QUANTITATIVE DETERMINATION OF BINDING OF ANTHRACYCLINE ANTIBIOTICS WITH DNA

T. A. Bogush, E. P. Baranov, S. V. Egudina, and N. I. Tankovich

UDC 615.33.015.44.07

KEY WORDS: doxorubicin; Hoechst dye; DNA; thymocytes

The development of a new method of intravital quantitative assay of accumulation of anthracycline antibiotics in cells was described in a previous publication [2]. However, there is as yet no strict quantitative assessment of the contribution of anthracycline binding directly with intracellular DNA to the accumulation process, although this is a decisive factor in the realization of the biological activity of this group of antitumor preparations. Extraction methods which can be used are quite crude and do not permit accurate determination of the anthracycline fraction found with DNA.

In the investigation described below, a new method of intravital assessment of intracellular binding of anthracycline with the cell DNA has been developed, using the dye Hoechst 33258, which binds specifically with DNA, during which the quantum yield of its fluorescence increases significantly [2]. In view of the hypothesis that anthracyclines compete with the Hoechst dye for landing sites on DNA [6], this means that on their addition to the Hoechst–DNA complex, displacement of the bound dye must take place, and in that case the quantity of antibiotic bound can be judged from the degree of fluorescence of the dye.

EXPERIMENTAL METHOD

Hoechst dye 33258 ("Aldrich," USA) and chicken erythrocyte DNA ("Reanal," Hungary) were used. Chromatographically pure doxorubicin was generously provided by Professor Yu. V. Dudnik (Research Institute for the Search for New Antibiotics, Academy of Medical Sciences of the USSR). Thymus cells from C57BL/6 mice aged 2 months, bred at the "Stolbovaya" Nursery, were used. The animals were killed by decapitation and the thymus was removed and transferred into Hanks' solution, without phenol red (Moscow Bacterial Preparations Factory, Ministry of the Medical and Biological Industry of the USSR), cooled to 4°C. To obtain a suspension of lymphocytes the stroma of the thymus was incised and the cells were eluted from it into incubation medium. The resulting cell suspension was washed twice by centrifugation for 5 min each time at 1200 rpm, and the residue was resuspended in Hanks' solution to a final concentration of 10^6 cells/ml. The experiments were conducted at a temperature of 20°C. The fluorescence measurements were made on a "Hitachi M-850" spectrofluorometer (Japan). The spectral width of the slits was 5 nm. Absorption spectra were recorded on a "Hitachi U-2000" spectrophotometer (Japan).

EXPERIMENTAL RESULTS

To determine the range of working concentrations of the Hoechst dye at which the fluorescence of the dye bound in cells with DNA is not dependent on its concentration, but is determined by the number of free landing sites

Research Institute of Experimental Diagnosis and Treatment of Tumors, Oncologic Scientific Center, Russian Academy of Medical Sciences, Moscow. Troitsk Branch, I.V. Kurchatov Institute of Atomic Energy, Moscow Region. (Presented by Academician of the Russian Academy of Medical Sciences, N. N. Trapeznikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 113, No. 6, pp. 636-638, June, 1992. Original article submitted October 25, 1991.

