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The Interaction of Adriamycin with Nuclear DNA: Evidence for a Drug Induced Compaction of Isolated Chromatin.

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Summary: Incubation of adriamycin with isolated chromatin converts the DNA to a form which exhibits an extremely fast sedimentation rate in sucrose gradients. This effect of adriamycin is abolished when chromatin is treated with pronase prior to the addition of the drug. Although both adriamycin and ethidium bromide intercalate into DNA the latter compound is without effect in this system. Examination of chromatin in the electron microscope demonstrates that in the presence of adriamycin there occurs an extensive condensation and compaction of the DNA fibers.

Introduction: Adriamycin, a member of the anthracycline class of antibiotics is currently being used as a chemotherapeutic agent in the treatment of patients with certain leukemias or solid tumors (1). Studies on the mechanism of action of adriamycin indicates that the in vivo target for the drug is probably nuclear DNA. The drug efficiently intercalates into native DNA (2) and when added to cells in culture inhibits both DNA and RNA synthesis (3, 4). Although the binding of adriamycin to native DNA has been carefully studied very little information has been obtained on the interaction of the drug with chromatin. Particularly, very little is known on the possible involvement of chromatin associated proteins in the mechanism of action of adriamycin. In the present report we show that adriamycin can induce a major conformational change in chromatin and that this effect requires the presence of proteins associated with the DNA.

Materials and Methods

Materials. Adriamycin was provided by the Developmental Therapeutics Program of the Division of Cancer Treatment, NCI. The drug was dissolved in 0.01 M Tris-HCL (pH 7.6) at a concentration of 1 mg/ml. Dilutions of the stock solution were made in 0.01 M Tris-HCl (pH 7.6).

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Cell Culture. Chinese hamster lung cells (HT-1) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Labeling of DNA was carried out by growing cells for 16-20 hours in medium containing 0.5 uCi/ml of [^{3}H]thymidine (70 Ci/mmole).

Preparation of Nuclei and Chromatin. Nuclei were prepared essentially as described by Hershey et al. (5). After removing the media from the culture dishes the cells were washed once with cold hypotonic buffer A (10 mM Tris-HCl (pH 7.6), 2.0 mM $MgCl_2$, 0.5 mM EDTA and 5 mM β-mercaptoethanol). Excess fluid was removed and the cells were collected from the dishes by scraping with a rubber policeman. The cells were allowed to swell for 10 min. at 0°C and thereafter lysed by 8-10 strokes in a tight fitting glass homogenizer. Nuclei were obtained after centrifugation of the lysate at 800 x g for 5 min. The nuclear pellet obtained from about 5 x 10^7 HT-1 cells was suspended in 1.5 ml of a solution containing 1 mM Tris-HCl (pH 7.6) - 1 mM EDTA - 1 mM β-mercaptoethanol. The nuclei were subjected to 8 strokes in a tight fitting glass homogenizer and the chromatin was collected after centrifugation at 10,000 x g for 10 min. The chromatin pellet was suspended in 0.4 ml of buffer A. A portion of the chromatin pellet was layered on a 5-20% sucrose gradient and centrifugation was carried out for 2 hours at 30,000 rpm in the Spinco SW50.1 rotor. The sucrose was prepared in 0.01 M Tris-HCl (pH 7.6) - 1 mM EDTA - 0.05% NP_{40}. At the end of the centrifugation fractions were collected from the bottom of the tube and an aliquot was used for radioactivity determination. The chromatin fractions sedimenting at about 40-60 S were collected and dialyzed overnight against 0.01 M Tris-HCl (pH 7.6) - 1 mM EDTA.

