The Effect of Terramycin on the Fine Structure of HeLa Cell Mitochondria*

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SUMMARY

Terramycin, in various concentrations, was added to culture media in which HeLa cells were proliferating. Bright-field and fluorescent microscopy revealed that terramycin is rapidly and specifically bound to the mitochondria. At low concentrations no changes in mitochondrial morphology were apparent. When concentrations were increased to 100 µg/ml or higher, the mitochondria became distended and rounded up.

Marked alterations in mitochondrial structure were observed at concentrations of 100 µg/ml with the electron microscope. These changes included vacuolation, compression of the cristae, and striking reduplication of the mitochondrial membranes. At 300 µg/ml no normal mitochondria were present, and degeneration of other cytoplasmic elements occurred. Concentrations of 1000 µg/ml proved lethal for all cells.

The tetracycline series of antibiotics is used in the treatment of bacterial infections in man and laboratory animals. In the laboratory, terramycin, one of the tetracyclines, is frequently added to tissue culture media to prevent the growth of bacteria and PPLO organisms. As with most antibiotics, the exact mechanisms of bactericidal action of the tetracyclines are unknown. It is, however, becoming increasingly apparent that they may also damage the cells of higher organisms. Earlier reports have indicated that certain antibiotics have deleterious effects on the growth and functions of cells in culture (4, 10, 14, 19); however, the degree of toxicity for bacteria is usually greater than it is for the mammalian cell.

Terramycin is readily detected in animal tissues soon after injection because of its brilliant yellow-green fluorescence (20, 23). Recent studies by DuBuy and Showacre (6) indicated that the tetracyclines were rapidly incorporated into cells, both in vivo and in vitro, and that these antibiotics were localized in the mitochondria. Biochemical studies indicated that the antibiotics caused a decrease in oxidative phosphorylation when added to isolated mitochondria (6). The present study is concerned with the specific alterations produced in the fine structure of HeLa cell mitochondria by the antibiotic terramycin.

MATERIALS AND METHODS

HeLa cells were grown on standard 3 “XI” micro slides in square French bottles for bright-field and UV studies. Control cultures and experimental cultures growing in varying concentrations of terramycin were rinsed in Hanks solution and mounted in control medium under coverslips sealed with paraffin and observed immediately with a Leitz fluorescent unit. Fixed cultures were stained by the aniline-acid fuchsin-methyl green method or the phosphotungstic acid-hematoxylin procedure for mitochondria (15).

For electron microscopy, stock HeLa cells were grown in a medium composed of 1 part calf serum and 2 parts medium 199 in stationary roller tubes. After monolayers had developed, terramycin (Pfizer brand of oxytetracycline) was added to the tubes in concentrations ranging from 100 to 1000 µg/ml culture medium. The pH of the media containing terramycin ranged between 7.1 and 7.4. After exposure to the antibiotic for 2–24 hours the cells were fixed in situ by exposure for 10 minutes to vapors from a 2 per cent osmic acid solution. Embedding was carried out in methacrylate as previously described (11). Thin sections were mounted on formvar films and stained with lead hydroxide (21) before examination.

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RESULTS

Bright-field microscopy.—The mitochondria in HeLa cells grown for 24–48 hours in media containing 5–10 \( \mu g/ml \) of terramycin were morphologically like those in cells grown in control media. Fixed and stained preparations contained many long, filamentous mitochondria. When the concentration of the antibiotic was increased to 100 \( \mu g/ml \), a shortening and swelling of the mitochondria occurred after 8–10 hours of incubation. Some cells also contained small numbers of spherical mitochondria. If the concentration of the antibiotic was increased to 300 \( \mu g/ml \) or higher, marked alterations in the morphology of the mitochondria occurred within 2 hours of incubation. The mitochondria distended and became spherical in shape. On continued incubation at these higher concentrations, no normal-appearing mitochondria were seen after 6 hours. Cell lysis was observed as early as 10 hours after the addition of these high concentrations of antibiotic, and all the cells appeared granular. Most of the cells had lysed after 24 hours’ incubation.

UV fluorescence.—Five minutes after the addition of 5 \( \mu g/ml \) of terramycin the mitochondria fluoresced bright green. At this, or slightly higher, concentration there were no alterations in the morphology of the mitochondria as observed in the fluorescent microscope. Morphological alterations similar to those in fixed sections were seen when the concentration of the antibiotic was increased to 100 \( \mu g/ml \) or higher. At all the concentrations fluorescence was limited to the mitochondrial elements.

