Intracellural Accretion of Free and Nanosphere-Encapsulated Doxorubicin

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It is shown that two new methods can be used to quantitate the intracellular accretion and interaction with DNA of any anthracycline antibiotics (AA) during short-term incubation of vital cells with preparation. The intracellular accretion of doxorubicin encapsulated in polymeric nanospheres (NS-DR) is significantly less than that of free DR. NS-DR and NS do not thereby affect the intracellular accretion of DR. Thus, qualitative and quantitative differences in the activity of free AA and their complexes with polymers may be related to the differences in their intracellular accretion and to the possibility of realizing biological effects of the complexes without penetrating inside the cell.

Key Words: *doxorubicin; doxorubicin-polymer complex; intracellular transport*

Anthracyclines bound with polymers are considered to be a promising class of antitumor preparations whose biological activity differs from the activity of free preparation in the majority of cases. For example, doxorubicin (DR) encapsulated in nanospheres (NS) exhibits markedly less total toxicity *in vivo* [5]. At the same time, NS may selectively intensify DR toxicity in DR-resistant tumor cells *in vitro* [7]. To clarify this phenomenon, the intracellular accretion of these complexes and binding of them with DNA should be studied, because it is precisely these processes which in most cases are crucial to the biological activity of anthracyclines. However, the use of routine methods of anthracycline study for this purpose is often a problem. For example, the usual biochemical methods involving cell destruction and extraction are inappropriate due to the low stability of complexes. On the other hand, direct spectrofluorometric measurements are often impossible because the fluorescence of an anthracycline-polymer complex is far less than that of free antibiotic. A daunorubicin-polyglutamic acid complex, in which antibiotic fluorescence comprises only 10% of the initial, is an example of such a case.

The aim of the present investigation was to demonstrate the feasibility and suitability of using two new methods of studying the interaction between AA-polymer complexes and cells. The first method, developed independently by us [1] and the French Garnier-Suillerot research team [10], permits assessment of the intracellular accretion and DNA binding according to the quenching of anthracycline fluorescence for short-term incubation with vital cells. The second approach, developed by us [2], allows for a study of weakly fluorescing anthracycline-polymer complexes and is based on the use of a specific DNA dye Hoechst 33258. DR encapsulated in NS is used in the study as a model anthracycline-polymer complex.

MATERIALS AND METHODS

Monolayer cultures of rat glioblastoma (the sensitive line C6 and the line with 6-fold multidrug resistant C6 0.001), human ovarian carcinoma (the

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sensitive line CaOv), and a suspended culture of human erythroleukemia cells (the line K562 sensitive to anthracyclines) were used in the study. The cells were cultured *in vitro* as described previously [3,6,9]. All experiments were carried out on cultures in the stationary phase of growth. The preparation of suspensions of normal CBA mouse thymocytes was described previously [2]. The final concentration of all cell types in the suspension differed in different experiments from 1 to 2×10^6 cells/ml. The cell suspension was prepared using 0.9% NaCl, pH 7.0. The number of cells was calculated using an automatic Coulter ZM hemocytometer microscopically in a Goryaev chamber.

DR hydrochloride (Newilly), NS isohexylcyanoacrylate and NS-DR (Bruxelles), and specific DNA dye Hoechst 33258 (Aldrich) were used in the study. All reagents were marked "analytical-grade". The reagents used in all experiments were as follows: a) 1×10^{-6} M DR; b) NS solution in a concentration equal to its content in 1×10^{-6} M NS-DR; c) NS-DR solution with an equimolar content of free antibiotic.

To assess the intracellular accretion and binding of anthracyclines with DNA, we used two spectrofluorometric methods described previously [1,2]. The intensity of anthracycline fluorescence was measured on Hitachi M-850 and Jobin Yvon JY 3CS spectrofluorometers. The experiments were performed with continuous mixing of the suspension. A 0.9% NaCl solution with 5 mM glucose added at pH 7.0 served as the incubation medium. Measurements were performed at wavelength 480 nm for DR and 347 nm for Hoechst dye at a slit width of 5 nm. The absorption wavelength was 590 nm for DR and 454 nm for Hoechst dye.

