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INTERACTION BETWEEN DAUNORUBICIN AND CHROMATIN FROM EHRLICH ASCITES TUMOR CELLS

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SUMMARY

Interaction of daunorubicin with chromatin from Ehrlich ascites tumor cells has been studied by spectrofluorimetry. Daunorubicin interacts with chromatin and displays at least two types of binding. The number of binding sites is reduced when compared to deoxyribonucleic acid. There is no difference in the overall structure of chromatins extracted from cells sensitive or resistant to daunorubicin.

INTRODUCTION

The anthracyclin antibiotic daunorubicin is a very active agent for the treatment of acute leukemias (1,2,3). Its activity has been attributed to its interaction with DNA (4,5,6,7) leading to the inhibition of DNA synthesis process. *In vitro*, inhibition of enzymes such as deoxyribonucleases, RNA polymerases and DNA polymerases is due to the formation of a drug-DNA complex and not to the direct action of the drug upon the enzyme (8,9,10,11). However informations about the interaction of daunorubicin with polymers other than polynucleotides are available and daunorubicin shows some affinity for proteins (12,13) and mucopolysaccharides (14).

The chemotherapeutic effect of daunorubicin is, in some cases, strongly reduced by an encountered resistance. Attempts to relate this resistance to a difference at the molecular level between cells sensitive and resistant to daunorubicin have not been successful yet. There is a decreased

SDS : Sodium dodecyl sulfate
PMSF : Phenyl-methyl-sulfonyl-fluoride

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uptake of the drug in resistant cells (15) but this diminution is not sufficient to explain resistance which could be due to several factors. The hypothesis that cellular proteins might play a role in the mode of action of daunorubicin and (or) the development of resistance cannot be ruled out. From this standpoint, we have in a preliminary step considered the influence of nuclear proteins upon the fixation of daunorubicin to DNA in chromatin.

The present study is concerned with the fixation of daunorubicin to DNA and chromatin from Ehrlich ascites tumor cells as well as the comparison between chromatins extracted from ascites tumor cells sensitive or resistant to the drug.

MATERIALS AND METHODS

Daunorubicin is a generous gift from Rhône-Poulenc, S.A., France. Solutions of daunorubicin were kept in the dark for one week at most. Concentration of daunorubicin was calculated using an extinction coefficient of 10,000 M^{-1} at 480 nm.

Ehrlich ascites tumor cells : CD 1 female mice (Charles River, France) 6-8 weeks old (20 g) were inoculated intraperitoneally with 10^6 cells. Ascites fluid was collected 8 days after inoculation and either used within the hour (chromatin preparation) or washed with phosphate-NaCl buffer and then frozen (DNA preparation). Resistance was developed by treatment with subtherapeutic doses of daunorubicin (0.25 mg/1 kg) given intraperitoneally for 5 days. Ascites fluid was collected 2 weeks after inoculation of resistant cells.

Ascites chromatin was prepared according to Paoletti (16). In this method, cells were washed (5 mn, 1,000 g) once with sucrose 0.25 M, EDTA 5 mM, pH 8 and four times with sucrose 0.25 M, EDTA 0.1 mM, pH 8. During this washing step, after each run, pH was adjusted to 8 by addition of NaOH 20 mM. Chromatin was then extracted as described by Noll et al. (17). Micrococcal nuclease (EC 3.1.4.7.) digestion was performed during 10 mn at 37° (20 units/ml). Our chromatin preparations contained about 70 % of proteins, 22 % of DNA and 8 % of RNA as determined according to ref. 18, 19, 20 respectively. Concentration of chromatin was calculated assuming an extinction coefficient of 7,300 M^{-1} cm^{-1} at 260 nm.

DNA preparation : Ascites DNA was prepared from purified nuclei according to Barthelemy-Clavey et al. (10). Concentration of DNA was calculated using an extinction coefficient of 6,800 M^{-1} cm^{-1} at 260 nm.

Electrophoresis : Chromatin was lyopholized, dissolved in Tris 0.02 M, pH 7.8, β-mercaptoethanol 5 %, SDS 2 % and submitted to electrophoresis in SDS according to Weintraub et al. (21). Proteins were stained with coomassie blue.

Binding experiments : Addition of nucleic acid (4) or chromatin to a solution of daunorubicin results in a decrease in the fluorescence and absorption intensities of daunorubicin. This decrease is dependent on the concentration of added nucleic acid or chromatin and can be used to determine the amount of bound dye.

1) Spectrofluorimetric titrations were carried out with a Jobin-Yvon spectrofluorimeter modified in the laboratory. All the experiments were performed at room temperature in Tris-HCl 0.01 M, NaCl 0.01 M, EDTA 0.2 mM, pH 7.5, buffer. Chromatin and DNA were dialyzed against this buffer. Each intensity reading was corrected by an appropriate blank with DNA, chromatin or buffer. Daunorubicin was excited at 485 nm and fluorescence emission was measured at 570 nm.

