluorescence in the cell sediment to the residual fluorescence of the antibiotic in the cell suspension after incubation was approximately the same for CaOv and P388 cells: 10-15% (Fig. 2, *a*).

The data presented in Fig. 2 indicate that the amount of DOX bound to DNA (the nuclear fraction of anthracycline) was 0.6×10^{-6} M and 0.4×10^{-6} M in CaOv and P388 cells, respectively. This is appreciably lower than the amount of intracellular DOX not binding to DNA (the cytoplasmic fraction of anthracycline), which was 0.08×10^{-6} M and 0.11×10^{-6} M for CaOv and P388 cells, respectively. The ratio of the DOX content in the nuclear and cytoplasmic fractions in CaOv and P388 cells was 7.5 and 3.6, respectively.

Hence, a simple method based on assessing the extinction of DOX fluorescence during incubation of the agent with a cell suspension and measurement of cell fluorescence after incubation permits a quantitative characterization of several parameters of anthracycline interaction with live cells, making it possible to: 1) follow up and assess the final level of interaction between DOX and intracellular DNA (the intracellular nuclear fraction of the antibiotic); 2) estimate the amount of intracellular DOX not binding to DNA (the intracellular cytoplasmic fraction of the antibiotic); and 3) determine the ratio of DOX bound or not bound to intracellular DNA, that is, the intracellular distribution of the antibiotic between the nucleus and cytoplasm.

The predominant intracellular accumulation of DOX in the nuclei of anthracycline-sensitive and weakly resistant tumor cells was previously demonstrated by much more laborious methods. For example, a comparative study of 4 resistant human pulmonary carcinoma cell lines by laser fluorescent microscopy revealed a clear-cut relationship between the level of resistance and the intracellular distribution of DOX. A transition from "predominantly nuclear to predominantly cytoplasmic" accumulation of DOX was observed in cells differing in level of resistance by 10 to 2000 times [8]. Similar data

were obtained in studies of K562 human leukemia cells: DOX was localized virtually entirely in the nuclei of sensitive cells, whereas in resistant cells appreciable accumulations of the anthracycline were seen in the cytoplasm [12]. Studies of the intracellular distribution of DOX in blast cells of patients with acute myeloid leukemia by laser scanning microscopy showed that the bulk of DOX is accumulated in the nuclei in primary untreated patients, similarly as in sensitive cells, and the ratio of nuclear to cytoplasmic fluorescence of the anthracycline is always more than 3. On the other hand, in blasts of patients with relapses of the disease DOX is accumulated in the cytoplasm and virtually does not penetrate into the nucleus, similarly as in highly resistant cell lines [7,10]. Modifiers of multiple drug resistance that increase sensitivity to the cytotoxic action of DOX alter the intracellular distribution of the antibiotic. After exposure to modifiers, the content of DOX in the nucleus markedly increases, whereas in the cytoplasm it drops [9,11].

Our findings permit us to conclude that, along with the total amount of anthracycline entering the cells, the intracellular distribution of the antibiotic is an important factor determining the sensitivity or resistance of cells to the antitumor agent. The most important parameter is probably the amount of anthracycline reaching the nucleus and binding DNA, as well as the ratio between the active nuclear fraction of intracellular antibiotic and the amount that is accumulated in the cytoplasm.

The simple approach developed and used in this study enabled us to assess this ratio in anthracycline-sensitive live tumor cells of mouse and man and demonstrated that the bulk of intracellular DOX forms a complex with DNA, in other words, an active nuclear fraction of anthracycline. This is in line with published data and confirms that the predominant accumulation of DOX in cell nuclei may be a characteristic of their antibiotic sensitivity.

The study was sponsored by the J. Soros International Research Foundation and by the Russian Foundation for Basic Research.

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