Intracellular Distribution of [\(^{14}\text{C}\)]Bleomycin and the Cytokinetic Effects of Bleomycin in the Mouse Tumor

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SUMMARY

The differential effects of bleomycin (BLM) on cycling and noncycling cells were investigated with a mouse ascites tumor in vivo. An i.p. injection of 37.0 or 111.1 \(\mu\)g BLM per g caused a decrease in tumor cell number but an increase in percentage of tumor cells in mitosis. There are no significant differences between the percentage labeled mitoses at various times after pulse labeling by tritiated thymidine of BLM-treated tumor cells and by that of an untreated control, except that the height of the second peak was significantly lower in the treated cells. Hence BLM may be cell cycle nonspecific, and the BLM-induced decrease in cell number, i.p., may stimulate some nondividing cells to reenter the division cycle. However, the fact that percentage of cells in mitosis versus time after the administration of BLM showed two peaks indicates the possibility that another cause of the increase in mitotic figures might be a relative increase of cycling cells due to higher sensitivity of noncycling cells to the agent.

Autoradiographic studies on the intracellular distribution of [\(^{14}\text{C}\)]BLM revealed the following. (a) There were few necrotic cells in mitosis that incorporated much [\(^{14}\text{C}\)]BLM into the cytoplasm at each time point and the mitotic figures gradually increased with time after i.p. injection of the isotope, while necrotic cells other than in mitosis, most of which were heavily labeled, increased in number with time. These findings seem to relate to the possibility that cycling cells may be less sensitive to BLM. (b) The mode of intracellular distribution of [\(^{14}\text{C}\)]BLM in mitotic cells changed with time and appeared to reflect the drug susceptibility depending on the cell cycle phase when labeled.

INTRODUCTION

Not all the cells in a neoplastic population are regularly participating in the mitotic cycle; a part of the population is out of cycle, and a fraction of this nonproliferative compartment seems to retain its proliferative potential to the extent of being open to recall into the cell cycle (7, 15). The data on the survival responses of cycling and noncycling mammalian cells after treatment with BLM are not consistent in the literature and at times are even contradictory. It was reported that in vivo this agent was at least as effective against noncycling EMT-6 tumor cells as against cycling tumor cells and that repair of potentially lethal lesions strongly reduces the cell-killing ability of BLM (8, 10).

Plateau-phase cells are a good in vitro model for noncycling tumor cells (9). There are 2 reports supporting higher sensitivity of plateau-phase mammalian cells to BLM (2, 14), but there are contradictory reports indicating that plateau-phase mammalian cells were more resistant to BLM than were log-phase cells (12, 18). The number of hematopoietic spleen colony-forming units of normal mice was reported to be only slightly reduced by BLM, but pretreatment of mice with endotoxin increased the sensitivity of these cells to this agent probably due to recruitment of colony-forming units into the proliferative cycle (3, 17).

The differential effects of BLM on cycling and noncycling cells may be related to the intracellular distribution of this agent. Previous autoradiographic studies (6) indicated that mouse tumor cells in mitosis do not incorporate much [\(^{14}\text{C}\)]BLM into themselves even 8 hr after i.p. injection of isotope, while the percentage of labeled cells other than in mitosis increased with time. Those findings seem to support higher sensitivity of noncycling cells to BLM. The present study was undertaken to determine whether BLM preferentially kills cycling or noncycling mouse tumor cells in vivo.

MATERIALS AND METHODS

Animals and Tumors. C3H/He mice were purchased from Nippon Clear Co., Ltd., Tokyo, Japan. They were maintained on laboratory chow and were used when they weighed approximately 25 g. A transplantable mouse ascites tumor (Fujimoto) was used. This tumor was derived from in vitro malignant transformations of C3H/He mouse embryo cells (5) and is maintained by passing through the strain of origin.

Growth Curves. The growth curves were checked between the 10th and 14th day after tumor inoculation. A single dose of 37.0 or 111.1 \(\mu\)g BLM per g body weight was given i.p. to mice bearing 10-day tumors. In these 2 groups, 3 mice per point were killed 24, 48, and 96 hr after the injection of BLM. The mice of the control group were given an i.p. injection of 0.9% NaCl solution. After the ascitic fluid was pipetted out and the peritoneal cavity was washed with 0.9% NaCl solution, the number of total tumor cells and the percentage of trypan blue-stained cells were determined.

Mitotic Index. BLM was injected i.p. into the mice bearing 11-day tumors at a dose of 37.0 or 111.1 \(\mu\)g/g body weight. In all 3 groups, including an untreated control, ascites was removed from 2 mice/pot at various time intervals after the injection of BLM and was smeared on glass slides.
Those smears were fixed with methyl alcohol and stained with Giemsa. At each time point, 1000 cells/mouse were observed for mitotic index.

**Cell Cycle Parameters.** Cell cycle parameters were studied by the percentage of labeled mitoses curve (4, 13) in 2 groups of mice.