Electron microscopy.—The fine structure of HeLa cells has been described previously (11). The mitochondria of HeLa cells vary considerably in morphology, ranging from short rods to long, filamentous structures (Fig. 1). Cristae often appeared irregularly oriented and were not abundant in the filamentous variety. The matrix had a relatively low electron density. These observations have been confirmed by other investigators (2, 7).

After exposure to 100 \( \mu g \) terramycin/ml for 12 hours all the cell organelles appeared as they did in control cultures except for the mitochondria. Although the structure of most mitochondria was definitely altered, a few appeared unaffected by the drug. The degree of mitochondrial alterations varied somewhat from cell to cell and may have depended upon the metabolic state of the cells. Slight damage was indicated by a localized distension or vesiculation. Many of the mitochondria were ring-shaped, probably representing horizontal sections through cup-shaped structures. Some mitochondria were rounded, with characteristic cristae displaced by stacks of membranes (Fig. 2).

At 200 \( \mu g/ml \) damaged and altered mitochondria were observed in the electron microscope in cells which had been incubated for as little as 2 hours. These damaged mitochondria appeared swollen; some retained a disorganized mass of internal membranes (Fig. 3), and other organelles contained a granular matrix enclosing heavy-walled vesicles (Fig. 4).

At 300 \( \mu g/ml \), mitochondrial damage was further accentuated; some of the mitochondria consisted of complex membranous whorls, and others had dense myelin-like inclusions located within the vacuoles. Some of these organelles were enormously swollen. In several instances the cristae were compressed toward one end of the mitochondria; in others the cristae appeared to have been replaced by dense granular material (Figs. 5, 6). At this stage, vacuolation of the endoplasmic reticulum was evident in many cells.

In later stages of degeneration, many of the mitochondria were transformed into large vesicles with translucent interiors surrounded by a thick periphery. Others retained their multivesicular nature, with vacuolar contents in various stages of degeneration. The nuclear structures appeared normal, but other signs of cell necrosis become apparent. There was a condensation of altered mitochondria and other cytoplasmic elements, which were interspersed with a fibrous material of unknown origin. Hyalinization of the cytoplasm at the cell periphery was also a noticeable feature (Fig. 7).

The effects of terramycin appear to be primarily evident in mitochondria. At a concentration of the antibiotic (100 \( \mu g/ml \)) at which marked changes in mitochondrial morphology were noted, other cytoplasmic structures did not appear to be altered. There was no evidence of nuclear changes or vacuolation of the endoplasmic reticulum. At the higher concentrations, evidence of cytoplasmic degeneration was widespread after 10–12 hours of incubation. These changes may, however, reflect secondary effects in cells deprived of normal mitochondrial function for extended periods of time.

DISCUSSION

Studies by other investigators have demonstrated that the tetracycline antibiotics are preferentially bound to mitochondria (6). Our experiments have shown that terramycin, oxytetracycline, in concentrations of 100 \( \mu g/ml \) or higher, rapidly produced striking alterations in the morphology of mitochondria in HeLa cells. The initial reaction involved vacuolation of the mitochondria, followed by compression of the cristae and/or redu-
plication of the mitochondrial membranes to form multilaminar bodies. As degeneration progressed, these changes were accentuated, the characteristic cristae were lost, and the organelles were converted to thick-walled vacuoles or myelin-like figures. At higher concentrations of the antibiotic, degeneration of the mitochondria was followed by cell lysis.

Mitochondrial alterations, similar to those described above, were among the structural changes seen in L cells following vaccinia virus infection (3), and adenoviruses have been reported to stimulate the reduplication of perinuclear membranes (9). Transformation of mitochondria into lamellar bodies has been described in epidermal (18) and HeLa cells (16), when the composition of the tissue culture media was varied. Cup-shaped mitochondrial profiles were observed occasionally in rat testicular interstitial cells (3). Formation of concentric lamellae has also been seen in inclusion bodies of alveolar phagocytes following ingestion of India ink (12).

In a rapidly multiplying population of cells a small percentage of the cells undergo degeneration. Morgan et al. (22) have observed vacuolation and compression of cristae in mitochondria, vesiculation of the endoplasmic reticulum, and, in more advanced stages, condensation of the cytoplasmic contents during spontaneous necrosis of cultured cells.

