Effect of Halogenated Pyrimidines on Radiosensitivity of Mouse Strain L Cells and Their Radioreistant Variant

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ABSTRACT

Incorporation of halogen analogs of pyrimidine into DNA of the mouse strain L cells enhanced their sensitivity to X-ray irradiation. The maximal radiosensitization was obtained either with 72 hours, incubation in the presence of BUdR alone or 24 hours' incubation in the presence of BUdR and aminopterin before X-ray irradiation. The replacement of thymidine by BUdR in these optimal conditions was approximately 30%, and a further increase of the replacement up to 50% did not intensify the radio-sensitization. These results may support the view that BU-labeling of single strand of DNA double helix is sufficient to confer significant radiosensitization. The radiosensitivity of the resistant L1 cells was restored by BUdR incorporation up to the same level as that of untreated original cells.

These observations would contribute to some extent towards the improvement of radiation therapy.

INTRODUCTION

The mechanism underlying the potentiation of radiation injury on mammalian cells has been the subject of considerable interest in radiobiology. The phenomenon...
of radiosensitization by halogenated DNA precursor appears to be one of the most intriguing and promising approaches to elucidate it. The increased sensitivity to radiation can be elicited by a variety of halogenated pyrimidine analogs incorporated into cellular DNA. According to Greer, Djordjevic and Szybalski, halogenation of DNA strands renders them more susceptible to radiation and this, in turn, results in an enhanced radiosensitivity of the carrier cells.

In the present study, the increased radiosensitivity with some pyrimidine halogen analogs will be presented, using the cell culture technique of the mouse strain L cells and their radioresistant variant, Lγ cells.

MATERIALS AND METHODS

The mouse fibroblastic strain L cells have been maintained in vitro at the Department of Experimental Radiology, Faculty of Medicine, Kyoto University were used in the present study. Lγ cells, resistant to 60Co γ-ray irradiation given fractionally up to the total dose of 14,000 r, were isolated by Horikawa et al. from the original mouse strain L cells. Both cells have been maintained through routine subculture every week at 37°C by monolayer technique, using YLH medium supplemented with 5% bovine serum and antibiotics.

Among several kinds of pyrimidine halogen analogs, 5-Bromo-deoxyuridine (BUdR) and 5-Iode-deoxyuridine (IUdR) were tested, which were supplied from Takeda Chemical Industries, Ltd.

For X-ray irradiation, a Toshiba X-ray machine was used, operating at 200 kvp and 25 mA., filtered with 1.0 mm of copper and 0.5 mm of aluminum. Dose rate of 75 r per minute and S.F.D. of 50 cm were routinely employed. During irradiation the cells were kept at room temperature.

In order to evaluate the effects of BUdR and IUdR on the radiation sensitivity of the cells, the dose-per cent survival curves of analog-labeled cells after irradiation with various doses of X-ray were compared with that of untreated cells.

The original mouse strain L cells are generally known not to form a colony. The number of viable cells after such treatment as irradiation with X-ray or exposure to pyrimidine analogs, therefore, can not be enumerated by the colony counting method developed by Puck et al. So the following method was devised: Two series of the cell suspensions in small test tubes containing 1 to 4 x 10⁴ cells, each series consisting of triplicate tubes, were incubated routinely for 7 days at 37°C after irradiation, one served as control and the other for treatment. Seven days of incubation was chosen, because the lag of the growth caused by irradiation is transient and can be recovered during this period. At the 7th day of incubation, multiplied cells suspended in tubes were stained by crystal violet in citric acid solution, a portion of the stained cell suspension were mounted on the Bürker type counting chamber, and the number of stained nuclei was counted under a microscope. On this occasion, cells without reproductive capacity must
be rejected. According to Puck, there are two types of cells which have no reproductive capacity. The first type, giant cells, were readily checked off by their extraordinary large monster-like appearances. The second type of cells without reproductive capacity which are fated to death, equivalent to the abortive colony forming cells by Puck, was discriminated from reproductive ones by faint staining and/or picnotic appearance of their nuclei. Dead cells were also easily rejected. The remainder which was discriminated from non-reproductive cells as mentioned above, was supposed to be viable and actively reproducible.