RESULTS

The first step was to compare free DR and NS-DR. It was shown in preexperiments that the fluorescence of the complex is only 50% lower than

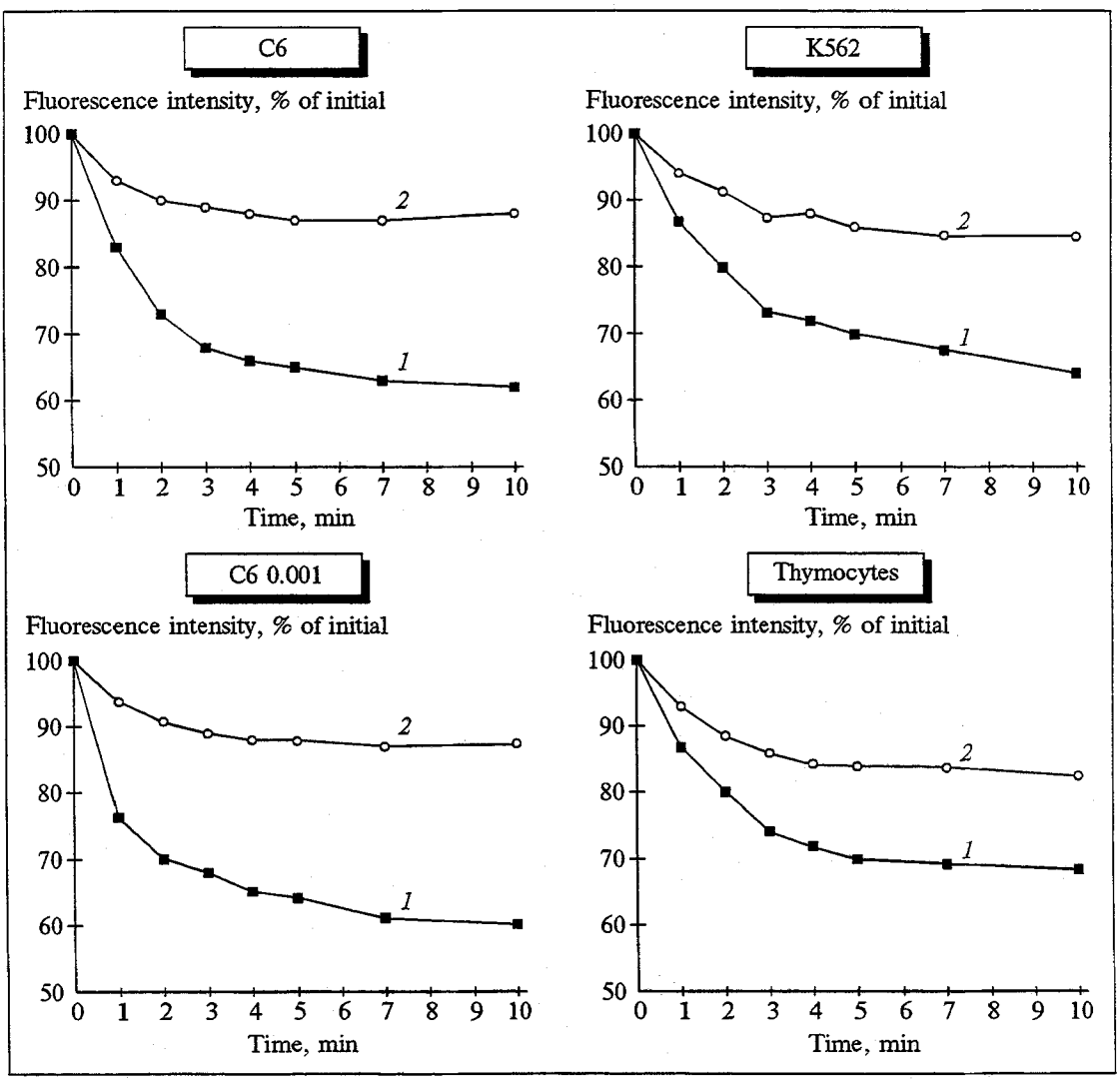

Fig. 1. Intracellular accretion of DR and NS–DR in various types of cells. 1.5×10^6 cells/ml were incubated with 2×10^{-6} M DR (*1*) or NS–DR with equimolar content of free DR (*2*) for 10 min.

with free anthracycline. Therefore, the intracellular accretion of NS-DR can be assessed according to the quenching of its fluorescence during incubation of cells with the complex. As was pointed out above, this approach was developed by our team [1] as well as simultaneously and independently by Garnier-Suillerot and co-authors [10]. Data on a marked decreases of anthracycline fluorescence for interaction with DNA but not with other cellular macromolecules formed the basis of this method.

Results of typical experiments with different kinds of sensitive or resistant tumor cells and mouse thymocytes are presented in Fig. 1. It is seen that the NS-DR fluorescence quenching during 10 min of incubation comprised around 10-15% in all studied cells. At the same time, DR fluorescence quenching was around 40% in tumor cells and about 30% in thymocytes. These results show that the intracellular accretion of NS-DR in various types of tumor and normal cells is much less than with free DR.

