10. M. Droller, M. Shneider, and P. Perelmann, Cell. Immunol., **39**, No. 1, 165 (1978).
11. P. May, E. Holmes, and S. Golub, Cancer Res., **45**, No. 1, 57 (1985).
12. B. Perussia and G. Trinchieri, J. Immunol., **126**, No. 2, 754 (1981).

COMPARATIVE ACCUMULATION OF THE FLUORESCENT PROBE HOECHST 33258 IN LEUKEMIA P388 CELLS SENSITIVE AND RESISTANT TO DOXORUBICIN

F. V. Donenko, N. B. Borovkova, S. V. Egudina, A. O. Kabieva, Sh. I. Kris'ko, and L. V. Moroz

UDC 616.155.392-07:616.155.3-02:615.332]-092.9

KEY WORDS: doxorubicin; Hoechst 33258; induced resistance.

Experimental studies have demonstrated a decrease in accumulation of certain preparations in tumor cells resistant to cytostatics of natural origin (doxorubicin, vincristine, actinomycin D, colchicine) compared with sensitive cells in systems both in vitro and in vivo [4, 10]. A decrease in doxorubicin accumulation in leukemia P388 cells with induced resistance to this anthracycline antibiotic was demonstrated previously [1]. However, on determination of the total content of the cytostatic in tumor cells, the question of the relative accumulation of the preparation actually in their nuclei remained unexplained, i.e., nothing could be stated about the accessibility of the nuclear DNA for the preparation in resistant and sensitive cells. It has been suggested that the cytostatic is located in resistant cells mainly in the cytoplasm [6]. There is evidence in the literature of an equal cytotoxic action of doxorubicin on resistant cells if present in equal intranuclear concentrations [5]. It is technically difficult, however, to measure penetration of doxorubicin inside the nucleus, for when it interacts with biological macromolecules, quenching of the fluorescence takes place.

We know that multiple drug resistance of tumor cells to cytostatics can be overcome with the aid of detergents in low concentrations [3]. In our own experiments, to overcome the drug resistance of P388 cells with induced resistance to doxorubicin, we used Triton X-100 in concentrations not destroying cell membranes.

In the present investigation we studied penetration of the fluorescent probe Hoechst 33258 into sensitive and resistant leukemia P388 cells. The reason for the investigation was as follows: 1) the Hoechst probe fluoresces only on interaction with the DNA molecule [2]; 2) the probe molecule is a heterocyclic compound, i.e., the Hoechst dye is similar in its molecular structure to the group of cytostatics of natural origin to which the tumor cells acquire crossed resistance; 3) doxorubicin and Hoechst 33258 are known to be competitive with one another for binding sites with purified DNA [9].

EXPERIMENTAL METHOD

Leukemia P388 cells within induced resistance to doxorubicin (P388/DXR) were obtained by selection from leukemia P388 cells (P388/0 — the initial strain, from the tumor strain bank of the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR) during treatment of animals with small doses of the antibiotic. To induce resistance, 35 passages were needed. To maintain the leukemia P388/0 and P388/DXR, male DBA/2 mice aged 2-3 months were used. The tumor cells were transplanted intraperitoneally in a dose of $1.0 \cdot 10^6$ cells in 0.2 ml of medium 199. The leukemia cells were isolated from

Department for the Study of New Antitumor Drugs, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Blokhin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 9, pp. 310-312, September, 1990. Original article submitted February 6, 1990.

 0007-4888/90/0009-1268$12.50

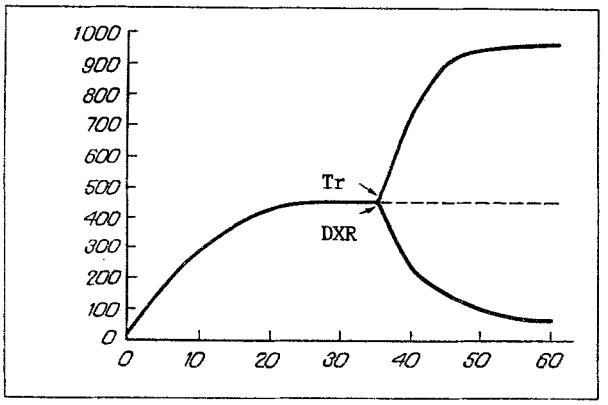

Fig. 1. Changes in intensity of fluorescence of Hoechst 33258 probe on its addition to leukemia P388/0 cells. Here and in Fig. 2, arrows indicate time of addition of Triton X-100 (Tr) or of DXR in concentrations indicated in "Experimental Method." Broken lines indicate intensity of fluorescence in control (without addition of modifiers). Abscissa, time (in min); ordinate, intensity of fluorescence (in r.u.).

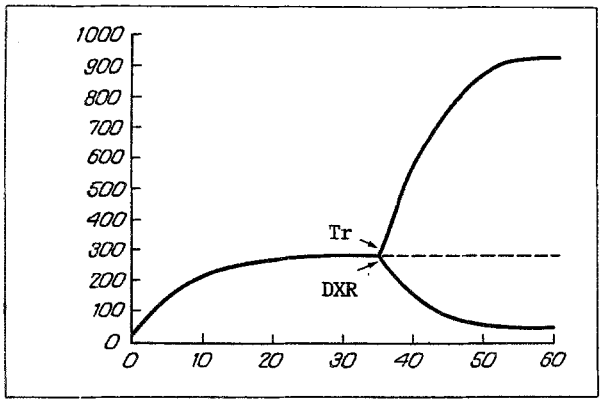

Fig. 2. Change in intensity of fluorescence of Hoechst 33258 probe on its addition to leukemia P388/DXR cells.

acetic fluid in Hanks' solution, and washed to remove erythrocytes by hemolytic shock. All operations to study transport of the preparations into leukemia cells were carried out in Hanks' solution with 10 mM glucose at room temperature. Cells were counted in a Goryaev's chamber. The Hanks' solution contained $5 \cdot 10^6$ cells in 1 ml. In the experiments the concentration of Hoechst 33258 was $5 \cdot 10^{-7}$ M, that of DXR $2 \cdot 10^{-6}$ M, and of Triton X-100 — 0.0001%.