The percentage of surviving reproductive cells after treatment to those untreated will be estimated by the following equation, provided that these reproductive cells could multiply at the same rate as the untreated reproductive cell.

\[
S = \frac{N_r}{N_c} \times 100 \%
\]

where \(N_r\) is the number of reproductive cells treated and \(N_c\) the number of these untreated after 7 days' incubation. Then the effect of various kinds of treatment would be judged quantitatively by the value of \(S\), which was plotted on a logarithmic scale against the dose of X-ray applied.

For the quantitative determination of purine and pyrimidine bases in DNA, the procedures developed by Tyner et al. \(^{14}\) were followed.

Further details in experiments will be described in the appropriate articles in the text of the paper.

RESULTS

1. Effect of \(BUdR\) and \(IUdR\) on multiplication of mouse strain L cells

Small test tubes containing routine medium supplemented with serial concentrations of \(BUdR\) or \(IUdR\) were inoculated with 1 to \(5 \times 10^4\) cells and incubated at 37° C. After 2, 4 and 7 days of incubation, the number of cells with intact reproductivity in the tubes was counted.

Apparently, multiplication of the cells was inhibited by adding \(BUdR\) or \(IUdR\) into medium. In the presence of 10 \(\mu g\) of \(BUdR\) per ml of medium, the rate of the cell multiplication was almost equal to that of the control, although short lag period was observed as shown in Table 1. It appears that both \(BUdR\) and \(IUdR\) have an inhibitory effect on the cell multiplication to almost the same extent.

<table>
<thead>
<tr>
<th>Concentration (BUdR) (\mu g/ml)</th>
<th>(IUdR) (\mu g/ml)</th>
<th>Average times necessary for doubling the number of cells (hours)</th>
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<tr>
<td></td>
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<tr>
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<td>56</td>
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<tr>
<td>10</td>
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<td>0</td>
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2. Effect of BUdR and IUdR on radiosensitivity

At first, the cells were irradiated with various doses of X-ray in the presence of BUdR, at concentration as high as 10 or 30 μg per ml. After 7 days’ incubation, no difference was observed in the slope of the dose-survival curves between BUdR-free (control) and BUdR containing medium (treated). The results indicate that BUdR has no radiosensitizing effect on the cells when it is administered simultaneously with the exposure to radiation.

Secondly, the cells were exposed to BUdR or IUdR prior to X-ray irradiation, that is, analog-labeled cells were prepared by cultivating the cells for various days in the presence of 10 μg of analogs per ml (pretreatment). When the pretreated cells were transferred to BUdR-free medium immediately before irradiation, then irradiated by serial doses of X-ray and the reproductive cells were counted after 7 days’ culture, the slope of dose-survival curves of the pretreated cells showed a tendency to be steeper than that of control, indicating the radiosensitizing effect of the analogs, as shown in Fig. 1. Although no detectable radiosensitization was observed by 24 hours’ pretreatment with BUdR, a slight sensitizing effect was found by 48 hours’ and maximal effect by 72 hours’ pretreatment, the period of which corresponds to the one generation time, the time interval necessary for twofold multiplication of the cells in the presence of BUdR at the concentration used.

In the case of IUdR, only the cells pretreated for 72 hours were examined. IUdR showed also sensitizing effect to X-ray irradiation on the mouse strain L cells, but was less effective than BUdR.

![Fig. 1 The dose-per cent survival curves of the mouse strain L cells cultured in the presence of BUdR and IUdR before X-ray irradiation](image)

3. Influence of aminopterin on radiosensitizing effect of BUdR

Aminopterin is a drug known to block the de novo synthesis of thymidylate. So, if this chemical is added to the medium in the presence of analogs, incorpora-
tion of analogs into DNA molecules of the cells is expected to be highly enhanced. In the present experiment an aliquot of 5 µg of aminopterin was added to the medium containing 10 µg of analogs for various time intervals. With 24 hours' pretreatment under this condition, the radiosensitivity of the treated cells was raised up to a similar level as that of the cells pretreated for 72 hours with BUdR alone, as shown in Fig. 2. There was no difference between the radiosensitizing effects of 24 hours' and 72 hours' pretreatment under this condition. That is, the radiosensitizing effect appeared to have been saturated by 24 hours' pretreatment and no further increase of the effect was achieved by longer pretreatment.