2) Absorption : Absorption spectra were recorded on a Cary 15 or a Beckman Acta III spectrophotometer. Binding experiments were performed by a procedure similar to that described by Müller and Crothers (22). Absorbance was measured at 480 nm.

Binding isotherms were determined at constant polymer concentration ($1.5\text{-}3 \times 10^{-5}$ M, expressed in nucleotide), the concentration of added daunorubicin varied between 5×10^{-7} and 1.5×10^{-5} M for DNA and between 1×10^{-7} and 5×10^{-6} for chromatin. Data were analyzed using Scatchard's relationship: $r/c = K(n-r)$ where r is the dye bound per DNA or chromatin phosphorus, c is the concentration of free dye, n and K are respectively the apparent number of binding sites and the apparent binding constant, both determined from a plot of r/c versus r.

RESULTS AND DISCUSSION

Protein content of ascites chromatin from sensitive cells is represented in fig. 1. The five histones are present along with a great number of non histone proteins. The same pattern is obtained with chromatin from resistant cells. Nevertheless, it has to be noticed that, so far, preparations of chromatin from resistant cells are more susceptible to proteolysis than preparations from sensitive cells and have to be used within four or five days. The integrity of both chromatins has been checked by tryptic digestion (21,23) and by circular dichroism (24,25).

It has already been shown that complexation of daunorubicin to DNA results in a red shift and a decrease in intensity of the absorption maximum (6). In fig. 2, one can see that the same phenomenon is observed with chromatin. An isobestic point for free and bound dye is found at 540 nm.

Fluorescence intensity of daunorubicin is strongly reduced by addition of increasing amounts of chromatin and almost totally quenched at high nucleotide/dye ratio (fig. 3).

Scatchard's plots obtained from fluorescence measurements are represented in fig. 4a. The curvature of the isotherm indicates that ascites chro-

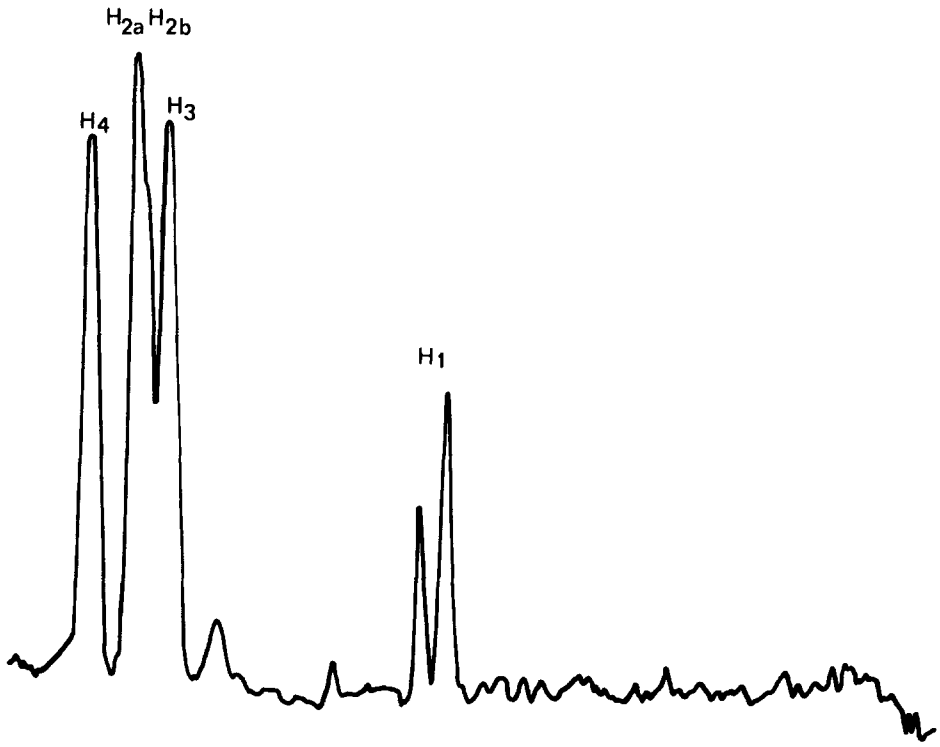

Fig. 1 - Densitometric scans of polyacrylamide gel electrophoresis of proteins from sensitive ascites chromatin.

matin displays at least two modes of binding. In a first approximation if one assumes that the binding sites are independent, without cooperativity or anti-cooperativity, the number of high affinity sites decreases from 0.17 for DNA to 0.08 for chromatin. There is only a slight change in the binding constant : $K = 5 \times 10^6\ M^{-1}$ for ascites chromatin and $K = 7 \times 10^6\ M^{-1}$ for ascites DNA. Complementary experiments carried out by spectrophotometry confirm these results (fig. 4a).

