Segregation of the Nucleolus Produced by Anthramycin¹

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SUMMARY

Anthramycin, an antibiotic possessing antitumor properties, was administered to rats and mice in a single intraperitoneal dose ranging from 1 to 10 mg/kg body weight. Light microscopy showed that, at the highest dose, anthramycin produced necrosis in the pancreas of rats but not in mice. By electron microscopy, nucleolar changes were observed in the spleen and kidney of both species by 1 hour. Acinar cells of the pancreas showed nucleolar caps and segregation of nucleolar constituents in both species at 3 to 4 hours. By 10 hours segregation of the nucleolus and focal cytoplasmic degradation were prominent in pancreatic cells of rats given higher doses but were not observed in hepatocytes of either species.

Anthramycin produced nucleolar alterations in Kupffer cells, endocrine and exocrine pancreatic cells, proximal tubular cells of the kidney, and in the reticuloendothelial cells of liver and spleen. The mechanism by which anthramycin acts is unknown, but its ultrastructural effects, as well as its structural similarity to actinomycin D, suggest that it may inhibit RNA synthesis at the DNA level. The functional consequences of segregation of the nucleolus have not been established.

INTRODUCTION

Recently, the effects of carcinogens and antitumor agents on nucleolar morphology and function have been subjects of renewed interest. Investigation of the acute ultrastructural and biochemical abnormalities following administration of pyrrolizidine alkaloids (44), aflatoxin (42), ethionine (22), actinomycin D (13, 15, 26, 36, 41), 4-nitroquinolinol-N-oxide (34), UV radiation (24), proflavin (38), and mitomycin (17) has indicated a variety of ultrastructural responses in the nucleus and has emphasized the critical role of the nucleus in cellular metabolism and inheritance. Particular emphasis has been given to the nucleolus because of its importance in ribosomal RNA (rRNA) synthesis and in the transfer of RNA from the nucleus to the cytoplasm (27–30).

Anthramycin, an antibiotic isolated from a thermophilic actinomycete (19), has in vitro antibacterial and in vivo antitumor activity (49). At concentrations effective as an antitumor agent against transplantable tumors in mice, anthramycin does not produce toxicity in the bone marrow and gastrointestinal tract (49). It possesses certain structural features in common with actinomycin D and the postulated biosynthetic intermediates of the actinomycins, 3-hydroxy-4-methylanthraniloyl peptides (3,47). Like kanamycin, neomycin, and other antibiotics (48), anthramycin permanently bleaches Euglena gracilis (11).

This report demonstrates that anthramycin is another agent which causes nucleolar changes in pancreatic cells, reticuloendothelial cells of liver and spleen, and in proximal tubular epithelium in the kidney.

MATERIALS AND METHODS

Thirty-six inbred Fisher-344 male rats weighing between 95 and 240 gm and fifteen C57 black mice weighing between 20 and 30 gm were used. Anthramycin (anthramycin methyl ether) dissolved in 100% ethanol was administered by a single intraperitoneal injection. The dosages used were 1.0, 1.7, 5, and 10 mg/kg body weight. At least two rats at each dose were sacrificed at 1, 4, 10, and 24 hr. Mice were sacrificed at 1, 3, 10, 12, and 24 hr.

Microscopic Studies. Small blocks of liver, pancreas, kidney, and spleen were fixed in s-collidine-buffered 1% osmium tetroxide for electron microscopy. The blocks were dehydrated in a graded series of alcohols and embedded in Epon 812 containing 5% araldite (20). Thin sections were cut with an LKB microtome using glass knives, stained with lead hydroxide and/or uranium acetate, and examined in an RCA 3B or 3G electron microscope. Semi-thin sections (0.5 to 1.5 /µ) of Epon-embedded tissue were stained with aqueous azure A in an equal volume of 5% sodium bicarbonate.

For light microscopy, portions of pancreas, liver, spleen, kidney, and duodenum were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin or by the periodic acid-Schiff procedure.

RESULTS

Light Microscopy

Loss of zymogen granules and necrosis were noted in the pancreatic acinar cells of rats but not in mice. Necrosis of duodenal crypts was present in both species. All pancreatic alterations were most pronounced with the highest dose (10 mg/kg) at 10 hr. No changes were observed in the liver, kidney, or spleen.