Mice bearing 11-day tumors were given an i.p. injection of 0.5 μCi [3H]TdR (Radiochemical Centre, Amersham, England) per g body weight 12 hr after i.p. injection of 37.0 μg BLM per g or without administration of BLM. In both groups, ascites was removed from 2 mice/point at various time intervals after the injection of [3H]TdR and was smeared on glass slides. Those smears were fixed with methyl alcohol and coated with Sakura NR-M2 emulsion (Konishiroku Photo Industrial Co., Ltd., Tokyo, Japan) by the dipping method. After 9 days of exposure, the slides were developed and stained with Giemsa. Fifty mitoses/mouse for each time interval were counted to establish the curves of labeled mitoses versus time.

The average duration of $T_{1/2} + 1/2M$ is the time between the injection of [3H]TdR and that at which 50% of the mitoses were labeled. $T_{1/2}$ is graphically determined by taking the time interval between the midpoints on the ascending and descending slopes of the labeled mitoses curves. $T_{1/2}$ is calculated as the difference between $T_{1/2}$ (time between the midpoints of 2 consecutive ascending curves) and the 3 other phases of the cell cycle.

**Autoradiography for [14C]BLM.** [14C]BLM, with a specific activity of 27.0 μCi/mg, was supplied by Nippon Kayaku Co., Ltd., Tokyo, Japan. An advantage of using [14C]BLM over tritiated BLM and the technique used has been previously described (6). The isotope was injected i.p. into mice bearing 12-day tumor at a dose of 1.0 μCi/g body weight (37.0 μg/g).

**RESULTS**

**Growth Curves.** A complete growth curve of Fujimoto ascites tumor is given in Chart 1. The effects of a single dose of 37.0 or 111.1 μg BLM per g on the tumor proliferation in vivo were checked between the 10th and 14th day after tumor inoculation. The results are shown in Chart 2. A single i.p. dose of 37.0 or 111.1 μg BLM per g caused a decrease in cell number until 48 hr after its injection, followed by no less active proliferation than the control in vivo.

**Mitotic Index.** The effects of a single dose of 37.0 or 111.1 μg BLM per g on the mitotic indices of the tumor in vivo are shown in Chart 3. The untreated control revealed that the effects of circadian rhythm are ruled out in this tumor.

The percentage of cells in mitosis of the tumor rose beyond the range of the untreated control, except for 16 hr, after 12 hr following i.p. injection of 37.0 μg BLM per g. At 111.1 μg BLM per g, the percentage of mitotic cells rose beyond the control level after 12 hr; the percentage fluctuated above 37.0 μg BLM per g after 16 hr.

**Cell Cycle Parameters.** Chart 4 shows the percentage of labeled mitoses of Fujimoto ascites tumor cells before and 12 hr after i.p. injection of 37.0 μg BLM per g, plotted against time after i.p. injection of [3H]TdR.

The results of the cell cycle parameters derived from the proportion of the labeled mitoses at various times in untreated tumor cells are: $T_{1/2}$, 18.4 hr; $T_{1/2}$, 3.4 hr; $T_{1/2}$, 10.8 hr; and $T_{1/2} + 1/2M$, 4.2 hr. The alternations in the percentage of labeled mitoses versus time of BLM-treated tumor cells were less well defined than those of the untreated control,
but there were no significant differences in the means of those cycle parameters between BLM-treated tumor cells and the untreated control.

**Intracellular Distribution of [\(^{14}\text{C}\)]BLM.** Intracellular distribution of [\(^{14}\text{C}\)]BLM in the tumor cells was investigated by means of autoradiography. The results are presented in Figs. 1 to 4 and in Table 1. Because a dry-mounting technique was used for detection of water-soluble [\(^{14}\text{C}\)]BLM, a rather high background was encountered in some of the autoradiographs. The cells in mitosis were considered labeled when more than 10 silver grains were seen on the chromosomes or when there was linear arrangement of grains on the cell membrane or a concentration of grains in the cytoplasm.

The mitotic figures gradually increased with time; no necrotic cells in mitosis were seen at 2.5 or 4.0 hr after injection of [\(^{14}\text{C}\)]BLM but they appeared in only 1% of cells in mitosis at 8 hr, and these incorporated much isotope into the cytoplasm. According to the previous report (6), necrotic cells other than in mitosis, most of which were heavily labeled, increased in number with time, reaching a mean of 25.6%. The percentage of labeled mitoses greatly increased from 2.5 to 4.0 hr after i.p. injection of [\(^{14}\text{C}\)]BLM but only slightly increased from 4.0 to 8.0 hr. Considering all the labeled mitoses, the percentage of the mitotic cells with label on the chromosomes decreased with time, but the percentage of the mitotic cells with label on the cell membrane increased.

