Specific Nuclear and Nucleolar Ultrastructural Lesions Induced by Proflavin and Similarly Acting Antimetabolites in Tissue Culture

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Summary

Proflavin induces a series of ultrastructural alterations in cultures of rat embryonic cells. Nonspecific cytoplasmic lesions include disorganization of endoplasmic reticulum and large cytoplasmic inclusions with osmiophilic material and myelin figures, similar to those described in HeLa cells treated with acridine orange and in liver and pancreatic acinar cells of rats fed with ethionine and azaserine. However, characteristic nuclear and nucleolar lesions such as clumping of the chromatin with unsticking from the nuclear membrane, disappearance of the nucleoplasmic matrix, and segregation of nucleolar components occur as treatment progresses in time and concentration. Two other antimetabolites, daunomycin and ethidium bromide, chemically unrelated to proflavin, produce identical nuclear and nucleolar lesions at adequate concentrations. Since proflavin, daunomycin, and ethidium bromide form complexes with DNA by intercalation between base pairs, the nuclear and nucleolar lesions may represent the morphologic expression for a specific molecular action. Possible explanations are proposed, taking into account the physicochemical properties of the drug-DNA complex and their effect on nucleic acid and protein synthesis.

Introduction

The remarkable progress achieved in the field of antimetabolite chemistry and the study of the molecular site of attack of these drugs has not yet been paralleled by an equal systematic investigation on the ultrastructural reactions of treated cells. It can be postulated that substances whose molecular action is similar should lead to similar specific cytoligic lesions, provided that dosage and duration of treatment are suitably chosen in order to dissociate primary target effects from nonspecific general toxicity involving the whole cell. A series of electron microscopic papers published in recent years already tends to show that this postulate is correct at least for certain groups of compounds. The highly selective action of actinomycin on the nucleolus can be cited as the best example (19, 20, 37, 42). Other drugs, such as 4-nitroquinoline N-oxide (38), mitomycin C (22), aflatoxin (3), and some other products, give rise to similar nucleolar changes which are now known as "nucleolar segregation." An hypothesis has been proposed by Simard and Bernhard (44) to explain the similarity of cytologic lesions produced by this group of chemically unrelated compounds.

The purpose of this paper is to present another type of characteristic ultrastructural change concerning both the nucleolus and chromatin and similarly induced by proflavin, daunomycin, and ethidium bromide.

Proflavin, a 3,6-diaminoacridine dye, is a mitotic inhibitor which interferes with nucleic acid synthesis in vitro (5, 10, 11, 16, 40, 43) and in vitro (2, 18). It is also a potent mutagen (12, 25, 30) that intercalates itself between adjacent base pairs of the DNA molecule, causing deletion errors on replication (24, 27). These effects are of interest for the study of generic action. But, to our knowledge, no ultrastructural study of the molecular action of proflavin has been made so far. Proflavin was included in a systematic investigation on the action of various kinds of antimetabolites on nucleolar fine structure (44). The lesions produced by proflavin in both the nucleus and the nucleolus proved to be so specific that special attention was given to this compound. Moreover, it was later found that 2 other compounds, the new antibiotic daunomycin, isolated from cultures of Streptomyces peucetius (8), and the trypanocidal drug, ethidium bromide, which also act by intercalation between base pairs of DNA (6, 49), produce similar, if not identical, alterations in the nuclear and nucleolar fine structure.

Materials and Methods

Rat embryos were obtained from the Wistar breeding colony of our Institute. They were extracted after 12-15 days of gestation. After trypsinization, the cells were transferred into a modified Eagle solution containing, respectively, 4 and 8 times the usual concentration of amino acids and proteins and supplemented with 20% calf serum. For our experiments, secondary cultures were utilized. The cells were grown with the same medium. Forty-eight hr after subculture, the cells had formed a monolayer; they were then changed to a maintenance medium containing 8% calf serum and treated with proflavin (1, 2, 10, and 20 \( \mu \)g/ml for periods of 1-72 hr), ethidium bromide (2, 5, and 10 \( \mu \)g/ml for 1, 6, and 24 hr), and daunomycin (0.5, 1, and 5 \( \mu \)g/ml for 1, 6, and 24 hr). For proflavin the same experiments were repeated with the BSC strain of monkey (Cercopithecus) kidney cells. For each period of time experimented, controls were made where cells were simply grown in the maintenance medium. Stock solutions of proflavin, ethidium bromide, and daunomycin were prepared at a concentration of 100 \( \mu \)g/ml by dissolving the powder in the maintenance medium. Shortly before use, this stock was diluted to the desired concentration.