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Electron Microscopy

Pancreas. At a dose of 5 mg/kg no changes were evident at 1 hr in the exocrine pancreas of rats. By 4 hr the endoplasmic reticulum of acinar cells showed marked distention and vesiculation which was sustained at later intervals. The nuclei of acinar cells separated into granular and fibrillar components (Figs. 1, 2) with a denser granular component occasionally present at the periphery. It is uncertain whether these denser granular components represent interchromatin granules, a newly-formed element, or a degradation of preexistent elements. Nucleoli of islet cells were fragmented but no cytoplasmic changes were noted.

Nucleolar segregation was also apparent in many of the nuclei at the lower dose (1 mg/kg) at 10 hr, and all nuclei examined were altered at the highest dose (10 mg/kg). Granules similar in size to interchromatin granules were increased in number and usually clustered in the center of the nucleus (Figs. 3, 4). Occasionally, the entire nucleoplasm appeared to be distinctly partitioned into two different densities (Fig. 5). Focal cytoplasmic necrosis of exocrine cells was present (Fig. 6).

At 24 hr (1 mg/kg), most of the endocrine and exocrine cells of the rat pancreas contained normal nucleoli but a round dense nucleolar remnant consisting only of the fibrillar component was present occasionally. Focal cytoplasmic necrosis persisted in some exocrine cells.

The nucleolar response of mouse pancreas to anthramycin was similar to that in rats. At 3 hr amidst acinar cells with normal nucleoli, there were cells with dispersed and disarranged nucleoli (Fig. 8). Nucleoli exhibited segregation at 4 and 12 hr (Figs 7, 9). The only cytoplasmic change was dilation of the endoplasmic reticulum.

Liver. Segregation of the nucleolus was not observed in either mouse or rat hepatocytes but granular aggregates measuring 0.2-0.4 μ and nucleolar plaques were present (Fig. 10). In both species, Kupffer cells showed nucleolar alterations at all intervals (Fig. 11). No remarkable changes were observed in the cytoplasm except the accumulation of fat droplets in Golgi vesicles of rat hepatocytes at 24 hr.

Kidney and Spleen. Anthramycin produced a variety of nucleolar changes in the kidney of rats. These alterations ranged from fragmentation of the nucleolus to complete separation of the nucleolar components (Fig. 12). In both species, there were nucleolar alterations in reticuloendothelial cells of the spleen (Figs. 13, 14).

DISCUSSION

Ultrastructure of the Normal Nucleus. The variation in structure of normal nuclei of mammalian cells has been reviewed by Davis (6), and attempts to unify the terminology of the nucleolus have been reported at a recent symposium (46). In general, the normal nucleolus is composed of (a) granules, 50-200 Å in diameter; (b) fibrils, 50-100 Å in diameter; and (c) “intranucleolar chromatin,” consisting of microfibrillar elements (2). The first and second components are sensitive to RNase digestion (21, 36) and the third component is partially digestible by trypsin. Marinozzi and Bernhard (21) divided the granular component into 2 types: one type resembles cytoplasmic ribosomes 100-150 Å in diameter and the second consists of irregular aggregates 50-100 Å in diameter. The nucleolus is surrounded, in part, by the nucleolus-associated chromatin which, like intranucleolar chromatin, contains DNA (2, 45). The morphology of normal nuclei show some degree of organ and species specificity. Nucleoli of rat and mouse pancreatic cells are similar in their ultrastructure but nucleoli of hepatocytes of several species of mice characteristically show slight separation of the fibrillar and granular components.

Organ Specificity, Mechanism of Action, and Biochemical Effects of Anthramycin. Nucleolar caps, produced by 4-nitroquinoline-N-oxide, was first described by Reynolds et al. (34). Like several other agents (39), anthramycin produces similar changes in the nucleolus. In general, the initial separation of nucleolar constituents is followed by a diminution of the granular component.

The various agents which cause nucleolar segregation demonstrate organ specificity. Actinomycin D produces this nucleolar change in the exocrine pancreas (13), cell culture (15), neurons (16), and hepatocytes (26, 41). Anthramycin causes similar abnormalities in nuclei of islet cells, the exocrine pancreas (Fig. 1), reticuloendothelial cells of the spleen (Figs. 13, 14), and in Kupffer cells (Fig. 11), while hepatocytes show only slightly altered nucleoli (Fig. 10). Aflatoxin (42), tannic acid (31), and pyrrolizidine alkaloids (44) affect nucleoli of hepatocytes and Kupffer cells but not those of the pancreas. Several agents produce nucleolar segregation in cell culture (15, 17, 34, 35, 38). Two of the most unusual stimuli related to nucleolar segregation are Mycoplasma (14) and Herpes simplex virus (40), since these are the only known instances of nucleolar segregation produced by microorganisms, though prominent nucleolar changes of a different type occur in liver cells infected with Ectromelia (18). A separation of the entire nucleoplasm into a lucent and a dense area was observed following anthramycin treatment (Fig. 7). Actinomycin D (13) and proflavin (38) produce similar partition of electron density of the nucleoplasm, and the same change has been observed following administration of cycloheximide (C. Harris, and D. J. Svoboda, unpublished results).