**DISCUSSION**

It was reported that cultured Chinese hamster cells at an earlier position in the cell cycle either fail to reach mitosis altogether at the concentration of 200 \(\mu\text{g}\) BLM per ml or enter mitosis and divide at a reduced rate at lower drug concentration (11, 16); also, 100 \(\mu\text{g}\) BLM per ml cause prolongation of S and G2 phase in cultured mouse L-cells (19). The rather lower rate of mitotic accumulation should be induced by BLM due to both interference with the progression of cultured cells from G2 to mitosis and completion of cell division without any delay (1, 11, 19).

In the present study, which was performed while the ascites tumor cells were in stationary phase (Chart 1), BLM apparently reduced the number of the tumor cells (Chart 2). However, the percentage of tumor cells in mitosis rose beyond the range of the untreated control later than 12 hr after i.p. injection of 37.0 or 111.1 \(\mu\text{g}\) BLM per g (Chart 3). The percentage of labeled mitoses versus time subsequent to pulse labeling by [\(^{3}\text{H}\)]TdR done 12 hr after i.p. injection of 37.0 \(\mu\text{g}\) BLM per g revealed the same peaks as those of the untreated control except that the height of the 2nd peak was significantly lower in the treated than in the untreated (Chart 4). In this case, it appears that BLM may be cell cycle nonspecific and that a BLM-induced (i.p.) decrease in cell number may stimulate some nondividing cells to reenter the division cycle (7, 15), followed by the increase in mitotic figures and the dilution of [\(^{3}\text{H}\)]TdR-labeled cells in mitosis.
That the percentage of cells in mitosis versus time after the injection of BLM showed 2 peaks (Chart 3) indicates the possibility of another cause of the increase in mitotic figures. Both of the 1st peaks were reached within a cell cycle time after the administration of BLM, which seems too early for recruitment of some nondividing cells into the proliferative cycle. The percentage of the labeled mitoses curve of BLM-treated tumor cells gave no indication of interference with the cell cycle progression in comparison with the untreated control (Chart 4). Hence the 1st peaks in the mitotic index curves of the BLM-treated tumor cells could be attributed to a relative increase of cycling cells due to possible higher sensitivity of noncycling cells to the agent.

Intracellular distribution of [14C]BLM has been investigated by autoradiography thus far up to 8 hr after i.p. injection of the isotope (at 37.0 μg/g; Figs. 1 to 4; Table 1). The division cycles in the ascites tumor cells are not synchronized, but the cells seen in mitosis must have been synchronous at the time of sacrifice. Integration of the differences between mitotic cells and cells other than in mitosis at each time point after [14C]BLM injection will give an outline of the changes due to the agent both in cycling and in noncycling cells. Few necrotic cells were seen in mitosis that incorporated much isotope into the cytoplasm at each time point; the mitotic figures gradually increased with time, while necrotic cells other than in mitosis, most of which were heavily labeled, increased in number with time according to the previous autoradiographic studies (6). Those findings seem to be related to the possibility that cycling cells may be less sensitive to BLM than are noncycling cells. The finding that the mode of intracellular distribution of [14C]BLM in mitotic cells changed with time coincides with the finding that cell cycle progression was not blocked (Chart 4). Considering that \( T_{1/2} + 1/2M = 4.2 \) hr and \( T_c = 10.8 \) hr, the finding that the percentage of mitotic cells with label on the chromosomes was much higher at 4.0 hr than at 8.0 hr after injection of [14C]BLM may be related to higher susceptibility of G2 phase to BLM than late S phase (1).

It has been reported (8, 10) that BLM-induced potentially lethal damage to EMT-6 cells was repaired in 22 hr. Repair of potentially lethal damage strongly reduces the cell-killing ability of BLM and seems to be related to the differential effects of BLM on cycling and noncycling cells. This mode of action may have some connection with an active proliferative action of the treated tumor cells which started 48 hr after BLM injection.

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REFERENCES

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Fig. 1. Autoradiograph prepared from the Fujimoto ascites tumor cells that were removed from a [14C]BLM-injected tumor-bearing mouse at 2.5 hr and stained, after development, with Giemsa. A cell in mitosis (m) is not labeled, while several cells other than in mitosis concentrate silver grains on the cell membrane. × 400.

Fig. 2. Autoradiograph at 4.0 hr. A cell in mitosis (m) has concentration of grains on the chromosomes. The cells other than in mitosis have label not only over the cell membrane but also over the nuclear membrane; n, necrotic cell other than in mitosis incorporating much isotope into the cytoplasm. × 400.

Fig. 3. Autoradiograph at 4.0 hr. A cell in mitosis (m) is not labeled. n, necrotic cell other than in mitosis incorporating much isotope into the cytoplasm. × 400.

Fig. 4. Autoradiograph at 8.0 hr. A cell in mitosis (upper m) has label both over the cell membrane and over the chromosomes. Another cell in mitosis (lower m) has a heavy concentration of grains on the cell membrane. Many cells other than in mitosis have label over the cell membrane, the nuclear membrane, or the cytoplasm. × 400.
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