Physiological and Biochemical Studies of Effects of Mitomycin C on Strain HeLa Cells in Cell Culture

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SUMMARY

Proliferation of HeLa cells cultured with 0.1 μg/ml mitomycin C for 24 hours and then transferred to normal medium was inhibited. HeLa cells treated with mitomycin C in serum-free medium were affected as well as those in serum-containing medium. Cells treated with mitomycin C in a nondividing phase in serum-free medium did not recover their ability to proliferate upon transfer to normal medium. No conspicuous decrease was found in the number of cells treated with mitomycin C at 4°C, even in a concentration as high as 50 μg/ml. This fact suggests that mitomycin C may show no inhibitory activity at such a low temperature or that this antibiotic may not penetrate through the cellular membrane in this condition. It was found by morphological observation that HeLa cell cultures with mitomycin C showed the appearance of giant cells, multinuclear cells, and an increase of the nuclear size. Chemical determination on protein and nucleic acid content showed that mitomycin C had a relatively strong inhibitory action on cell division and DNA synthesis, and a moderate action on protein and RNA synthesis. These effects seem to fit in with the observations on the appearance of giant cells.

Mitomycin C, which is an antibiotic isolated from Streptomyces caepitoaua (2), has been demonstrated to have strong antitumor action not only on solid, ascitic, and leukemic tumors of the mouse, the rat, and the hamster (3, 10, 16, 18), but also on various types of human cancer (14, 17). It was also shown by Perlman and his associates (8) that this antibiotic was highly toxic against the mouse fibroblast strain L cells cultured in vitro. Smith et al. (15) showed that mitomycin C was an active antibiotic, 0.025 μg/ml of which were sufficient to cause 50 per cent inhibition in the culture of the strain KB cells (human epidermoid carcinoma). In Escherichia coli B, Shiba et al. (12) presented evidence that mitomycin C completely inhibited synthesis of deoxyribonucleic acid (DNA), whereas it did not affect ribonucleic acid (RNA) and protein formation. In the present paper, cytotoxic effects of mitomycin C on strain HeLa cells cultured in various conditions and its effects on the synthesis of nucleic acids and of protein in these cells were described.

MATERIALS AND METHODS

The strain of HeLa cells used throughout this work was supplied by the National Institute of Health in Tokyo. These cells were derived from Subline A, which was a colonial clone of its parent stock after 387 transfers. After fifteen transfers, in the Research Institute for Microbial Diseases, Osaka University, in a medium consisting of 15 per cent bovine serum and 0.5 per cent lactalbumin hydrolysate in Earle’s salt solution, and after ten transfers in the present laboratory in the following medium, the cells were used for the present experiment.

Culture medium for the stock cells and the experimental cells consisted of 15 per cent bovine serum and 0.5 per cent lactalbumin hydrolysate (NBC) in a modification of Earle’s salt solution (CaCl₂·2H₂O, 0.265 mg/ml; MgCl₂·6H₂O, 0.175 mg/ml; NaH₂PO₄·2H₂O, 0.14 mg/ml; NaHCO₃, 0.5 mg/ml); 63 μg/ml (400 units/ml) of combined penicillin G crystal and 100 μg/ml of dihydrostreptomycin sulfate were added to the medium. Mitomycin C, dissolved in salt solution by being heated at 80°C for 5 minutes, was sterilized through a Seitz filter, added to the culture medium, and the various final concentrations were obtained by gradual dilution. The initial pH of the media was adjusted to 7.4.

In stock cultivation square bottles have been used and incubated under stationary conditions.

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In experimental culture the simplified replicate tissue culture method was employed (4, 5). Cell suspension was prepared by treating a culture at 37°C for 2 minutes with 0.25 per cent trypsin (Difco Laboratories Inc.) in Hanks balanced salt solution deficient in Ca++, Mg++, and PO4−. After centrifugation at 350 × g for 5 minutes, sedimentary cells were resuspended in the culture media and delivered into short test tubes. Each tube received 0.75 ml of cell suspension and 0.75 ml of culture fluid, respectively. It was found in preliminary experiments that the optimum inoculum size was in a range from 10,000 to 20,000 cells, except in special cases. In each culture group consisting of ten tubes, three tubes were terminated, respectively, after 2, 4, and 7 days of cultivation; the number of cell nuclei in these groups was determined following shaking for 30 minutes in a solution of 0.1 M citric acid and 0.05 per cent crystal violet at 37°C. One remaining tube served as a spare in each group.

