EFFECTS OF MITOMYCIN-C ON HELA CELLS AT THE VARIOUS STAGES OF DIVISION CYCLE*1

(Plates XII-XIV)

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Synopsis

Effects of Mitomycin-C on various stages of the division cycle of Hela cells were investigated by autoradiography with tritiated thymidine.

Regarding the first division cycle after Mitomycin-C treatment, the latter half of the G1 period and the first half of the S period of the division cycle seemed to be highly sensitive to Mitomycin-C.

INTRODUCTION

Shiba et al.27) reported that Mitomycin-C specifically inhibited the DNA synthesis of E. coli even at a concentration which failed to inhibit the synthesis of protein or RNA. Since then, evidences13,25) have accumulated indicating that the primary action of this antibiotic was closely related to the synthesis of DNA. However, it has remained unclarified whether DNA synthesis of the cell is affected to the same degree by Mitomycin-C regardless of the stage of its division cycle.

There are many reports on the radiosensitivity of cells in various stages of division cycle,4-6,9,14,15,20,21,26,30,32,34,35) but only a few papers are available on the sensitivity of cells in different stages of division cycle to antitumor agents.7,12,33)

The present experiments were carried out to clarify the effect of Mitomycin-C on the various stages of division cycle of HeLa cells.

MATERIALS AND METHODS

Mitomycin-C Mitomycin-C was supplied through the courtesy of Kyowa Hakko Kogyo Co. Each ampule contained 2 mg of crystalline Mitomycin-C, which was dissolved and diluted with Hanks' balanced salt solution immediately before use.

HeLa Cells HeLa cells had been supplied by Dr. Katsuta, The Institute for Infectious Diseases, University of Tokyo, and maintained in our laboratory for several years.

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Culture Method HeLa cells were cultivated in TD-40 flasks, at 37°C. The culture medium consisted of 15 parts of bovine serum and 85 parts of Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate, $1 \times 10^4$ units/L of penicillin, 100 mg/L of streptomycin, and a small quantity of Phenol Red. The medium was renewed every other day. A four- or five-day-old monolayer cells were harvested with trypsin treatment (0.25%, Difco, 1:250) and used for the experiment.

Evaluation of Growth-inhibitory Effect of Mitomycin-C on HeLa Cells Fifty or seventy thousand HeLa cells were inoculated in short test tubes. After incubation at 37°C for about 24 hrs., the test tubes were divided at random into several groups. Each group was treated with the same medium containing various amounts of Mitomycin-C except for one group which was not treated with Mitomycin-C and used as control. After incubation for a definite length of time, depending on experimental conditions, the medium containing Mitomycin-C was discarded, the cells were washed twice with Hanks' solution, and then cultivated with a Mitomycin-free medium up to 6th day of cultivation. The medium was renewed every other day. On the 4th and 6th day of cultivation, the cell number of three tubes per each group were counted with a hemocytometer.

Effect of Mitomycin-C on the Incorporation of Tritiated Thymidine into DNA of HeLa Cells About $2 \times 10^6$ HeLa cells were inoculated in each TD-40 flask. After 24 hrs. of cultivation, they were treated with the medium containing 1 or 5 μg/ml of Mitomycin-C for 1 hr. Following two rinsing with Hanks' solution to minimize residual Mitomycin-C, a fresh medium without Mitomycin-C was added, and cultivation was continued. At regular intervals following Mitomycin-C treatment, a DNA fraction of HeLa cells was extracted by the Schmidt-Thannhauser method immediately after pulse labeling for 1 hr. with tritiated thymidine (specific activity 3.8 Ci/mM) (The Radiochemical Centre, Amersham). The radioactivity of the DNA fraction for each group was counted by a gas-flow counter. DNA was measured by the Beckmann spectrophotometer at 540 mμ after being treated with diphenylamine.

Autoradiography Small square bottles with cover slips were used for autoradiographical experiments. Each bottle was added with $2 \times 10^8$ HeLa cells. The experiments were performed after a 24-hr. cultivation, when the cells were assumed to be in a logarithmic phase. At certain intervals following pulse or continuous labeling with tritiated thymidine, the cover slip was removed and fixed with ethanol for 1 hr. immediately after being rinsed with Hanks' solution. Autoradiography was carried out by a dipping method with an NTB-2 Kodak emulsion. After 5 or 7 days of exposure, specimens were developed and stained with Giemsa. One thousand cells for each specimen were counted for the calculation of labeling index and mitotic index. In measuring the rate of labeled mitotic cells, 100 mitotic cells were counted. The number of grains per labeled cell was counted for 80 labeled cells.