The described approach was also used by us for a study of the interaction of DR, NS, and NS-DR on the intracellular transport of agents in various types of tumor cells (K562, C6, C6 0.001) and in mouse thymocytes. The basis for this consisted of data on the biodegradation of the NS-DR complex in the organism [8]. This points to the possibility of a simultaneous interaction of both free anthracycline and polymer and their complex with cells in administration of NS-DR *in vivo*. Results of a typical experiment on C6 cells are given in Fig. 2. We see that preincubation of cells with DR does not affect the NS-DR fluorescence quenching. And vice versa, preincubation of cells with NS-DR does not affect the fluorescence quenching of free DR. Analogous experiments on the other above-mentioned cell types demonstrated the absence of a mutual influence of the preparations on their fluorescence quenching. In addition, no differences in the fluorescence quenching between DR and the DR+NS mixture (data not presented) were noted for incubation with tumor cells and thymocytes. That means that the NS-DR complex as well as the free polymer do not affect the intracellular transport of DR and, vice versa, free DR does not affect the intracellular transport of the NS-DR complex.

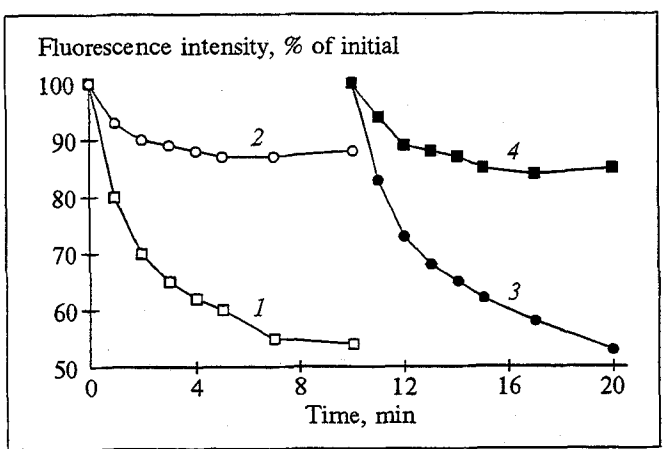

Fig. 2. Effect of preincubation with DR and NS–DR on intracellular accretion of NS–DR and DR, respectively, in sensitive cells of rat C6 glioblastoma. 2×10^6 cells/ml were incubated with DR (*1*) or NS–DR (*2*) for 10 min. DR (*3*) or NS–DR (*4*) were then added to the cell suspension preincubated with NS–DR or DR, respectively, and incubated for 10 min. DR concentration is 1×10^{-6} M, NS–DR with an equimolar content of free DR.

To assess the informativeness of the two methods used, we performed a comparative determination of intracellular accretion of free DR and DR-NS in the same experiment according to the quenching of the fluorescence proper of the preparations and that of the Hoechst dye for incubation of a CaOv cell suspension with anthracycline.

The data in Fig. 3 show that the intracellular accretion and DNA binding of NS-DR are significantly less as compared to free DR. However, when the effect of DR, NS-DR, NS, NS+DR mixture, and NS+(NS-DR) on the background Hoechst fluorescence was assessed in 0.9% NaCl solution, the quantitative correctness of the results in the experiments with Hoechst dye was placed in doubt. The background fluorescence of 2×10^{-6} M Hoechst dye in 0.9% NaCl solution comprised around 15% of its fluorescence after 15 min incubation with the CaOv cell suspension (10^6 cells/ml). The addition of 1×10^{-6} M DR to the dye solution practically did not affect this parameter. At the same time, NS-DR with 1×10^{-6} M free DR, NS in a concentration equal to its content in 1×10^{-6} M NS+DR solution, as well as an NS-DR mixture with an approximately equal concentration of components caused a nearly two-fold increase of the dye's background fluorescence. On the basis of the known data it is difficult to explain the mechanism of the polymer's effect on the background fluorescence. However, the phenomenon shows that the study of intracellular accretion of the NS-DR complex using Hoechst dye is not strictly quantitative. Thus, direct measurement of the fluorescence quenching of this anthracycline for incubation with cells is probably the most accurate method of quantifying its intracellular accretion and DNA binding. These findings demonstrate the limitations in using Hoechst dye when studying complexes of anthracyclines with NS (and probably other polymer carriers). Therefore, not only the intensity of antibiotic complex fluorescence, but also the possible interaction of the poly-