The mechanism by which anthramycin acts is unknown. It has been found to inhibit RNA synthesis in Ehrlich tumor cells (G. Zbinden, personal communication). It possesses structural features found in the actinomycins and their biosynthetic intermediates (3, 47). Leimgruber et al. (19) suggested that “anthramycin could be transformed in vivo to ‘actinomycin analogs’, which actually may be responsible for the observed anti-tumor activity.”

Although the majority of agents which cause nucleolar segregation bind DNA and/or RNA (39), some DNA-alkylating agents such as dimethylnitrosamine (7), diethylnitrosamine (23), and ethionine (22) do not cause the same nucleolar alterations. Work in progress in this laboratory indicates that, in acute stages, dimethylnitrosamine, ethionine, and 3'-methyl-dimethylaminoazobenzene, when given in high doses, produce a form of nucleolar segregation, suggesting that the nucleolar effects of agents which alkylate nucleic acids are dependent upon dose and time.
Segregation of the Nucleolus by Anthramycin

Although many of the nucleolar segregating agents are carcinogens, there is no evidence implicating the related alterations in nucleolar ultrastructure to carcinogenesis (43). Indeed, the methylation or ethylation of nucleotide bases, as with the nitrosamines, may be meaningless in terms of transmission of genetic information by DNA.

A recent comparative study of antimetabolites by Simard and Bernhard (39) suggests that nucleolar segregating agents “...bind to DNA and interfere with its template activity for the conduct of DNA-directed RNA synthesis by RNA polymerase.” This hypothesis is compatible with the biochemical mechanism of aflatoxin (9) and the proposed mechanism for nucleolar caps induced by UV irradiation of the non-nucleolar nucleoplasm (24). The recent finding in our laboratory that cycloheximide can produce nucleolar segregation indicates that an alteration in protein synthesis is an important facet of nucleolar segregation (C. Harris and D. J. Svoboda, unpublished results). In this context, it is of interest that the primary action of cycloheximide appears to be in the cytoplasm and the drug interferes with protein synthesis at a step more distal in the protein synthetic pathway than the site(s) of interference or inhibition by other agents which cause nucleolar segregation.

The synthesis of rRNA seems to be particularly sensitive to low concentrations of DNA-binding agents. As noted by Chiga et al. (5), 80% of the nucleoli of hepatocytes undergo segregation after administration of concentrations of actinomycin D (13 μg/100 gm body weight) which inhibit only a fractional amount of DNA-dependent RNA synthesis. At a slightly higher concentration of actinomycin D (20 μg/100 gm body weight), Jacob et al. (12) have indicated that the synthesis of the initial rRNA species (45 S rRNA) is completely blocked within 30 seconds.

The protein matrix, of which 30% is histone, comprises approximately 70% of the nucleolar dry weight (4, 10). Histones appear to play an important role in RNA synthesis (1,4) and, conceivably, changes in the structure or viscosity of the nucleolar protein matrix could produce secondary morphologic alterations, such as segregation, in nucleoli. This consideration seems all the more pertinent when one considers the stabilizing effect of histones on nucleic acids (4) and the effects of cycloheximide on nucleolar ultrastructure. Freedman et al. (8) found that the inhibition of RNA synthesis by actinomycin D resulted in a 50% decrease in synthesis of the lysine-rich histone fraction.

Anthramycin-induced changes parallel some of the morphologic effects of actinomycin D. Actinomycin D produces nucleolar segregation and inhibits DNA-dependent RNA synthesis by binding the guanine residues of DNA (32, 33). As suggested by Perry (29), actinomycin D would be expected to inhibit the synthesis of high molecular weight guanine + cytosine-rich RNA, 45 S rRNA species, before low molecular weight RNA, such as soluble RNA, would be inhibited. Indeed, the 45 S rRNA has been found to be sensitive to low concentrations of actinomycin D (12). The possibility that nucleolar segregating agents, such as actinomycin D, may degrade or alter newly synthesized nucleolar rRNA cannot be overlooked (30). Schwartz and Garofalo (37) found little or no evidence of migration of labeled 18 S and 28 S rRNA into the cytoplasm following actinomycin D treatment.