Proflavin is a product of Nutritional Biochemicals Corporation (Cleveland, Ohio). Daunomycin was kindly provided by Farmitalia (Milan, Italy), and ethidium was generously given by Boots Pure Drugs (Nottingham, England).

For electron microscopy, the cells were fixed in \( \textit{in situ} \) by the addition of 2% osmium tetroxide phosphate buffered according...
to Millonig (29) at pH 7.3 for 1 hr or 2.5% glutaraldehyde phosphate buffered at pH 7.5 for 15 min. At the end of the fixation time, the cells were scraped off the glass surface with the help of a rubber policeman and centrifuged for 10 min at 8000 rpm. The pellet obtained was cut into small cubes with a razor blade. Aldehyde-fixed cells were embedded in glycolmethacrylate (23) and polymerized under an ultraviolet lamp at 4°C. Osmium-fixed cells were embedded in Epon. Thin sections were cut with a Huxley ultramicrotome, using glass knives. They were stained with 2% uranyl acetate, 20 min, followed by lead citrate, 10 min (36), or 2% uranyl acetate in 50% alcohol, 30 min–1 hr. The sections were examined with a Siemens Elmiskop I at 80 kv, with an objective aperture of 50 μ.

Results

Ultrastructure of Untreated Rat Embryonic Cell

Monolayers of rat embryonic cultures consist of elongated and rather pleomorphic small cells. Occasionally, the cytoplasm displays phagocytic vacuoles and a fair amount of glycogen. Mitochondria are oval-shaped, and their internal cristae are rudimentary. The Golgi apparatus is poorly developed. Ergastoplasmic membranes are rare, but free ribosomes are abundant. These cells in general are undifferentiated in exponential growth, but fibroblasts predominate as cultures grow older, that is, after 48 hr.

The nucleus is rather large as compared with the size of the cell. There is little chromatin fixed to the nuclear membrane or associated with the nucleolus. Perichromatinic granules are rare; they measure 300–350 Å in diameter and are surrounded by a clear halo of 600–700 Å as described by Watson (50). The nucleoplasms are composed of a fine fibrillar material with scattered granules, occasionally grouped in clusters and measuring 200–250 Å in diameter. These granules have been described by Swift (45) and by Granboulan and Bernhard (13) and are called “interchromatinic granules.” Rarely, special inclusions called “nuclear bodies” are seen within the nucleoplasm of rat embryonic cells; they have been found in a wide variety of cells, either pathologic or normal, since their 1st description (7). The nucleolus shows irregular contours and is often located close to the nuclear membrane. The bulk of the nucleolar mass consists of dense granules, 150–200 Å in diameter, embedded in a loose reticular network of fibrils 50 to 80 Å in diameter and an electron-dense matrix. This nucleolar network is known as the “nucleolonema” (Fig. 1). Enzymatic digestions have shown that granules and fibrils are digested by RNase, whereas the amorphous matrix is attacked by pepsin (28).

Alterations Observed in Proflavin-treated Cells

Cytoplasmic Lesions. At a concentration of 1 μg/ml, the 1st lesions are observed after a treatment of 6 hr. The rat embryonic cells show a slight tendency to increase in size and to round up. Cytoplasmic lesions include an increase in vacuoles and fat droplets. The Golgi apparatus, although hyperplastic in some cells, undergoes no major transformation throughout the treatment. Some mitochondria are swollen and several clumps of glycogen particles appear. After a treatment of 24 hr, the most striking lesions are the number of cytoplasmic inclusions containing dense osmiophilic material. These inclusions are membrane-bound and appear to form by condensation of several vacuoles containing patchy or membranous material with occasional lamellate myelin figures (Fig. 2). The rough-surfaced endoplasmic reticulum develops a whorled arrangement with focal separation of the membrane system and dilatation of the cisternae (Fig. 3). Free ribosomes become moderately abundant. As the concentration increases up to 10 μg/ml, these cytoplasmic alterations are more pronounced, but there is no other remarkable transformation. Cytoplasmic inclusions undergo progressive focal degradation (46). The separation between the membranes of the endoplasmic reticulum increases remarkably, and the interspace is filled with an amorphous substance of moderate electron density (Fig. 5). The cisternae transform into separated vesicles covered by ribosomes. Free ribosomes are more abundant than in normal cells (Fig. 6).