Results

Growth-inhibitory Effect of Mitomycin-C on HeLa Cells

Fig. 1 shows the growth curves for a non-treated group and for groups treated with various concentrations of Mitomycin-C for 1 hr. It was observed that the cells in the non-treated culture multiplied exponentially with its doubling time ranging between...
Fig. 1. Growth curves of HeLa cells treated with various concentration of Mitomycin-C for 1 hour

K: Control
A: 5 μg/ml
B: 1 μg/ml
C: 0.2 μg/ml
D: 0.04 μg/ml
E: 0.008 μg/ml
F: 0.0016 μg/ml

Cell growth (%) = \frac{\text{No. of cell nuclei in Mitomycin-C administered group}}{\text{No. of cell nuclei in control group}} \times 100 \text{ (on the 6th day of cultivation)}

Fig. 2. Effect of Mitomycin-C on the proliferation of HeLa cells at various concentrations and treatment periods
25 and 30 hrs., while the cells in the treated culture were inhibited to various degrees depending on the concentration of Mitomycin-C.

Fig. 2 shows percentage of cell growth against control on the 6th day of cultivation following exposure of HeLa cells to various concentrations of Mitomycin-C over different periods of time. The curves showed almost a similar pattern despite their shift as the period of treatment differed. However, no further shift of the curve was observed when the cells were treated longer than 48 hrs. It was observed that Mitomycin-C could be effectively added until a certain critical point which might be referable to the doubling time (Fig. 1), beyond which no further growth inhibition was observed.

**Effect of Mitomycin-C on the Incorporation of Tritiated Thymidine into DNA of HeLa Cells**

At 0, 3, 6, 12, and 24 hrs. after treatment with 5 or 1 µg/ml of Mitomycin-C for 1 hr., tritiated thymidine was added at a concentration of 1 µCi/ml for 1 hr. Fig. 3 shows the specific activity of DNA for each group expressed in terms of percentage of activity to that of the control group. Each column is an average of duplicated tests.

![Incorporation rate graph](image)

Incorporation rate = \(rac{\text{cpm per } \mu g \text{ DNA of Mitomycin-C treated cells}}{\text{cpm per } \mu g \text{ DNA of non-treated cells}} \times 100\)

Fig. 3. Incorporation of tritiated thymidine into DNA of HeLa cells following Mitomycin-C treatment

Only a slight inhibition of incorporation was observed immediately after Mitomycin-C treatment and a maximum inhibition occurred 6 hrs. later. After 12 hrs., however, the incorporation was considerably restored.

The above experimental data suggest that HeLa cells might be highly sensitive to Mitomycin-C at some stages in their division cycle. In order to confirm this assumption further study was carried out by autoradiography.
Estimation of Generation Time and Each Stage of Division Cycle of HeLa Cells

At regular intervals after 1 hr. of labeling with tritiated thymidine, the mitotic index, labeling index, and rate of labeled mitotic cells were examined (Fig. 4). The interval "a" between the first rise of the rate of labeled mitotic cells and the second rise indicates the generation time of HeLa cells. It proved to be 28 hrs. with an apparent, good coincidence with the doubling time estimated from the growth curve of HeLa cells.

\[ n(t, \tau) = a N(t) e^{a\tau} = a N_0 e^{a\tau} e^{a(\tau-T)} \]

where \( N(t) \) denotes the total cell number at time \( t \), \( a = \frac{\ln 2}{T} \), and \( T \), the generation time.

a) Length of mitotic period (M):

\[ \text{mitotic index (\%)} = \frac{\int_0^M n(t, \tau)d\tau}{100} \approx e^{aM} - 1 \approx aM, \quad M \ll T \]

Therefore

\[ M = \frac{\text{mitotic index (\%)} \times T}{100 \times \ln 2} \]

This equation gives a value of \( M \) of 2 hrs., because \( T \) is 28 hrs., an average mitotic index being 4.9%.
b) Length of DNA synthetic (S) and postsynthetic period (G2):

Assuming that thymidine is added at time \( t = 0 \), labeling index \( \left( L(t) \times 100 \right) \) at time \( t < G_2 + M \) is given by

\[
L(t) = \frac{1}{N(t)} \int_{G_2 + M}^{G_2 + S + t} n(t,0) \, dt = e^{a(G_2 + M + S)} - e^{a(G_2 + M - t)}
\]

(1)

Assuming that all the cells move round the mitotic cycle at the same rate, changes in the rate of labeled mitotic cells following a short exposure to thymidine are theoretically represented by the black line in Fig. 5. The curve obtained experimentally (dotted line) seems to be less angular due to a variation in the rate of movement of the cells round the mitotic cycle.