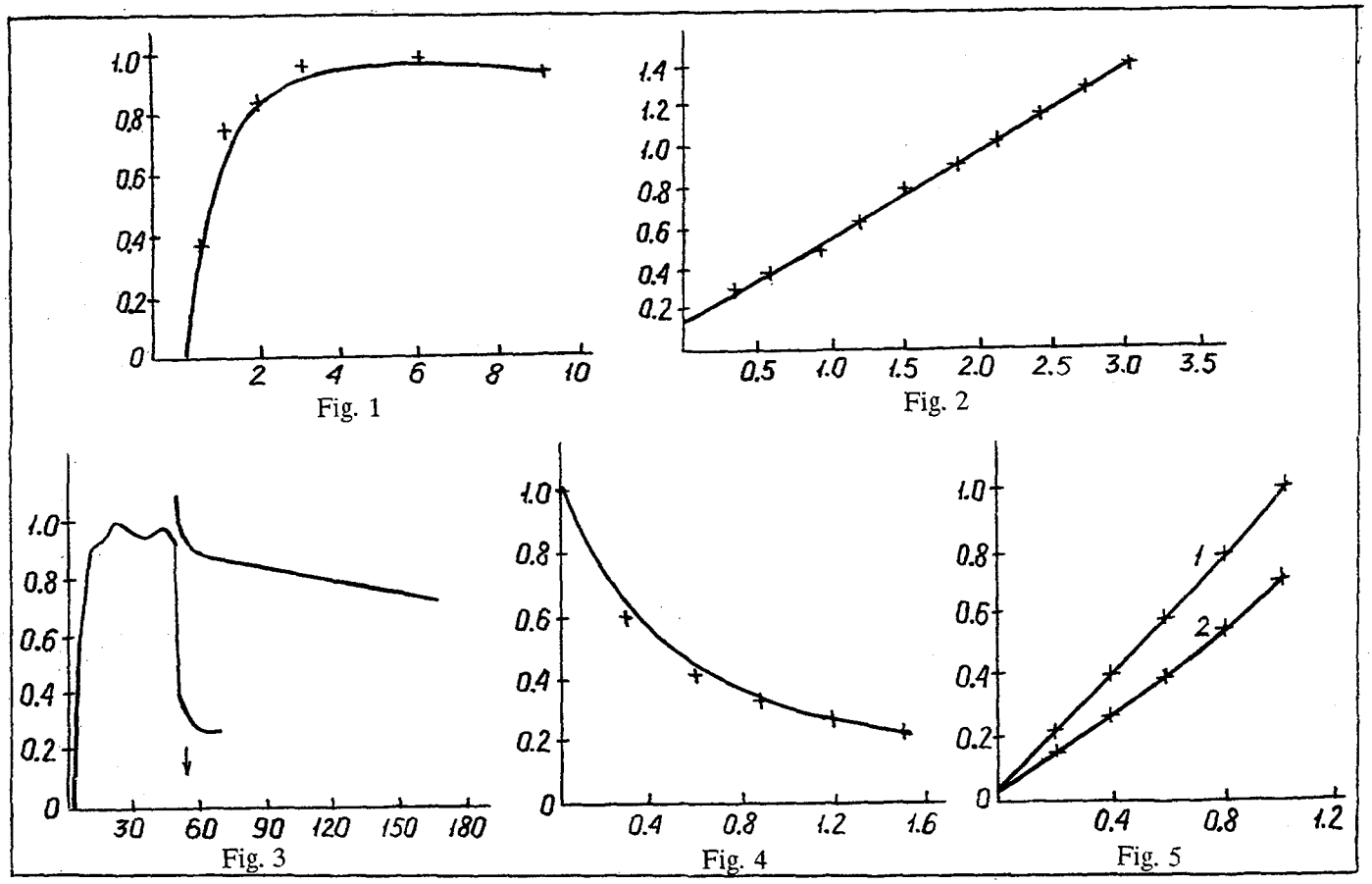

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 1. Dependence of intensity of fluorescence of Hoechst 33258 on its concentration on addition of dye to thymocyte suspension (10^6 cells/ml) after establishment of stationary level of fluorescence. Abscissa, concentration of Hoechst 33258 (in μM) Ordinate, intensity of fluorescence (relative units).

Fig. 2. Dependence of intensity of fluorescence of Hoechst 33258 ($3 \cdot 10^{-6}$ M) on DNA concentration in solution. Abscissa, DNA concentration (in μM base pairs). Ordinate, intensity of fluorescence (relative units).

Fig. 3. Dependence of intensity of fluorescence of Hoechst 33258 ($3 \cdot 10^{-6}$ M) on time after its addition to thymocyte suspension (10^6 cells/ml) and effect of doxorubicin on this process (arrow indicates time of addition of antibiotic to medium in concentration of 10^{-6} M). Left axis corresponds to intensity of fluorescence of dye (curve joining points), right – doxorubicin (curve joining crosses). Abscissa, time (min). Ordinate, intensity of fluorescence (relative units), left – at wavelength of 454 nm, right – 590 nm.