Nuclear and Nucleolar Lesions. Early nuclear and nucleolar lesions appear after 6 hr of treatment at 1 μg/ml. The nucleus increases in size and turns into an egg-shaped mass with occasional cytoplasmic invaginations. The nucleolus has rounded up. The granules increase in number, and the nucleolonema gains electron density as fragmentation of its reticular aspect occurs (Fig. 4). Few other changes are observed whether in the chromatin or in the nucleolus after 12 or 24 hr at 1 μg/ml. Reversibility of the lesions is then possible if the cells are changed to a maintenance medium for 24 hr.

At a concentration of 10 μg/ml for 1 hr, proflavin-treated cells undergo major transformations. The chromatin gains density and migrates toward the periphery of the nucleus. Spotty electron-dense aggregates are scattered throughout, while the nucleolus forms an opaque spherical mass where granules and fibrils are difficult to discern. The reticular network of the nucleolonema is completely lost (Fig. 7).

Shortly after, at 10 μg/ml for 6 hr, striking changes take place in the nucleus without any other form of transition. The bulk of the chromatic mass forms electron-dense osmiophilic aggregates standing out in a nucleus that otherwise keeps its size and shape. There is an unusual unsticking of the chromatic clumps from the nuclear membrane (Fig. 8). After 24 hr at the same concentration, the clumping and unsticking of the chromatin increases as the nucleoplasm has lost considerable material and electron density (Fig. 9). Aldehyde-fixed and glycolmethacrylate-embedded cells show that the nucleoplasm, usually described as fibrillar does not entirely disappear but forms precipitates around the chromaticic clumps with intermixing tiny threads (Fig. 10). The perichromatinic granules are totally lost in the treatment, while the interchromatinic granules are grouped in clusters: their size has increased considerably (300–350 Å in diameter) and their shape is still very irregular (Figs. 6, 10).

After 24 hr the nucleolus has considerably decreased in size and shows a complete separation of its components. In proflavin-treated cells, however, the lesion has to be carefully looked for since it is not as frequent and as typical as with actinomycin-treated nuclei. The granular zone usually stands out, and there is considerable variation in the increased size (200–250 Å in diameter) and forms of the granules, which frequently overlap the fibrillar zone. The amorphous zone and the contrasted zone are seen only occasionally in early lesions. The regular sequential appearance of the lesions that have been described for antimetabolites which specifically affect the nucleolus (44) has never been observed in proflavin-treated cells (Figs. 11–14).
As the treatment progresses in time (10 and 20 μg/ml for 48 and 72 hr), the sparsity of the chromatin increases remarkably, leaving the sticky-looking aggregates in a poorly defined fibrillar network. The size of the nucleolus decreases with persistence of the granules. Cytoplasmic alterations are characterized by intense vacuolization and final necrosis. No mitosis was ever observed in treated cells. Reversibility of the lesions proved to be impossible after 24 hr at concentrations of 10 μg/ml.

The same nuclear and nucleolar lesions were observed with proflavin-treated BSC cells at the same concentrations.

Alterations in Daunomycin- and Ethidium Bromide-treated Cells

Similar lesions were produced by daunomycin and ethidium bromide with a different sequence. In cells treated with these 2 compounds, nucleolar segregation is the 1st lesion to appear at 1 μg/ml (daunomycin) and 2 μg/ml (ethidium bromide) for a treatment of 6 hr (Figs. 13, 15).