Assuming that the interval \( "b" \) from labeling to the first peak of the curve obtained experimentally is equal to the interval between the labeling and the midpoint of the upper line of the theoretical curve, the time \( "b" \) is given by

\[
b = G_2 - \frac{S + t - M}{2} \quad \text{and} \quad G_2 = b - \frac{S + M - t}{2}
\]

(2)

from equations (1) and (2),

\[
S = \frac{2T}{\log 2} \log \left[ \frac{L(t)}{e^{a(b + M/2)}} \right] \sqrt{1 + \left( \frac{L(t)}{e^{a(b + M/2)}} \right)^2} - 2T - t
\]

(3)

\( t = 1, T = 28, b = 11, M = 2, L(t) = 0.48 \)

Therefore, \( S = 13.6 \) hrs., and \( G_2 = 3.7 \) hrs. from equation (2).

c) Length of presynthetic period (G1):

From equation \( G_1 = T - (M + S + G_2) \), \( G_1 = 8.7 \) hrs.
Effect of Mitomycin-C on Each Stage of Division Cycle of HeLa Cells

Gradual changes in mitotic index, labeling index, and number of grains per labeled cell following Mitomycin-C treatment for 1 hr. are shown in Fig. 6.

![Graph showing changes in mitotic index and labeling index](image)

Fig. 6. Changes in mitotic index and labeling index following a pulse labeling with tritiated thymidine (1 μCi/ml, 1 hr.) immediately after Mitomycin-C treatment (1 μg/ml, 1 hr.)

The mitotic index of Mitomycin-C treated HeLa cells showed almost the same value as non-treated cells until 9 hrs. after the treatment, followed by a sudden decrease after 12 hrs. without recovery until 30 hrs. after the treatment.

The labeling index of Mitomycin-C treated HeLa cells was normal in value immediately after Mitomycin-C treatment but was followed by a gradual decrease, reaching a minimum in 6 hrs. Then it regained a normal value in 9 hrs. reaching above normal between 12 and 18 hrs. after the treatment.

The distribution of grains showed no remarkable change in the non-treated group, but it showed significant changes in the experimental group (Fig. 7). A mean grain number per labeled cell decreased, well reflected in the decrease of the incorporation of tritiated thymidine (Fig. 3); a minimum value was observed 6 hrs. after the treatment, followed by a recovery. However, not a few cells with low grain count were observed even after the mean grain count had recovered (Fig. 7).

The data so far obtained are summarized in Table I. The changes observed in mitotic index suggested that the cells subjected to 1 hr. of treatment within 9 hrs. before the mitosis were in normal cycle without being apparently disturbed. It was strongly suggested that the cells more than 12 hrs. before mitosis had been significantly impaired. From the data on the length of each stage of division cycle, it seems that Mitomycin-C
Fig. 7. Distribution of frequency of grain number per labeled cell immediately after a pulse labeling with tritiated thymidine (1 µCi/ml, 1 hr.) following Mitomycin-C treatment (1 µg/ml, 1 hr.)

Bar with a number indicates a mean grain number per labeled cell

Fig. 7. Distribution of frequency of grain number per labeled cell immediately after a pulse labeling with tritiated thymidine (1 µCi/ml, 1 hr.) following Mitomycin-C treatment (1 µg/ml, 1 hr.)
Table I. Changes in Mitotic Index, Labeling Index, and Mean Grain Number per Labeled Cells immediately after Pulse Labeling with $^3$H-Thymidine (1 μCi/ml, 1 hr.) following Mitomycin-C treatment (1 μg/ml, 1 hr.)

<table>
<thead>
<tr>
<th>Hours after Mitomycin-C treatment</th>
<th>Mitotic index</th>
<th>Labeling index</th>
<th>Mean grain number/ labeled cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>normal</td>
<td>normal</td>
<td>nearly normal</td>
</tr>
<tr>
<td>3</td>
<td>normal</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td>6</td>
<td>normal</td>
<td>minimum</td>
<td>minimum</td>
</tr>
<tr>
<td>9</td>
<td>nearly normal</td>
<td>recovered to normal</td>
<td>slightly recovered</td>
</tr>
<tr>
<td>12</td>
<td>decreased</td>
<td>increased</td>
<td>fairly recovered</td>
</tr>
<tr>
<td>18</td>
<td>decreased</td>
<td>increased</td>
<td>fairly recovered</td>
</tr>
</tbody>
</table>

did not disturb the flow of cells at either the G2 period or the latter half of the S period by treatment.