With regard to biochemical lesions produced by tetracyclines, several reports have noted that oxidative phosphorylation is particularly sensitive to these antibiotics. Loomis (16) first noted that aureomycin specifically depressed phosphorylation without inhibiting respiration of mitochondria. DuBuy et al. (6) found oxygen consumption unchanged but a decreased efficiency of oxidative phosphorylation in the presence of tetracycline and terramycin as well as aureomycin. Brody et al. (1) studied a series of tetracyclines and concluded that in vitro inhibition of oxidative phosphorylation may be due to the chelating ability of the tetracyclines. They postulated that tetracyclines combine with and effectively remove Mg++, which is necessary for the coupling reaction to proceed. If the binding of terramycin to cell structures also depends on its chelating ability, then its preferential localization in mitochondria would appear to indicate binding with the Fe++ of the cytochromes.

From extensive studies of fractionated mitochondria, Green (8) found that the double-membrane structure is necessary for coupling oxidation to phosphorylation and stated that the respiratory enzymes are rigidly organized and integrated within the architecture of the mitochondria. Because of this close interrelationship of structure and function in mitochondria, it is difficult to determine exactly where terramycin has its effect. The initial effect may be on the enzymatic machinery, and the consequent loss of respiratory activity would lead to degeneration of mitochondrial organization. Inhibition of the energy-producing mechanisms of the mitochondria then would block the metabolic processes of the cell involved in carbohydrate metabolism and eventually lead to cell destruction. It is less probable that the tetracyclines directly modify mitochondrial structure upon which enzymatic activity depends, thus resulting in cell destruction.

The addition of as little as 5 μg/ml of terramycin to our tissue culture medium resulted in the fluorescence of the mitochondria of living HeLa cells which were exposed to UV light. Since higher concentrations are often used in tissue culture media, one may presume that levels below those needed to produce cytotoxic effects may interfere with the "normal" function of the mitochondria. In some instances, cells used for measuring metabolic activities were grown in media containing four different antibiotics (17). With the possible exception of penicillin, which is apparently nontoxic to growing cells even at high concentrations, it appears that most antibiotics have specific effects on the host cell as well as infecting bacteria. One should, therefore, be cognizant of these inhibitory effects when interpreting data on metabolic rates derived from cells grown in media containing antibiotics.

REFERENCES


**Fig. 1.—Control HeLa cell.** Part of the nucleus (nuc) appears at lower left, and the cell periphery with a few microvilli courses along the top of the micrograph. Hyaline central area which consists of a network of filaments corresponds to glycogen (gly). The irregularly arranged cristae are evident in the mitochondria scattered through the cytoplasm. X22,000.
Fig. 2.—Portions of three cells from a culture exposed to 100 μg terramycin/ml for 18 hours. Nucleus (nuc) of one cell appears at upper right. Alterations in mitochondrial structure are readily apparent. These changes include localized vesiculation (1), ring-shaped forms (2), and multiplication of membranes with compression of the cristae (3). Several mitochondria in this field still retain normal structure. X22,000.
FIG. 3.—Part of a HeLa cell from a culture grown in 200 µg terramycin/ml for 2 hours. This section contains examples of mitochondria with a localized vesiculation (1), a ringlike shape (2), and a swollen organelle in which the cristae are present as a tangled network (3). X2,000.

FIG. 4.—Another example of mitochondrial damage produced in cells grown in media containing 200 µg terramycin/ml for 2 hours. Cristae have disappeared from these mitochondria which have a granular matrix and thick-walled vesicles (1), one of which contains a large vacuole (2). X16,000.
FIG. 5.—Micrograph of HeLa cells after exposure to 300 μg terramycin/ml for 24 hours. The nucleus (nuc) appears at lower left. Mitochondrial destruction is widespread. Some appear as multilaminar bodies (1), others are vacuolated and contain dense granular material (2). Vesiculation of the endoplasmic reticulum becomes evident (3). Few, if any, normal mitochondria are present at this concentration of terramycin. X28,000.
Fig. 6.—Section through the cytoplasm of a HeLa cell grown in 300 μg terramycin/ml for 24 hours. Numerous mitochondria have assumed a multilaminar appearance (1). Another mitochondrion is composed of compartmentalized vacuoles and encloses a dense myelin-like figure (2). The vacuolar distension of one mitochondrion has compressed the cristae to one side (3). ×28,000.
Fig. 7.—Low-power micrograph of HeLa cell from a culture exposed to 300 µg terramycin/ml for 24 hours. The cytoplasmic contents are condensed and consist of many vacuolated and granular structures, derived from degenerate mitochondria and vesiculated endoplasmic reticulum, enmeshed in fibrous material. There are, as yet, no discernible changes in the structure of the nucleoli and nucleoplasm (nuc). X10,000.
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