Results obtained with DNA are in good agreement with previous spectrofluorimetric studies on daunorubicin and calf thymus DNA (26). The difference observed in daunorubicin interaction when one deals with chromatin instead of DNA could be expected from studies on ethidium bromide-chromatin interaction (27,28). We can assume that the reduced number of intercalating dye binding sites in chromatin is explained by a less accessibility of its DNA when it is engaged in a chain of nucleosomes.

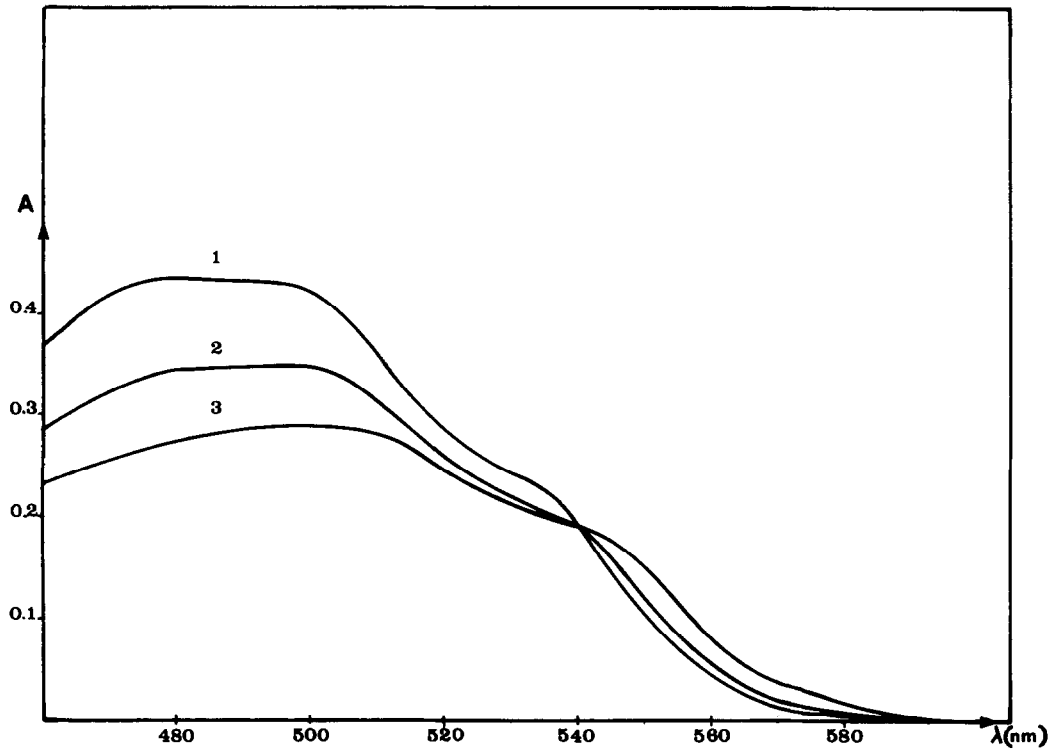

Fig. 2 - Visible absorption spectra of daunorubicin-chromatin complexes.
1. Free daunorubicin
2. Chromatin 1.5×10^{-4} M
3. Chromatin 5.3×10^{-4} M.

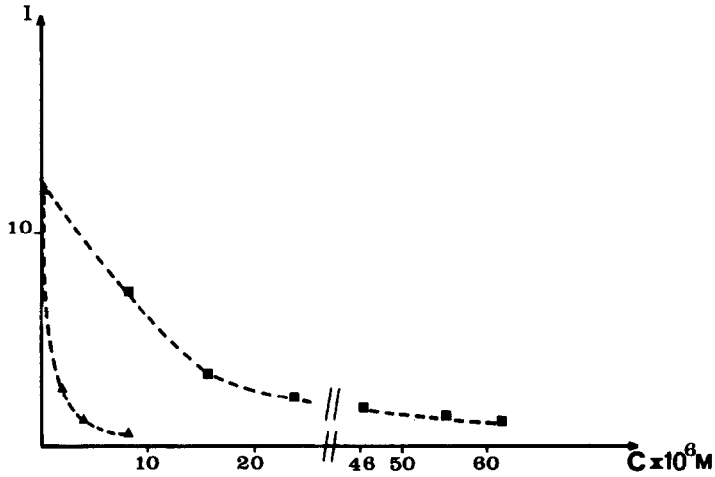

Fig. 3 - Decrease of daunorubicin fluorescence intensity upon addition of chromatin and DNA.
▲ DNA
● Chromatin
C = nucleotide concentration
Daunorubicin concentration : 10^{-7} M.