Nucleic acids and protein were chemically determined as follows. The cells from ten tubes were washed 5 times in balanced salt solution. The preliminary experiment showed that this washing procedure was enough to remove any detectable contamination from the serum protein in the medium. The acid-soluble fraction was extracted in cold 0.5 N perchloric acid (PCA) for 20 minutes. After centrifugation at 1,500 × g for 10 minutes the precipitate was resuspended in cold 0.5 N PCA, and extraction of the acid-soluble fraction was repeated again. The sediment was suspended in 0.5 N PCA, and the nucleic acid fraction was extracted at 90°C for 15 minutes. Total nucleic acids contained in the supernatant fluid were measured by reading the optical density at 260 mμ with a Beckman spectrophotometer. The precipitate containing protein was dissolved in 1 N NaOH, and the amount of protein was determined colorimetrically by Folin’s method (6). DNA in the total nucleic acid fraction, soluble in hot PCA, was determined by a modification of Burton’s method (1), with diphenylamine. The amount of RNA in the hot PCA-soluble fraction was determined by Mejbaum’s method (7). Since this value of RNA agreed closely with the value obtained by subtracting DNA from total nucleic acid amounts, RNA was usually calculated by the latter method.

RESULTS

Effects of concentrations of mitomycin C in the medium.—The media containing mitomycin C in the following concentrations were prepared to examine the effects of mitomycin C on the proliferation of HeLa cells: 0.01 μg/ml, 0.1 μg/ml, 1.0 μg/ml, 3.0 μg/ml, 10.0 μg/ml, and finally 50.0 μg/ml. The results obtained from the cultivation for 7 days are represented in Chart 1.

In concentrations of more than 3.0 μg/ml the decrease in the number of cell nuclei was evident after culturing for 2 days. In the concentrations 0.1 μg/ml, 0.3 μg/ml, and 1.0 μg/ml, the number of cell nuclei was increased after 2 days and then decreased gradually with further cultivation. In the concentration of 0.01 μg/ml, proliferation of HeLa cells after 2 days was comparable to that in the control culture (not containing mitomycin C); but an inhibitory effect of mitomycin C was observed after 4 days.

Effects of the duration of treatment with mitomycin C.—In concentrations of 0.1 μg/ml, 0.3 μg/ml, and 1.0 μg/ml, the number of cells increased in the first 2 days but decreased thereafter. The duration of treatment with mitomycin C varied as follows: 1 day, 2 days, 3 days, 4 days, and 7 days. After the cells were treated with mitomycin C during these periods the test medium was replaced with normal medium. The effects of duration of treatment with 0.1 μg/ml of mitomycin C are shown in Chart 2.

As seen in Chart 2, proliferation of HeLa cells was inhibited by treatment with 0.1 μg/ml of mitomycin C for 1 day, although some increase in the number of cell nuclei was observed for 3 days.
The treatment of 0.1 \( \mu g/ml \) mitomycin C for more than 2 days had the effect of decreasing the number of the cell nuclei.

**Effects of mitomycin C on HeLa cells in serum-free media.**—To ascertain whether mitomycin C has an inhibitory action on cells in the resting phase or those in the dividing phase, the effects of various concentrations of mitomycin C on HeLa cells in serum-free media were examined. Without serum HeLa cells did not proliferate even in the absence of mitomycin C. These results are shown in Chart 3.

It can be seen that in serum-free media, as well, the higher the concentration of mitomycin C, the more pronounced was the decrease in the number of cell nuclei. This suggests that mitomycin C may have a cytotoxic effect both on resting or non-dividing cells as well as on proliferating cells.

To investigate whether nondividing cells treated with mitomycin C could recover their proliferative activity, cells treated with 0.1 \( \mu g/ml \) mitomycin C in serum-free medium for a given time were returned to normal medium. The preliminary experiment showed that incubation of HeLa cells in serum-free medium without mitomycin C for less than 2 days had the effect of increasing the number of cell nuclei.