This segregation follows the regular pattern, although here again the lesion is less frequent and less typical than with actinomycin D. At these concentrations it is important to note that the only lesion observed is the nucleolar segregation (Figs. 15, 17). But if concentration and time of treatment increase (5 μg/ml for 24 hr with daunomycin and 10 μg/ml for 24 hr with ethidium bromide), the morphologic appearance of the treated cell is entirely superposable to the nuclear and nucleolar lesions induced by proflavin. The chromatin clumps into electron-dense aggregates and unsticks from the nuclear membrane. The nucleoplasm loses totally its already low electron density. The perichromatin granules are lost, and the interchromatinic ones form scattered clusters (Figs. 16, 18). The cytoplasmic lesions induced by daunomycin and ethidium bromide will not be further described here since they grossly correlate the lesions already described in proflavin-treated cells.

Discussion

Molecular Action of Proflavin, Daunomycin, and Ethidium Bromide

Peacocke and Skerrett (31) have shown that aminoacridines are bound to DNA mostly by a 1st order combination which saturates at 1 molecule bound per 4 or 5 nucleotides. Later, it was established that the most satisfactory mode of bindings is intercalation of the dye into the helix between adjacent base pairs by extension of the DNA molecule (24). The plausible sites of intercalation would be in decreasing order (a) on an AT pairing and below, (b) on an AT pair above and a GC below, and (c) on a GC pair above and below (47). This binding results in marked extension of the DNA molecule, enhanced viscosity, and decrease of the sedimentation coefficient (24); it accounts probably for the high mutagenic action of proflavin, causing replication errors of insertion and deletion of nucleotides.

The biochemical action of proflavin is comparable to that of actinomycin D (33–35), that is, inhibition of enzymatic reactions leading to RNA and DNA synthesis (18). But proflavin does not show any specificity for inhibition of DNA and RNA formation, whereas actinomycin blocks selectively DNA-directed RNA synthesis by RNA polymerase. Reich (32) has proposed that, since actinomycin is assumed to bind in the minor groove (14), it seemed logical to expect that this groove would be the specific template site for RNA polymerase and subsequently for RNA synthesis. According to the conjecture proposed by Lerman (24, 25), proflavin affects both the major and the minor groove, and this might explain its unspecificity.

The antibiotic daunomycin is an antimetabolite with cytotoxic and antimitotic activity (8). Like proflavin, daunomycin is believed to bind to DNA by intercalation between base pairs, and the complex daunomycin-DNA results in an increased relative viscosity and a decreased sedimentation rate (6). Daunomycin inhibits RNA synthesis regardless of the base composition of the DNA template (48), and its selectivity for the inhibition of RNA synthesis is best explained by a larger ring system of its molecule and also by its amino-sugar side chain that projects in the minor groove, causing steric obstruction for the conduct of RNA synthesis (6).

The phenanthridine drug ethidium bromide is also known to form reversible complexes with both DNA and RNA and inhibits nucleic acid synthesis and nucleic acid polymerases (9, 48). This compound binds with DNA regardless of its base composition (48) by intercalation between base pairs of the DNA double helix (49).

Tentative Correlations with Their Morphologic Effects

The cytoplasmic injuries produced by proflavin are characteristic but rather unspecific. The whorled appearance, separation of membranes, and dilution of the cysterna of the endoplasmic reticulum in proflavin-treated cells are similar to those already described in rat liver and pancreatic acinar cells treated with azaserine and ethionine (1, 15, 17). Similar lesions have also been observed in polio-infected HeLa cells (4) and in culture cells treated with several antimetabolites such as puromycin, chloramphenicol, and 8-azaguanine (44). They are probably related to a severe disturbance in protein synthesis preceding cell death and part of a nonspecific defense mechanism of the cells against an insulating agent. Cytoplasmic inclusions containing lamellate membranes are strikingly similar to those observed in acridine orange-treated HeLa cells (39) and azaserine-treated rats (17). Evidence has been put forward that these inclusions represent segregated dyes or other colloidal particles in cytosomes (41) or lysosomes (21).