The labeling index and the mean grain number per labeled cell proved to be minimum 6 hrs. after the treatment. Thus, Mitomycin-C seemed to affect severely those cells which would be in the S period 6 hrs. after Mitomycin-C treatment; that is, in the latter half of the G1 period and the first half of the S period during the treatment.

**Effect of Mitomycin-C on Depolymerization of DNA of HeLa Cells**

As a preliminary experiment, the time required for labeling of all cells with tritiated thymidine was determined. Most cells were labeled until about 20 hrs. and 3~5% of the cells remained unlabeled (Fig. 8).

![Fig. 8. Labeling index of HeLa cells in a continuous labeling with tritiated thymidine (1 μCi/ml)](image)

Following a 24-hr. continuous labeling, HeLa cells were rinsed 3 times with a culture medium without the labeled precursor and incubated for 12 hrs. in order to minimize the radioactivity of the acid-soluble fraction. Then the cells were treated with Mitomycin-C at a concentration of 1 μg/ml for 1 hr., and further incubated in a medium without the labeled precursor. Zero to 24 hrs. after the Mitomycin-C treatment, radioactivity of the culture media, the acid-soluble, and DNA fractions was counted.
by a liquid scintillation counter. As a result, no significant radioactivity was observed either in the acid-soluble fraction of HeLa cells treated with Mitomycin-C or in the culture medium of the treated group (Table II).

This result suggests that DNA is not depolymerized into such small fragments as found in the acid-soluble fraction, so far as the present experimental conditions are concerned.

### Table II. Radioactivities in the Acid-soluble and DNA Fractions from ³H- Thymidine labeled HeLa Cells and in Culture Media following Treatment with Mitomycin-C

<table>
<thead>
<tr>
<th>Hours after Mitomycin-C treatment</th>
<th>DNA fraction (cpm/μg)</th>
<th>Acid-soluble fraction (cpm/10⁶ cells)</th>
<th>Culture medium (cpm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Mitomycin-C treated</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>120.0</td>
<td>153.0</td>
<td>nil</td>
</tr>
<tr>
<td>3</td>
<td>109.0</td>
<td>138.3</td>
<td>nil</td>
</tr>
<tr>
<td>6</td>
<td>112.2</td>
<td>149.6</td>
<td>nil</td>
</tr>
<tr>
<td>9</td>
<td>108.1</td>
<td>120.8</td>
<td>nil</td>
</tr>
<tr>
<td>12</td>
<td>119.8</td>
<td>138.0</td>
<td>nil</td>
</tr>
<tr>
<td>18</td>
<td>83.5</td>
<td>116.8</td>
<td>nil</td>
</tr>
<tr>
<td>24</td>
<td>94.9</td>
<td>106.6</td>
<td>nil</td>
</tr>
</tbody>
</table>

### DISCUSSION

Regarding the sensitivity of cells in different stages of division cycle to irradiation, various conclusions have been presented depending on different experimental methods and/or materials. The severest damage is found in cells (1) in the mitotic period, (2) in the G₂ period, (3) in the S period, and (4) in the G₁ period.

One of the most important factors which cause such discrepancy seems to be the difference in irradiation dosage. Lajtha, and Pelc and Howard reported that the DNA synthesis of cells in the S period was inhibited by a large dosage of irradiation, but the DNA synthesis of cells in the S period was not markedly inhibited with small dose irradiation, while the cells in the G₁ period were considerably affected in their DNA synthesis by this treatment.

When studying the effect of a cytotoxic agent in vitro, experimental conditions which reflect events in vivo are preferable. To find out the stage of division cycle of cells most sensitive to Mitomycin-C, care should be taken to avoid too high a concentration and too long an exposure of the agent lest all the cells should be severely damaged, ignoring their different sensitivity. The present experimental conditions (Mitomycin-C 1 μg/ml, 1 hr.) did not lead to the complete inhibition of cell proliferation (Fig. 1), but the dosage employed may be applicable in clinical cases.

The generation time and the length of each stage of division cycle of various cells have been calculated by various methods. The present estimate of generation time of HeLa cells is a little longer than that reported by Terasima and by Yamada. This discrepancy is probably due to the difference in the medium and kinds of HeLa cells employed. In their work, HeLa S3 clone was used. The present estimate of the mitotic period is longer than that reported by other investigators for reasons unknown. The present estimation method for G₂ is different from a routine one, in which the G₂ period

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is calculated from the interval from zero time to the time when 50% of mitotic cells are labeled. In practice, however, these two methods have given similar results.