The Triton X-100 and fluorescent dye Hoechst 33258 were obtained from Serva, West Germany; chromatographically pure DXR was generously provided by Yu. V. Dudnik (Institute for the Search for New Antibiotics, Academy of Medical Sciences of the USSR).

The fluorescent measurements were made on a Hitachi M-850 fluorescent spectrophotometer (Japan). Accumulation of the dye was measured no fewer than 3 times at different passages of the tumors. The intensity of fluorescence of the Hoechst dye was measured at λ_{ex} = 347 nm and λ_{em} = 454 nm (at the maxima of absorption and fluorescence of the bound probe).

EXPERIMENTAL RESULTS

On the addition of the Hoechst 33258 probe to leukemia P388/0 cells the intensity of fluorescence of the dye due to its binding with DNA increased. It will be clear from Fig. 1 that the intensity of fluorescence rose from 18 to 450 relative units

(r.u.); the time of flattening out at a stationary level in this case was 25 min. The effect of Triton X-100 and of DXR on the intensity of fluorescence of Hoechst 33258 in P388/0 cells was studied. To do this, the detergent or cytostatic was added to the leukemia cells in the stationary phase of fluorescence of the probe (at the 35th minute). Triton X-100 caused a sharp decrease in the intensity of fluorescence from 450 to 960 r.u., evidence of a greater degree of binding of the dye with DNA. Incidentally, this action on sensitive cells of substances abolishing multiple drug resistance is probably linked with the existence of "spontaneous" active transport of the preparations from sensitive cells [8]. Addition of DXR to P388/0 cells led to a decrease in the intensity of fluorescence of the Hoechst 33258 to 40 r.u., evidence of displacement of the Hoechst dye from the DNA molecule by DXR.

Dependence of the intensity of fluorescence of the Hoechst probe on time after its binding with DNA of P388/DXR cells is shown in Fig. 2. Clearly the intensity of fluorescence also increased and flattened out on a plateau after 25 min of observation. However, the level of fluorescence, the content of sensitive and resistant cells in the samples being equal, was significantly lower in samples with resistant cells, namely 290 r.u., i.e., 60% of the level of fluorescence with P388/0 cells. Addition of Triton X-100 in the stationary phase of fluorescence of the dye, bound with DNA of P388/DXR cells, also led to a sharp rise of the level of fluorescence to 920 r.u. The level of fluorescence for P388/0 and P388/DXR cells after addition of the detergent were similar, i.e., binding of the Hoechst dye with DNA was the same in the sensitive and resistant cells. Triton X-100 is a nonpolar detergent and, in low concentrations (which were used in the work) it increases cell membrane permeability for low-molecular-weight substances, and this evidently explains the results.

Addition of DXR to P388/DXR cells also led to a decrease in fluorescence of the Hoechst dye to 60 r.u.

A difference in penetration of the dye Hoechst 33342 into sensitive and resistant human lung cancer cells has been described in the literature. However, the authors cited did not observe any marked action of verapamil, a modifier of multiple drug resistance, on accumulation of the dye in resistant cells. Moreover, in the work in question competition for binding sites with DNA between DXR and the dye was not investigated, although, as was stated above, it significantly limits the use of their technique for the study of the phenomenon of multiple drug resistance [7].

The following conclusions can thus be drawn from the investigations described above: 1) resistant cells of leukemia P388/DXR binds 30% less Hoechst 33258 in nuclei than sensitive cells; 2) Triton X-100 in low concentrations abolishes differences in the accumulation of the Hoechst dye in sensitive and resistant cells; 3) competition for binding sites on DNA is observed between the Hoechst dye and doxorubicin in both types of cells, and this fact can evidently be utilized for the quantitative determination of the antibiotic when bound with DNA.

LITERATURE CITED

1. L. V. Moroz, F. V. Donenko, N. B. Borovkova, and S. M. Sitdikova, Byull. Éksp. Biol. Med., **108**, No. 12, 714 (1989).
2. D. J. Arndt-Jovin and P. M. Jovin, J. Histochem. Cytochem., **25**, No. 3, 585 (1977).
3. G. Bradley, P. F. Juranka, and V. Ling, Biochim. Biophys. Acta, **948**, No. 1, 87 (1988).
4. R. Ganapathi, H. Schmidt, D. Grabowski, et al., Brit. J. Cancer, **58**, No. 3, 335 (1988).
5. M. Gigli, T. W. D. Rasoanaivo, J. M. Millot, et al., Cancer Res., **49**, No. 2, 560 (1989).
6. H. G. Keizer, G. J. Schuurhuis, H. J. Broxterman, et al., Cancer Res., **49**, No. 11, 2988 (1989).
7. S. A. Morgan, J. V. Watson, P. R. Twentyman, and P. J. Smith, Brit. J. Cancer, **60**, No. 3, 282 (1989).
8. A. A. Neyfakh, T. V. Dmitrevskaya, and A. S. Serpinskaya, Exp. Cell Res., **178**, No. 2, 513 (1988).
9. H. D. Preisler, Cancer Treat. Rep., **62**, No. 8, 1393 (1962).
10. J. R. Riordan and V. Ling, Pharmacol. Ther., **28**, No. 1, 51 (1985).