Fig. 4. Dependence of fluorescence of Hoechst 33258 ($3 \cdot 10^{-6}$ M), bound with DNA ($2 \cdot 10^{-6}$ M base pairs) on concentration of doxorubicin in medium. Abscissa, concentration of doxorubicin (μM). Ordinate, intensity of fluorescence (relative units).

Fig. 5. Intensity of fluorescence of doxorubicin (1) and of doxorubicin in the presence of DNA ($2 \cdot 10^{-6}$ M base pairs) (2), depending on concentration of antibiotic. Abscissa, concentration of doxorubicin (in μM). Ordinate, intensity of fluorescence, relative units.

on DNA, dependence of the intensity of fluorescence of the Hoechst dye on its concentration in the thymocyte suspension was recorded (Fig. 1). Clearly, within the dye concentration range of 0.5 to $3 \cdot 10^{-6}$ M the intensity of its fluorescence increased with an increase in its concentration in the incubation medium. Starting with a concentration of the dye of $3 \cdot 10^{-6}$ M the intensity of its fluorescence remained virtually constant. This means that within this con-

centration range all landing sites accessible for the dye on DNA were occupied, for the intensity of fluorescence of Hoechst 33258 in the cells is determined only by its binding with DNA.

In order to determine, under the experimental conditions chosen, the quantity of DNA accessible for the dye, model experiments were carried out to assess dependence of the intensity of fluorescence of the dye in a concentration of $3 \cdot 10^{-6}$ M on the content of pure DNA in the incubation medium (Fig. 2). Comparison of the curves shown in Figs. 1 and 2 shows that it was $2 \cdot 10^{-6}$ M, for at this DNA concentration in the solution the intensity of fluorescence of Hoechst 33258 corresponds to maximal fluorescence of the same amount of dye in incubation medium containing 10^6 cells/ml.

Since, according to data in the literature [6], Hoechst dye and anthracycline antibiotics can compete for landing sites on DNA, addition of anthracyclines to cells incubated with Hoechst 33258 ought to lead to quenching of fluorescence of the latter. A characteristic quenching curve obtained in our experiments is shown in Fig. 3.

For every point on this kinetic curve the quantity of antibiotic which must be added to pure DNA, taken in the chosen concentration (Fig. 2) in order to obtain the same decrease in the intensity of fluorescence of bound Hoechst 33258 can be determined. These data are given in Fig. 4. On the other hand, at any concentration of the antibiotic in the solution, that part of it which is bound with DNA can be determined from the quenching of its fluorescence, for we know [1] that bound molecules "do not fluoresce." By comparing the curve of dependence of the intensity of fluorescence of doxorubicin in solution and in the presence of DNA, at different concentrations of the antibiotic, the fraction of the antibiotic bound with DNA can be determined from their difference (Fig. 5).

The method we have developed, unlike the previous method [2], thus enables not the total level of intracellular accumulation of anthracyclines, but the quantity of antibiotic directly bound with DNA at any given moment of time after addition of the preparation to the cells, to be determined. This marks a real advance toward the solution of an important problem, namely the preclinical assessment of the sensitivity of cells, including tumor cells, to the cytotoxic action of antitumor preparations. The grounds for this conclusion are given by the abundant evidence that intracellular accumulation and binding of anthracyclines with DNA correlate directly with the sensitivity of the cells to the action of antibiotics [4, 5].

LITERATURE CITED

1. E. P. Baranov, V. Kh. Brikenshtein, T. V. Dmitrevskaya, et al., Antibiotiki, No. 3, 208 (1984).
2. T. A. Bogush, E. P. Baranov, A. B. Kozorez, et al., Antibio. Khimioter., **35**, No. 11, 16 (1990).
3. D. J. Arndt-Jovin and P. M. Jovin, J. Histochem. Cytochem., **25**, No. 3, 585 (1977).
4. A. A. Ilindenburg, M. A. Barker, E. Gleyzer, et al., Cancer Res., **47**, 1421 (1987).
5. S. Nagoaka, S . Kawasaki , Y. Kario et al., Eur. J. Cancer Clin. Oncol., **23**, 1297 (1987).
6. H. D. Preisler, Cancer Treat. Rep., **62**, 1393 (1978).