However, the nuclear lesions induced by proflavin, daunomycin, and ethidium bromide proved to be characteristic, as they were not observed with 21 other antimetabolites studied on rat embryonic cells (44). The relationship between the morphologic effect of these compounds on the nucleus and their biologic and biochemical activities can be speculated upon as follows: (a) As a result of their complex formation with DNA, they alter the physicochemical properties of chromatin, including its stainability with osmium and uranyl acetate, its affinity for the binding sites on the nuclear membrane, and its distribution in the normal interphase nucleus. (b) Because of the inhibition of DNA synthesis, the sparsity of chromatin increases as the treatment progresses in time and concentration. (c) The secondary inhibition of pro-

The following abbreviations are used: A, adenosine; T, thymidine; G, guanosine; and C, cytidine.
tein synthesis results in the almost complete loss of nucleoplasmic matrix, leading to this empty-looking appearance.

The increase in size and cluster formation of interchromatinic granules and the disappearance of the perichromatinic granules are difficult to interpret, since their exact nature has not yet been elucidated.

Early nucleolar lesions, such as increase of granular components, fragmentation, and condensation of the fibrillar network, found in proflavin-treated cells have been observed at the optical (26) as well as at the ultrastructural level in cells treated with various compounds, some of which may finally give rise to nucleolar segregation (44). They probably represent disturbances in nucleolar function but have no specificity whatsoever.

The nucleolar segregation caused by proflavin, ethidium bromide, and daunomycin presents some variations from the typical lesion induced by actinomycin D. In proflavin-treated cells especially, the lesion is not constant and is characterized by a smaller nucleolus with a prominent overlapping granular zone. Apart from the 4 above-mentioned compounds, 7 other antimetabolites produce nucleolar segregation: they are 4-nitroquinoline-N-oxide, mitomycin C, aflatoxin, nogalamycin, chromomycin A3, azaserine, and echinomycin. A working hypothesis was proposed that encompasses the following points: (a) binding to DNA, (b) inhibition of DNA-directed RNA synthesis by RNA-polymerase, and (c) obstruction of the minor groove of helical DNA, induce segregation, redistribution of nucleolar components, and exhaustion of the nucleolus (44). This specific lesion is produced at low concentration for short periods of time for antimetabolites that conform exactly to these postulates. The ultrastructural effect is often proportional to their biochemical action. For proflavin, ethidium bromide, and daunomycin, higher concentrations are required, probably because a larger number of molecules is necessary for the steric obstruction of the minor groove and interference with the template activity of DNA for the conduct of RNA synthesis by RNA-polymerase. These speculations can be verified experimentally in the future with combined autoradiographic, cytochemical, and biochemical techniques.

Acknowledgments

The author gratefully acknowledges the helpful advice and stimulating suggestions of Dr. Wilhelm Bernhard throughout the course of this work and the preparation of the manuscript, Prof. Paul Tournier of the Department of Virology, Miss Marie-France Bourali for the tissue culture work, and Miss Françoise Yven for secretarial assistance.

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FIG. 1. Untreated rat embryonic cell. The nuclear chromatin (chr) is mostly associated with the nuclear membrane or the nucleolus. Perichromatinic granules (→) and interchromatinic granules (ig) are present as well as a nuclear body (nb). The nucleolus consists of dense granules (g) embedded in a loose fibrillar reticular network, the nucleolonema (nu). Between the meshes, small cavities are filled with light amorphous material (p). All figures represent osmium-fixed, Epon-embedded material stained with uranyl acetate and lead citrate, except as otherwise noted. × 45,000.
FIGS. 2–4. Initial lesions in proflavin-treated rat embryonic cells.

Fig. 2. Proflavin, 1 μg/ml, 24 hr. The large vacuolated cytoplasmic inclusions (ci) contain patchy or lamellate osmiophilic material with myelin figure (→) and are membrane-bound. Vacuoles (v) are also present. No nuclear lesion is evident. × 30,000.

Fig. 3. Proflavin, 1 μg/ml, 24 hr. The endoplasmic reticulum shows separation of its membranes (→) and dilation of the cisternae (C). × 75,000.

Fig. 4. Proflavin, 1 μg/ml, 6 hr. The nucleolus has rounded up: granules (g) increase as fragmentation of the nucleolonema (nu) occurs. × 30,000.
Figs. 5, 6. Advanced cytoplasmic lesions in proflavin-treated cells.