The present results summarized in Table I suggested that the latter half of the G₁ period and the first half of the S period were most sensitive to Mitomycin-C, as already mentioned. These results may not be reasonably explained by an assumption that Mitomycin-C affects either of these two stages. Damage of the cell in the latter half of the G₁ period alone is incompatible with a decrease in the mitotic index 12 hours after the treatment. Likewise, a depression of DNA synthesis of the cells in the first half of the S period alone cannot account for the absence of cells with a grain count as high as in non-treated cells 6 hours after the treatment.

It is considered that a decrease in the labeling index 6 hours after the treatment can be explained by an inhibition of the passage of some cells from the G₁ to the S period and a decrease in the grain count is due to a depression of DNA synthesis of the cells in the first half of the S period and the latter half of the G₁ period during the treatment (Fig. 9, refer to 6 hours after the treatment). The mitotic index remains almost normal for 9 hours after the treatment, because damaged cells do not yet reach the M period (Fig. 9, refer to 9 hours after the treatment). If damaged cells were to go around their cycle at a normal pace, they would reach the M period 12 hours after the treatment, but the slow progress of the damaged cells caused a decrease in the mitotic rate at that time. Besides, the labeling index is increased above that of non-treated cells, because depression of the DNA synthesis causes a prolongation of the S period, resulting in an accumulation of the cells in the S period.
Grain count is considerably recovered because of the progress of the cells into the S period in the first half of the G₁ period in Mitomycin–C treatment, and of the cells which were born after the Mitomycin–C treatment (Fig. 9, refer to 12 hours after the treatment).

Thus, it is most probable that Mitomycin–C severely affects the cells in the latter half of the G₁ and the first half of the S period, regarding the first division cycle after Mitomycin–C treatment. Mitomycin–C seems to damage cells which are in a stage closely related to the DNA synthesis. This finding is in good accordance with the results of biochemical studies which indicate a specific inhibition of the DNA synthesis by Mitomycin–C. However, inhibition in the incorporation of tritiated thymidine is manifested 6 hours after the treatment. This finding seems to suggest that Mitomycin–C severely affects the cells which are going to initiate or just initiated the DNA synthesis rather than the cells which have already synthesized a considerable amount of DNA.

At present, no reasonable interpretation can be given for the absence of decrease in the grain count immediately after treatment with Mitomycin–C.

Regarding radiosensitivity of the cells, these two stages are also most sensitive to irradiation according to several workers.4, 5, 14, 16, 21, 26, 30, 32) Walker and Helleiner,33) Hishimoto,7) and Kosaka12) reported similar results with nitrogen mustard, alanine-nitrogen mustard, and Mitomycin–C, respectively, by different methods.

It was reported that a process leading to deoxyribonucleoside triphosphate was not inhibited by irradiation.3) Mitomycin–C also failed to inhibit activity of DNA polymerase. Iyer and Szybalski,10) and Matsumoto and Lark17) reported that a cross-linking between complementary strands of DNA was caused by Mitomycin–C. Such cross-linking in DNA molecules might cause a depression of priming activity of DNA resulting in the inhibition of DNA synthesis as reported by Pricer and Weissbach.22) A possible explanation may be given for the fact that cells in the latter half of the S period are free from inhibition of the DNA synthesis by Mitomycin–C in the first division cycle after the treatment. The Mitomycin–C–induced cross-linking may occur in the cells approaching the end of DNA replication, mainly between newly synthesized double strands of DNA which serve as a primer in the second division cycle.

It has been demonstrated that the acid-soluble fraction of cells is accumulated after a treatment with Mitomycin–C11, 18, 23, 25) or by irradiation.2, 15, 19) Some workers claimed that accumulation of the acid-soluble fraction resulted from an inhibition of the DNA synthesis.25) Other workers reported that depolymerization of DNA was responsible for the accumulation.11, 18, 23) The present results support the former assertion.

In the present experiments, the changes of cells in the first division cycle after the Mitomycin–C treatment were analysed and discussed, but further observation is required to clarify the fate of the cells treated in a less sensitive stage with reference to the recovery of cell proliferation.

The authors wish to express their sincere thanks to Prof. Dr. Dennosuke Jinnai, Director of the Department of Surgery, Osaka University Medical School, for his encouragement in carrying out this work.

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REFERENCES


EXPLANATION OF PLATES XII-XIV

Photos 1 and 2. Autoradiograms of HeLa cells immediately after Mitomycin-C treatment; 1. experimental, 2. control (Mitomycin-C not added)

Photos 3 and 4. Autoradiograms of HeLa cells 6 hours after Mitomycin-C treatment; 3. experimental, 4. control (Mitomycin-C not added)

Photos 5 and 6. Autoradiograms of HeLa cells 12 hours after Mitomycin-C treatment; 5. experimental, 6. control (Mitomycin-C not added)