Ultrastructural evidence indicates that anthramycin is a typical nucleolar segregating agent which produces separation of nucleolar constituents followed by a diminution of the “granular component,” 100–200 Å in diameter. These nucleolar granules and the 60 S ribosomal precursors observed in ultracentrifugation studies are most likely one and the same (25).

In conclusion, nucleolar segregation is the ultrastructural reflection of nucleolar dysfunction, that is, alterations in the synthesis of ribosomal precursors by various agents which bind DNA and inhibit the synthesis of rRNA. Alterations in pre-existing nucleolar rRNA and protein synthesis may also be partly responsible for the ultrastructural changes observed.

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Figs. 1–14. All electron micrographs are from sections stained with uranyl acetate and lead.

Fig. 1. Rat pancreas, 4 hr after administration of anthramycin, 1 mg/kg body wt. The fibrillar component (f) and the granular component (g) show complete segregation. Ill-defined granular densities (arrows) are adjacent to the segregated nucleolus. × 72,000.

Fig. 2. Rat pancreas, 4 hr after administration of anthramycin, 1 mg/kg body wt. The granular component (g) is diminished and segregated from the two contrasted zones of the fibrillar component (f₁ and f₂). A denser granular component (arrow) is present elsewhere in the nucleoplasm. × 66,000.

Fig. 3. Rat pancreas, 10 hr after administration of anthramycin, 5 mg/kg body wt. The nucleolus (nuc) contains only the fibrillar component and a constellation of granules (bracket) is centrally located. × 16,000.

Fig. 4. Rat pancreas, 10 hr after administration of anthramycin, 5 mg/kg body wt. The granular aggregate appears to be composed of at least two different sizes of granules (f and 2). × 61,000.

Fig. 5. Rat pancreas, 10 hr after administration of anthramycin, 5 mg/kg body wt. Distinct partition (marked by x) of the nucleoplasm into regions of contrasting density is present. × 5,800.

Fig. 6. Rat pancreas 10 hr after administration of anthramycin, 5 mg/kg body wt. Foci of cytoplasmic necrosis containing mitochondria (m) and endoplasmic reticulum (ER) are prominent in acinar cells after high doses of anthramycin. × 6,700.

Fig. 7. Mouse pancreas, 4 hr after administration of anthramycin, 10 mg/kg body wt. The nucleolus is segregated into granular (g) and fibrillar (f) components. The denser granular component (arrow) adjacent to the nucleolus is similar to those found in Figs. 3 and 4. × 29,000.

Fig. 8. Mouse pancreas, 3 hr after administration of anthramycin, 1.7 mg/kg body wt. The nucleolus shows fragmentation and partial segregation of the granular (g) and fibrillar (f) components. × 39,000.

Fig. 9. Mouse pancreas, 12 hr after administration of anthramycin, 1 mg/kg body wt. Segregation of the fibrillar (f) and granular (g) components of the nucleolus is present in the majority of the cells observed at 12 hr. × 19,000.

Fig. 10. Rat liver, 24 hr after administration of anthramycin, 1 mg/kg body wt. Hepatocytes show little response to anthramycin. Nucleolar plaques (p) and granular aggregates (arrows) are noted with increased frequency. × 19,000.

Fig. 11. Rat liver, 24 hr after administration of anthramycin, 1 mg/kg body wt. Kupffer cell nucleoli demonstrate partial separation of components at all intervals tested. × 29,000.

Fig. 12. Rat kidney, 12 hr after administration of anthramycin, 1 mg/kg body wt. Nucleolar alterations in the proximal tubule cells are evident at all intervals beginning at 1 hr. Large microbodies (mb) containing dense nucleoids are seen. × 7,000.

Fig. 13. Rat spleen, 12 hr after administration of anthramycin, 1 mg/kg body wt. The fibrillar component (f) is partially encircled by a ring of dense granules (g). × 72,000.
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Fig. 14. Rat spleen, 12 hr after administration of anthramycin, 1 mg/kg body wt. Nucleolar plaques (arrows) are present. × 72,000.
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