**Chart 2.**—Effects of duration of treatment with 0.1 \( \mu g/ml \) mitomycin C on HeLa cells. The cells were initially cultured with 0.1 \( \mu g/ml \) mitomycin C, then the culture medium was changed into normal medium after 1 day, 2 days, 3 days, 4 days, and 7 days of cultivation. The numbers of cell nuclei in each culture group represent the means of those of three culture tubes. Vertical lines on the upper two curves indicate the range of variation.

**Chart 3.**—Effects of mitomycin C in various concentrations on HeLa cells in serum-free medium. The cells were cultured with mitomycin C in various concentrations indicated here in serum-free medium. The numbers of cell nuclei in each group represent the means of those of three culture tubes.

**Chart 4.**—Effect of 0.1 \( \mu g/ml \) mitomycin C on HeLa cells in serum-free medium which were further cultured in the normal medium. The cells preincubated in the normal medium for 1 day were treated with 0.1 \( \mu g/ml \) mitomycin C in the serum-free medium for 2 days. Then the medium was replaced with normal medium to culture for further 3 days. The numbers of cell nuclei indicated here represent the means of those of three culture tubes. Vertical lines on each curve indicate the range of variation.
than 2 days had no harmful effect on their further proliferation when transferred to normal medium. After the cells were preincubated in normal medium for 1 day so that they would adhere to the glass surface, they were incubated for 2 days in the serum-free medium containing 0.1 μg/ml mitomycin C. After this treatment the cells were again cultured in normal medium. The results are shown in Chart 4.

As shown in this chart, cells treated with 0.1 μg/ml mitomycin C in serum-free medium as well as in medium containing 15 per cent serum seemed unable to proliferate when placed in conditions favorable for growth. 

Effect of mitomycin C on HeLa cells at 4° C.—The cells were treated with mitomycin C in various concentrations at 4° C. in a refrigerator. These results are shown in Chart 5.

No conspicuous decrease was found in the number of cell nuclei in cultures at 4° C. treated with various concentrations of mitomycin C, although proliferation was not observed even after 7 days in the control culture. This fact suggests that the metabolic activity of cells may decrease at 4° C. and therefore not be affected by mitomycin C or that the incorporation of mitomycin C through the cellular membrane may decrease at low temperatures.

Morphological observations of HeLa cells treated with mitomycin C.—HeLa cells cultured with mitomycin C in various concentrations in Carrel flasks were fixed in Carnoy's fluid and stained in methylgreen-pyronine or Giemsa solution to examine the morphological changes of the affected cells. In the culture with 0.01 μg/ml mitomycin C, increase in the size of the cells and especially the appearance of giant cells and of multinuclear cells were observed after 7 days of cultivation. In the culture with 0.1 μg/ml mitomycin C numerous giant cells with giant nuclei were observed after 7 days, followed by detachment of the affected cells from the glass surface of the culture flasks. The higher the concentration of mitomycin C used, the more pronounced was the effect observed at the earlier time of cultivation. In the culture with 1.0 μg/ml mitomycin C, detachment of the affected cells was already observed after 2 days of cultivation.

Effect of mitomycin C on protein and nucleic acids content in HeLa cells.—Sixty culture tubes which received inoculations of HeLa cells were incubated in medium containing 0.01 μg/ml mitomycin C. Fifteen tubes were terminated, respectively, at 0, 2, 4, and 6 days of cultivation. In three of these the number of cell nuclei was determined; ten tubes were used for determining protein, DNA, and RNA content; the remaining two tubes served as spares. The number of cell nuclei, and the content of protein, DNA, and RNA in HeLa cells treated with 0.01 μg/ml mitomycin C are shown in Table 1.

In the presence of 0.01 μg/ml mitomycin C there was a gradual increase in protein, DNA, and RNA content, comparable with that in control cultures; on the other hand, the increase in the number of cell nuclei was clearly inhibited after

**DISCUSSION**

HeLa cells cultured with mitomycin C from 0.1 μg/ml to 1.0 μg/ml showed an increase in the number of cell nuclei during the initial 2 days of cultivation, followed by a gradual decrease with
further cultivation. Even when the cells treated with 0.1 µg/ml mitomycin C were transferred to normal medium on the 2d day of treatment, their normal capacity to proliferate could not be restored. These facts indicate that the increase in cell nuclei during the first 2 days may depend on proliferating activity initiated before the onset of treatment with the antibiotic and that mitomycin C incorporated into the cells during the first 2 days may have continued to exert a cytotoxic division and DNA synthesis but a moderate action on protein and RNA syntheses. The appearance of giant cells in cultures treated with mitomycin C seems to be also explained by the inhibition of cell division and DNA synthesis.