Fig. 2 The dose-per cent survival curves of the mouse strain L cells cultured in combination of BUdR and aminopterin before X-ray irradiation

Fig. 3 The dose-per cent survival curves of radioresistant Lr cells cultured in the presence of BUdR before X-ray irradiation
4. Radiosensitization of Lγ cells by BUdR

While the radioresistant cells, Lγ, were less sensitive to X-ray irradiation than the original mouse strain L cells, enhanced radiosensitivity by pretreatment with BUdR was also observed on these Lγ cells. The radiosensitivity of Lγ cells was increased to the same level as the original cells by the pretreatment as shown in Fig. 3.

5. Relative radiosensitizing effect

Degree of sensitizing effect of various pretreatments can be estimated by the steepness of the slope of dose-per cent survival curves. In order to express the relative steepness of the slope, the sensitizing factor was introduced, which refers to reciprocal of the ratio of 50 per cent survival dose of the treated cells to that of the control. The reason why 50 per cent survival dose was chosen, is that errors in counting the cells with reproductive capacity may be minimum at that per cent. Table 2 summarizes the values of the sensitizing factors calculated for various treatments.

| Table 2 50 per cent survival dose of X-ray and sensitizing factor under various conditions |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell lines | Concentrations | Hours of incubation before irradiation | 50% survival doses (r) | Sensitizing factor** |
| µg BUdR/ml | µg aminopterin/ml | | | |
| L | 0 | 0 | 0 | 280 | 1.00 |
| 10 | 0 | 0 | 260* | 1.08 |
| 10 | 0 | 24 | 280 | 1.00 |
| 10 | 0 | 48 | 240 | 1.16 |
| 10 | 0 | 72 | 100 | 2.78 |
| 10 | 0 | 96 | 120 | 2.33 |
| 10 | 5 | 24 | 120 | 2.33 |
| 10 | 5 | 72 | (120) | 2.33 |
| Lγ | 0 | 0 | 0 | 590 | (1.00) 0.47 |
| 10 | 0 | 72 | 290 | (2.04) 0.95 |
| µg IUdR/ml |
| L | 10 | 0 | 72 | 180 | 1.56 |
| 10 | 0 | 120 | 350 | 0.80 |

* Irradiation in the presence of BUdR
** Sensitizing factor = \( \frac{1}{\frac{50\% \text{ dose of treated cells}}{50\% \text{ dose of untreated cells}}} \)

6. Rate of BUdR incorporation into DNA

The rate of replacement of thymidine by BUdR was determined by chromatographic procedures, as mentioned previously. After 3, 5 and 7 days' cultivation in the presence of 10 µg of BUdR per ml, the replacement was found to be about 33, 51 and 44 per cent respectively.
DISCUSSION

Puck et al. (10-13) developed a method for a quantitative radiobiological study on somatic mammalian cells, in which it was suggested that a cell with reproductive capacity forms a colony and a cell, which lost reproductive capacity, tends to form a giant cell or an abortive colony. To evaluate the effect of radiation, he used the dose-per cent survival curve, in which the ratio of number of formed colonies on the treated plates to that on the control plates was plotted against the dose of irradiation employed. In our experiment, giant cells are easily checked off and cells which correspond to an abortive-colony-forming cells by Puck et al. were rejected by their morphological appearances and staining characters. The remainder of cells which is assumed to retain reproductive capacity corresponds to those which form colonies by Puck's method. If this is so, the dose-per cent survival curves would be able to be delineated by making reasonable assumption that cells with the reproductivity intact after various treatments can multiply at the same rate as the untreated reproductive cells.

Because, when $R_r$ is the number of cells with reproductive capacity in the original suspension of the treated series, $N_r$ the number of cells initially inoculated in the control series, $N_c$ and $N_e$ the number of the treated cells and of the untreated cells after 7 days' incubation respectively, $N_c/N_e$ means an apparent multiplication ratio of reproductive cells. So, $R_r$ which indicates the number of cells with reproductivity is calculated by the following equation:

$$R_r = N_r \times N_e / N_c$$

The value of $R_r$ would be a true estimation only when $N_e$ cells in the original control suspension are all viable and reproductive. The ratio, $R_r/N_e$, however, would not be influenced even when the assumption mentioned above is not the case. The ratio $R_r/N_e$ or $N_r/N_e$, means the per cent survival of reproductive cells in the treated series and is considered to correspond to the per cent survival in the Puck's colony counting method. Although there is no direct evidence on reproductivity unlike the Puck's method in which a reproductive cell formed a colony, the dose-per cent survival curves in our method would be useful to evaluate the radiosensitivity of the cells.