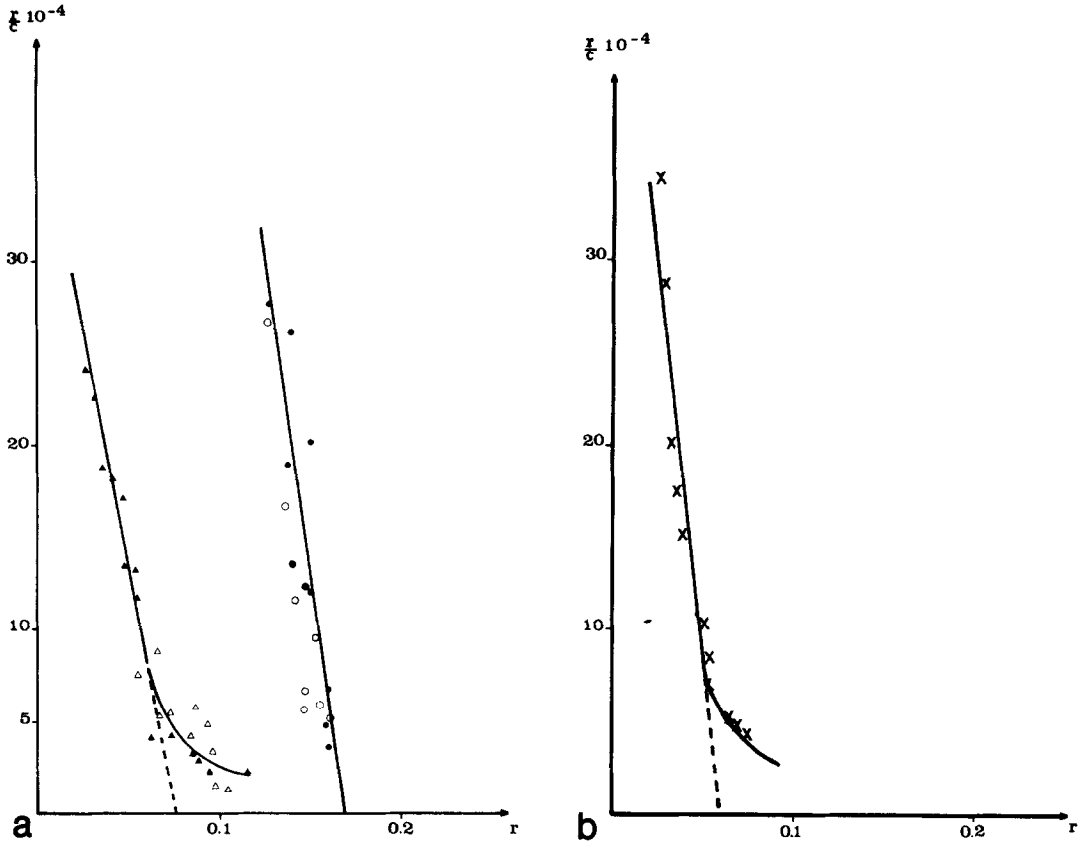

Fig. 4 - Scatchard's plots for the binding of daunorubicin to chromatin and DNA.
a) Ascites chromatin from sensitive cells : ▲ fluorescence, Δ absorption.
Ascites DNA : ● fluorescence, o absorption.
b) Ascites chromatin from resistant cells : x fluorescence.

If one compares the behaviour of daunorubicin towards chromatins prepared from sensitive and resistant cells, one can see (fig. 4b) that the number of binding sites as well as the value of the binding constant are the same. One can argue that Scatchard's plots can only give an order of magnitude for the binding constant and the number of binding sites and that our experiments cannot distinguish between small differences at the nucleosome level in chromatin. Fluorescence polarization studies of ethidium bromide with the two chromatins confirm the similarity observed with daunorubicin (D. Genest et al. to be published).

There is recent evidence that some non-histone proteins are part of the nucleosome (29,30). Experiments were performed with a preparation of resistant chromatin containing the full set of histones but depleted by proteolysis of a great amount of non-histone proteins : no difference has been observed in daunorubicin binding. This result suggests that the bulk of non-histone proteins is not required for the maintenance of a polynucleosome like structure. Besides, it seems worth noticing that limit digests of histones H_1 and H_3 still preserve a nucleosome like structure.

We have shown that daunorubicin which is well known to interact with DNA interacts also with DNA engaged in a nucleoprotein complex. The same interaction is observed with chromatin extracted from cells sensitive or resistant to the drug. Daunorubicin binding to DNA in chromatin does not seem to depend upon the presence of non-histone proteins. This observation does not rule out the hypothesis that some non-histone proteins or other cellular proteins do not participate in daunorubicin action or in the phenomenon of resistance. Presently, work is in progress on the proteic composition of the two chromatins and on the subcellular localization of the proteolytic activity contaminating resistant chromatin.

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