Effect of adriamycin on the sedimentation rate of chromatin. Isolated chromatin was incubated in a reaction mixture containing 0.03 M Tris-HCl (pH 7.6) and varying concentrations of adriamycin in a final volume of 0.12 ml. Control tubes contained identical reaction constituents except 0.01 M Tris-HCL (pH 7.6) was added in place of drug. After an incubation period for 20 min. at 37°C the entire reaction mixture was layered on a 4 ml. 5-20% neutral sucrose gradient which overlaid a 0.7 ml. CsCl shelf (1.2 grams CsCl/ml of 20% neutral sucrose). The sucrose solutions were prepared as described above. Centrifugation was carried out for 30 min. at 30,000 rpm and 4°C in the Spinco SW50.1 rotor. At the end of the centrifugation fractions were collected from the bottom of the tube directly into scintillation vials containing glass fiber filter paper.

Results and Discussion: The molecular events occurring during the interaction of adriamycin with chromatin are largely unknown. It has been previously shown that the drug is capable of interacting with chromatin to produce DNA regions which are single-stranded (6). Previous studies have also demonstrated that the drug when added to cells in culture is capable of bringing about the formation of DNA strand breaks (7-9). In the present study evidence is presented that adriamycin induces an additional conformational change in isolated chromatin. The results show that the drug is capable of bringing about an extensive condensation and compaction of the isolated DNA. In these studies isolated chromatin was incubated with varying concentration of adriamycin and

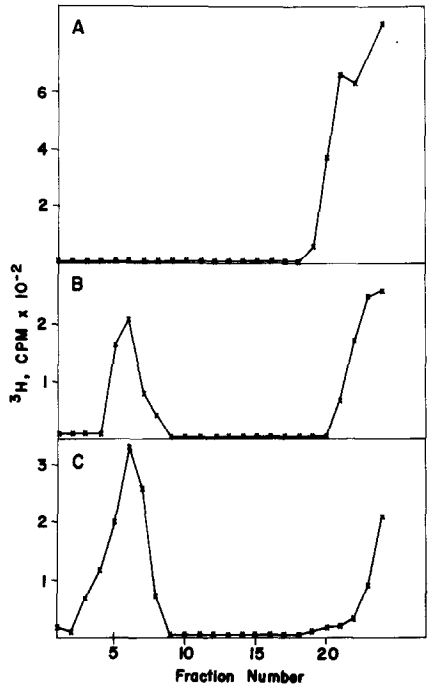

Figure 1: Effect of adriamycin on the sedimentation rate of isolated chromatin. Isolated chromatin was incubated under standard conditions in the absence (panel A) or presence of adriamycin. The final concentrations of adriamycin were 7 µg/ml (panel B) or 28 µg/ml (panel C). Sedimentation in neutral sucrose gradients was carried out as described in Methods.

the effect of the drug on the sedimentation rate of the DNA was determined. As shown in Figure 1, adriamycin at a concentration of 7 µg/ml converts about 40% of the DNA to structures which exhibit an extremely fast sedimentation rate in neutral sucrose. Increasing the concentration of adriamycin to 28 µg/ml results in the conversion of about 80% of the DNA to fast sedimenting forms (Figure 1,C). Although these experiments were carried out by incubating drug and chromatin in low ionic strength Tris-HCl buffer identical results have been obtained when 0.15 M NaCl is included in the reaction mixture. Essentially identical results to those described above have also been obtained when adriamycin is incubated with chromatin which has not been purified in sucrose gradients. However, if this material is stored for periods longer than two weeks at 4°C adriamycin is unable to convert the DNA to fast sedimenting forms.

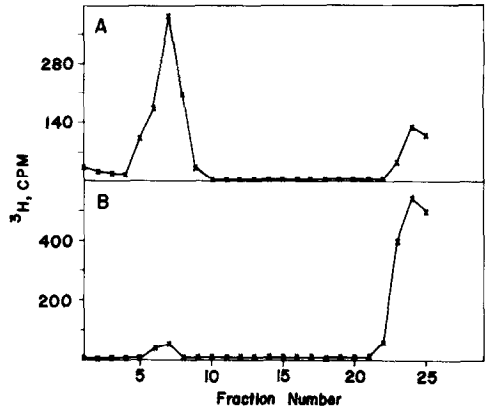

Figure 2: Effect of adriamycin on the sedimentation rate of pronase treated chromatin. Isolated chromatin was incubated in the absence (panel A) or present of 20 ug of pre-digested pronase for 20 min. at 25°C (panel B). At the end of the incubation adriamycin was added to each reaction mixture to a final concentration of 28 ug/ml. Incubation was continued for an additional 20 min. at 37°C and the solutions were thereafter centrifuged in neutral sucrose as described in Methods.