Potentiation of cellular radiosensitivity with pyrimidine analog was first observed by Greer (7), who demonstrated a striking increase in sensitivity to ultraviolet irradiation by thymine analog BU, using E. coli strain 15 T-, a thymineless mutant. Djordjevic and Szybalski (8) also reported that the incorporation of BUdR and IUdR into mammalian cells cultured in vitro, using strain D98S derived from human sternal marrow, enhanced the sensitivity to ultraviolet and X-ray irradiation of the cells. In the present study, also the enhanced radiosensitivity to X-ray irradiation was confirmed, using the mouse strain L cells, and a value greater than 2.0 in term of the sensitizing factor was obtained under optimal condition.

Maximal radiosensitization was observed with 72 hours' pretreatment by BUdR.
alone or 24 hours' pretreatment with combination of BUdR and aminopterin, and in these conditions the per cent replacement of the thymidine by BUdR was about 30%. The increase in per cent replacement did not exhibit the increased radiosensitization, that is, the radiosensitizing effect was not increased in proportion to the rate of incorporation of BUdR into cellular DNA.

A number of authors reported diverse values of per cent replacement of thymidine by its analogs. Szybalski et al.8)15) reported that the maximal radiosensitization was observed at 45% replacement of thymidine by BUdR in human cell line D98S and at 60% in its variant, D98/AG. Kaplan et al.16) demonstrated that, using E. coli B and B/r, radiosensitization was maximal at the end of log-phase growth of these microorganisms and then, 40 to 50% substitution of BU for thymine was observed. Berry and Andrews17) reported, however, that the maximum incorporation of either IUdR or BUdR was barely 2% in ascitic lymphocytic leukemia cells of the mouse in vivo. Delihas et al.18) also showed that, in H. Ep 1 cells, incorporation of BUdR into cellular DNA was found to be approximately 1%. These diversity in values of replacement might be due to the differences of cells used and/or of methods of determination employed.

Szybalski et al. claimed that labeling of both strands of DNA molecule with BUdR, "bifilar labeling" as they termed, is essential for enhancement of ultraviolet sensitivity of mammalian cells. Kaplan et al. demonstrated, however, that "unifilar labeling" is sufficient for enhancement of sensitivity both to X-ray and ultraviolet irradiation, although degree of sensitization was higher with "bifilar" than "unifilar" labeling. In the present experiment, 72 hours' incubation in the presence of BUdR alone was the optimal condition for radiosensitization, and the period of 72 hours corresponds to one generation time of the L cells in the presence of BUdR. If the mode of transfer of DNA from parent to offspring in the cell line used is "semi-conservative" as Simon (19) demonstrated, it is logical to conclude that "unifilar" labeling was sufficient to increase radiosensitivity in our experiment, in accordance with the view suggested by Kaplan. And the fact that the increase in per cent replacement of thymidine by BUdR did not improve radiosensitivity of the cells, also supports the above-mentioned view.

There is little information as regards the mechanism by which pyrimidine halogen analogs increase the radiosensitivity of the cells which incorporated them. Szybalski (20) suggested the most possible explanation for it; when 5-Bromo-deoxyuridylic acid is incorporated in place of thymidine in the DNA strand, a repulsive force is produced between the negatively charged halogen atom and proximate phosphate radicals within the DNA double helix, and the repulsion imposes a considerable strain on the DNA strands at the phosphate ester bond, rendering it prone to breakage with even reduced levels of radiation shear. Thus, the increase in radiosensitivity is considered to be attributable to their instability of the phosphate ester bond of DNA replaced by halogen analogs for the thymine. Stahl et al. (21), however, suggested the possibility that sensitization might be due
to the inhibition of certain enzymes, using $T_{2u}$ and $T_{2u} +$ bacteriophage and similar conclusion was obtained by Howard-Flanders et al. (22), using $T_1$ phage.

Judging from our present data, it can not be concluded which mechanism is working principally, but the possibility of radiosensitization by virtue of inhibition of certain enzymes can not be ruled out.

The sensitivity of radioresistant $L_1$ cells to X-ray was restored with BUdR up to the same level as that of unlabeled original strain. Although the rate of replacement of thymine by BUdR in this variant remains to be studied, the data presented here would contribute to the elucidation of mechanism of radiation effect on living cells, which would provide a fundamental principle for radiation therapy.

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