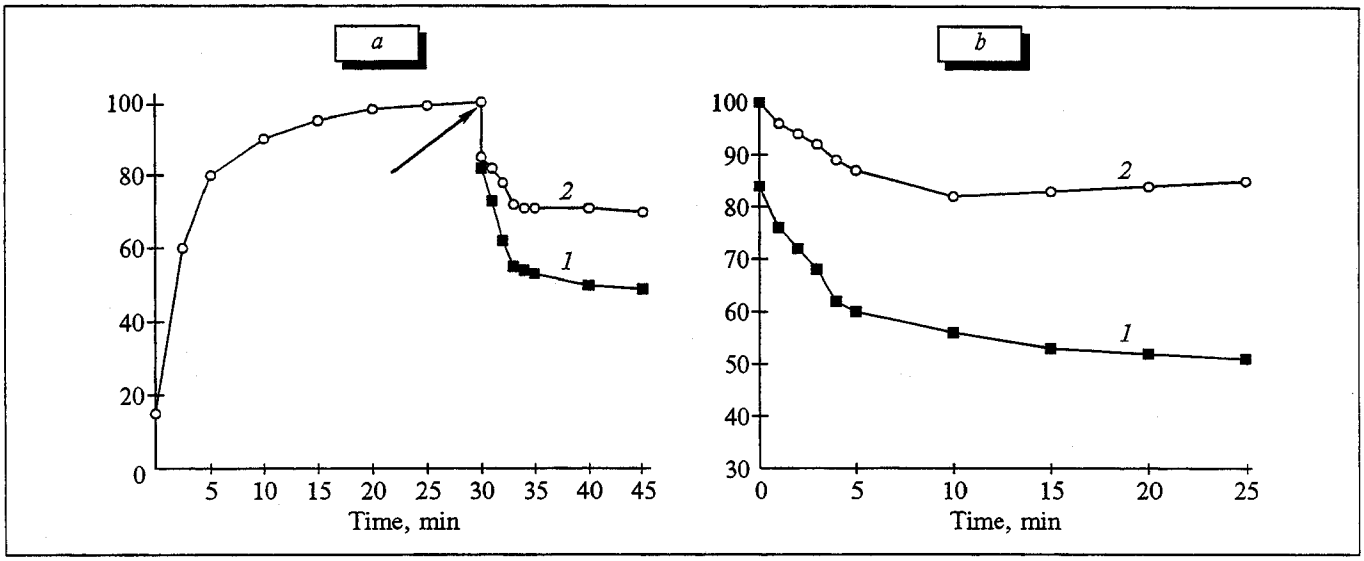

Fig. 3. Intracellular accretion of DR and NS–DR in sensitive CaOv tumor cells. Ordinate: fluorescence intensity of Hoechst dye (*a*) and AA (*b*), % of initial. *a*) 1×10^6 cells/ml were incubated with 1×10^{-6} M Hoechst dye for 30 min, and then 1×10^{-6} DR (*1*) or NS–DR with an equimolar content of free DR (*2*) was added to the cell suspension and incubated for 15 min. *a*) an arrow (anthracycline) marks the addition of DR or NS–DR to the cell suspension incubated with Hoechst dye; *b*) 1×10^6 cells/ml were incubated with 1×10^{-6} M DR (*1*) or NS–DR with an equimolar content of free DR (*2*) for 25 min.

mer with the dye may be a factor in choosing one or the other of the methods.

The findings demonstrated a significant lesser intracellular accretion of the NS-DR complex and binding with DNA as compared to free DR. Previously the same phenomenon was shown by Robert and co-workers in the same and different types of tumor cells using the extraction method [1].

It is to be noted that free NS do not exhibit modifying activity, and the intracellular accretion of DR in the DR+NS mixture is exactly the same as of free DR. This is in agreement with the data on the absence of biological activity of free NS, namely, anthracycline acquired the ability to overcome drug resistance only when there was a strong binding of DR and NS in the complex [4]. The findings suggest that the NS-DR complex and NS liberated in the organism should not affect the intracellular accretion of preparations with the same mechanism of transport as DR (vinca alakaloids, epidophilotoxins, acridines, taxol, and others). Therefore, NS-DR should not lower the biological activity of these drugs when they are administered in combination with anthracycline.

Analyzing the results, we may assume that despite the high biological activity, the intracellular accretion of NS-DR is far less than of free DR. Only 10-15% of the polymer-anthracycline complex is incorporated in vital normally functioning cells and binds with intracellular DNA. Hence, at least some biological effects of anthracyclines bound with polymers may probably be realized at the level of the cell membrane, without penetrating into the cell. Another explanation for the qualitatively different high efficiency of anthracycline-polymer complexes was proposed previously by Robert, who speculated upon a possible modification of the intracellular distribution of the drug [4].

Finally, it should be stressed that the new approaches to the study of NS-DR interaction with cells used in the present investigation shed light on the biological activity not only of the substance in question, but also of a fundamentally new class of antitumor preparations, namely, complexes of anthracycline antibiotics with polymer carriers.

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