It was found by Rueckert and Mueller (9) that HeLa cells cultured with amethopterin showed an unbalanced growth. The cells which were made thymidine-deficient by treatment with this agent showed inhibition of DNA synthesis, of prolifera-

### TABLE 1

**EFFECT OF 0.01 µG/ML MITOMYCIN C ON PROTEIN, DNA, AND RNA CONTENT PER TEN TUBES IN HELa CELLS**

<table>
<thead>
<tr>
<th>Mitomycin C added (µg/ml)</th>
<th>Culture (days)</th>
<th>No. cell nuclei</th>
<th>Protein content (µg)</th>
<th>DNA content (µg-P)</th>
<th>RNA content (µg-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.0X10⁶</td>
<td>1.020</td>
<td>1.4</td>
<td>2.8</td>
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<tr>
<td></td>
<td>2</td>
<td>11.8X10⁶</td>
<td>2.063</td>
<td>2.6</td>
<td>6.5</td>
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<tr>
<td></td>
<td>4</td>
<td>23.3X10⁶</td>
<td>3.059</td>
<td>4.3</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25.6X10⁶</td>
<td>3.137</td>
<td>4.8</td>
<td>3.0</td>
</tr>
<tr>
<td>0.01 µg/ml</td>
<td>0</td>
<td>4.7X10⁶</td>
<td>969</td>
<td>1.4</td>
<td>2.6</td>
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<tr>
<td></td>
<td>2</td>
<td>10.0X10⁶</td>
<td>2.109</td>
<td>2.5</td>
<td>6.5</td>
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<td></td>
<td>4</td>
<td>15.2X10⁶</td>
<td>3.005</td>
<td>3.4</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>14.1X10⁶</td>
<td>3.111</td>
<td>3.6</td>
<td>2.2</td>
</tr>
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</table>

### TABLE 2

**EFFECT OF 0.1 µG/ML MITOMYCIN C ON PROTEIN, DNA, AND RNA CONTENT PER TEN TUBES IN HELa CELLS**

<table>
<thead>
<tr>
<th>Mitomycin C added (µg/ml)</th>
<th>Culture (days)</th>
<th>No. cell nuclei</th>
<th>Protein content (µg)</th>
<th>DNA content (µg-P)</th>
<th>RNA content (µg-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.7X10⁶</td>
<td>247.2</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.6X10⁶</td>
<td>338.0</td>
<td>1.3</td>
<td>2.5</td>
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<tr>
<td></td>
<td>4</td>
<td>9.8X10⁶</td>
<td>574.0</td>
<td>2.3</td>
<td>4.3</td>
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<tr>
<td></td>
<td>6</td>
<td>16.0X10⁶</td>
<td>561.0</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1 µg/ml</td>
<td>0</td>
<td>1.7X10⁶</td>
<td>200.8</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.8X10⁶</td>
<td>249.0</td>
<td>1.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.8X10⁶</td>
<td>387.0</td>
<td>1.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.0X10⁶</td>
<td>561.0</td>
<td>0.9</td>
<td>2.0</td>
</tr>
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</table>

It is interesting to note that the cells in serum-free medium as well as those in the serum-containing medium were affected by mitomycin C.

The results of the chemical determination of protein, DNA, and RNA showed that mitomycin C had a relatively severe inhibitory action on cell division, and later of RNA synthesis. Protein was synthesized slowly in such a condition. These relatively slight effects on protein and RNA synthesis are comparable with the results obtained in the present experiments.

In Escherichia coli B, it was indicated by Shiba et al. (12) that synthesis of DNA was completely inhibited in the presence of mitomycin C. However, it was shown by Sekiguchi and Takagi (11) that the impaired synthesis of DNA in cells treated with mitomycin C could be promptly restored by infection with bacteriophage T4r. They considered the possibility that an alternative pathway of
formation of DNA, resistant to mitomycin C, might be present in the infected cells. In HeLa cells treated with mitomycin C the synthesis of DNA was inhibited more strongly than those of RNA and protein. However, this inhibitory action on the synthesis of DNA was not so complete as shown in bacteria.

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