Additional studies have been carried out to examine the effect of adriamycin on the sedimentation rate of pronase treated chromatin. As shown in Figure 2 removal of protein from the DNA essentially abolishes the ability of adriamycin to convert chromatin to fast sedimenting forms. Further studies have also shown that adriamycin at a concentration of 28 μg/ml is unable to convert native T7 DNA to fast sedimenting structures (not shown).

Experiments have also been conducted to compare the effect of adriamycin and ethidium bromide on the sedimentation rate of chromatin. Since both of these compounds can efficiently intercalate into DNA this type of study provides a relative measure of the contribution of this event to the drug induced conformational change in chromatin. Under conditions where adriamycin converts 85% of the chromatin to fast sedimenting forms ethidium bromide had no similar effect on the sedimentation rate of the DNA. In the presence of ethidium bromide the DNA has a sedimentation rate similar to that observed with chromatin incubated with buffer (not shown). It is thus indicated that the action of adriamycin in this system is not completely dependent on the ability of the drug to intercalate into DNA.

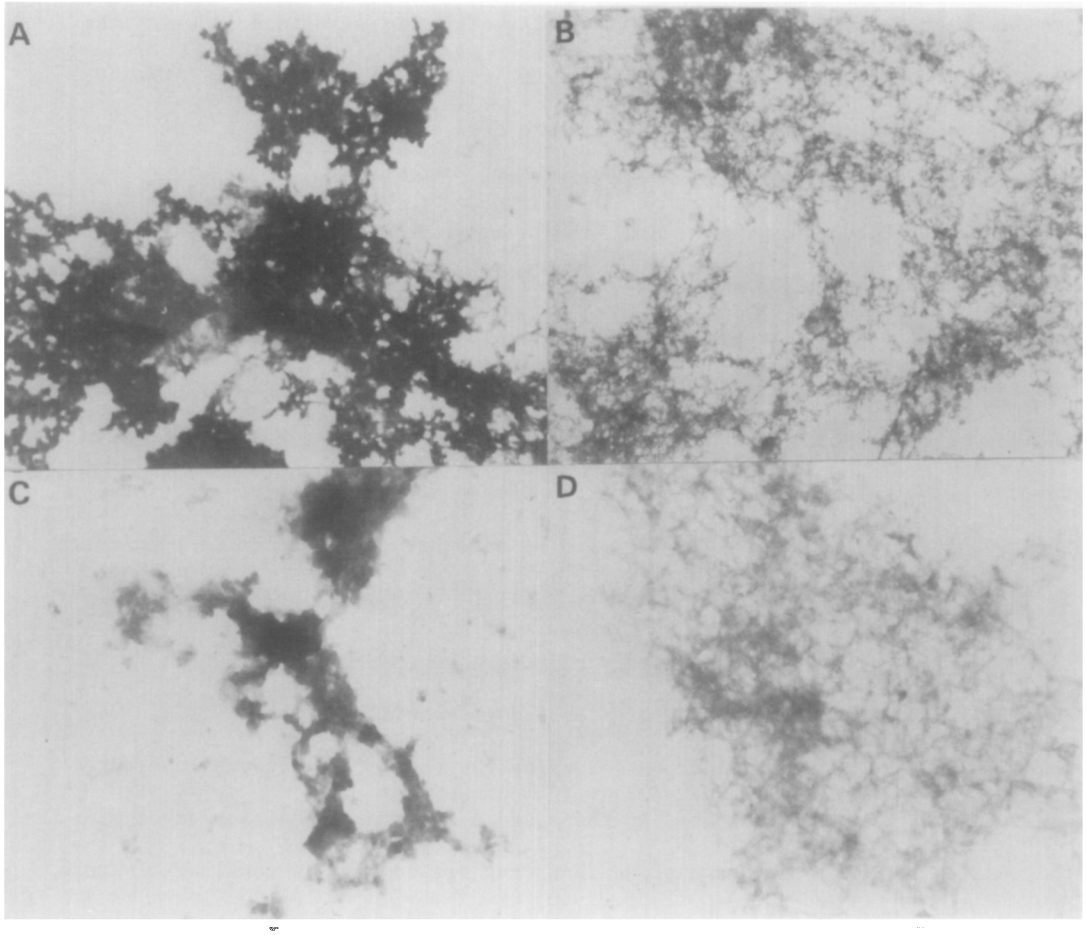

Figure 3: Electron microscopy of chromatin treated with adriamycin. Chromatin contained in the pellet fraction after centrifugation of disrupted nuclei (A, B) or chromatin isolated after sucrose gradient centrifugation (C, D) was incubated in the absence or presence of adriamycin. The concentration of adriamycin was 28 µg/ml. Drug treated chromatin is shown in A and C. At the end of the incubation period, EDTA was added to 0.01 M and an aliquot of the reaction mixture was spotted on a parlodion coated grid. After 1 min. the grid was blotted, stained with 2% uranyl acetate (in water) and washed briefly in water. The dried grids were visualized in a Philips 201 electron microscope. Final magnification: A, C, D x 13,500; B x 7,200.

To determine the nature of the structures formed after incubation of adriamycin with chromatin the drug treated DNA was examined in the electron microscope. In these studies we analyzed chromatin isolated from sucrose gradients (Figure 3, C,D) and also chromatin contained in the pellet fraction obtained after disruption of nuclei (Figure 3, A,B). The chromatin incubated