Fig. 5. Proflavin, 10 µg/ml, 6 hr. The endoplasmic reticulum is markedly dilated and forms intercommunication channels (→) containing a moderately osmiophilic substance. Early sequestration of vacuoles (v) is seen on the left. Cytoplasmic inclusions (ci) show focal degradation. × 60,000.

Fig. 6. Proflavin, 10 µg/ml, 48 hr. The cisternae (C) form vesicles studded with ribosomes. The abundance of free ribosomes in Figs. 5 and 6 are noteworthy. × 75,000.
Figs. 7, 8. Nuclear and nucleolar lesions in proflavin-treated cells.

Fig. 7. Proflavin, 10 µg/ml, 1 hr. The nucleus shows margination of the chromatin (chr), toward the nuclear membrane. Spotty aggregates (→) are seen in a nucleoplasm (N) that has lost electron density. The nucleolus is an opaque spheric mass with complete loss of its normal architecture. × 30,000.

Fig. 8. Proflavin, 10 µg/ml, 6 hr. The whole appearance of the nucleus has changed totally. The chromatin forms electron-dense clumps (cc) with early unsticking from the nuclear membrane (→). Within a nucleoplasm (N) of low electron density, clusters of interchromatinic granules are seen (ig). × 15,000.
FIGS. 9, 10. Advanced nuclear lesions in proflavin-treated cells.

FIG. 9. Proflavin, 10 μg/ml, 24 hr. The chromatinic clumps (cc) are completely unstuck from the nuclear membrane, and their sparsity increases. Tiny filaments (→) are seen in an otherwise empty nucleoplasm (N). Two nucleoli in segregation are seen. × 15,000.

FIG. 10. Proflavin, 10 μg/ml, 24 hr. Glutaraldehyde-fixed and glycolmethacrylate-embedded cells show a better preservation of the amorphous nucleoplasmic matrix (N) with intercommunicating precipitates around clusters of interchromatinic granules (ig) and chromatinic clumps (cc). × 45,000.
Figs. 11-14. Nucleolar segregations induced by proflavin. The prominent granular zone (g) occasionally overlaps the fibrillar zone (f). The contrasted zone (sc) is seen in Fig. 12. In glutaraldehyde-fixed and glycolmethacrylate-embedded cells, the amorphous zone (p) appears more clearly (Figs. 13, 14).

Fig. 11. Proflavin, 20 μg/ml, 12 hr. X 45,000.
Fig. 12. Proflavin, 20 μg/ml, 24 hr. Note the tiny filaments (→) in the nucleoplasm. X 45,000.
Fig. 13. Proflavin, 10 μg/ml, 24 hr. X 30,000.
Fig. 14. Proflavin, 10 μg/ml, 24 hr. The granules (g) are very irregular in size (200–250 Å) and shape. X 90,000.
Figs. 15, 16. Nuclear and nucleolar lesions produced by ethidium bromide in rat embryonic cells.

Fig. 15. Ethidium bromide, 2 μg/ml, 6 hr. Nucleolar components are segregated in a granular zone (g), a fibrillar zone (f), and a contrasted zone (sc). X 35,000.

Fig. 16. Ethidium bromide, 10 μg/ml, 6 hr. Clumping of the chromatin occurs (cc) with unsticking from the nuclear membrane (→). The nucleoplasm (N) has totally lost its electron density. Nucleolar segregation is present although somewhat different from that seen in Fig. 15. X 45,000.
Figs. 17, 18. Nuclear and nucleolar lesions produced by daunomycin in rat embryonic cells.

**Fig. 17.** Daunomycin, 1 μg/ml, 6 hr. Segregation of nucleolar components into granular (g), fibrillar (f), and contrasted (sc) zones occurs, but the chromation is evenly distributed. × 75,000.

**Fig. 18.** Daunomycin, 5 μg/ml, 6 hr. Chromatinic clumping occurs with unsticking from the nuclear membrane (→). Cluster of interchromatinic granules (ig) is seen in a rather clear nucleoplasm (N). × 30,000.
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Cancer Res 1966;26:2316-2328.

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