in the absence of drug appears as a spongelike fibrous structure in which the DNA fibers are contained in a highly complex network (Figure 3 B,D). When the DNA is incubated in the presence of the drug a profound change occurs in the structure of the chromatin (Figure 3 A,C). The type of structure observed varied somewhat but there was essentially a complete disappearance of chromatin such as that shown in Figure 3 B,D. In the presence of drug the DNA was more heavily stained and in many cases the chromatin fibers were considerably thickened (Figure 3 A,C). Many regions of the chromatin were highly condensed and single fibers could not be observed. Attempts to detect chromatin in various stages of condensation by varying drug concentration have thus far been unsuccessful. The results of these studies have also indicated that there is little evidence for any drug induced aggregation of the isolated DNA.

The results of the present study therefore suggest that adriamycin is capable of inducing a compaction of isolated chromatin. Further evidence for this has recently been obtained by measuring the rate of Micrococcal nuclease digestion of chromatin incubated in the absence and presence of adriamycin. The results of this study have shown that drug treated DNA is considerably more resistant to nuclease cleavage than is chromatin which has not been incubated with the drug (unpublished results). These results therefore suggest that as the chromatin is compacted nuclease susceptible sites such as linking regions between nucleosomes (10, 11) are now protected from the action of the enzyme. Of interest is the finding that ethidium bromide is inactive in this system thus suggesting that DNA intercalation is not a major factor in the compaction reaction. In contrast to the present system ethidium bromide is, however, as active as adriamycin in inducing the formation of single-strand regions in isolated chromatin (6). Thus the ability of adriamycin to induce chromatin compaction and DNA single-strand regions may not be related. The mechanism by which adriamycin induces a condensation of chromatin is unknown. Possibly the ability of adriamycin to intercalate into DNA and to also form protein dependent interstrand cross links (9) leads to the formation of an unstable

structure which undergoes compaction. Whether the drug induced folding of chromatin is also occurring in vivo and contributes to the cytotoxic effect of adriamycin remains to be determined. Previous studies have shown, however, that the addition of adriamycin to isolated human leukocytes results in the formation of chromosomes with a variety of morphological aberrations (12). Possibly these events occurring in vivo are related to the ability of adriamycin to induce chromatin condensation in vitro.

Acknowledgement

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