Effects of Bleomycin on Progression through the Cell Cycle of Mouse L-Cells

Michinori Watanabe, Yosinobu Takabe, Takesi Katsumata, and Toyozo Terasima

First Department of Medicine, Chiba University School of Medicine, Inohana, Chiba, Japan [M. W., Y. T., T. K.], and Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, Chiba, Japan [T. T.]

SUMMARY

The effect of bleomycin on progression of mouse L-cells through the cell cycle was studied. In the presence of bleomycin, the beginning of DNA-synthetic (S) phase occurred at the same rate as that of the untreated control, but the duration was prolonged. In cells treated pulse-wise with bleomycin at the G1 phase, the prolongation of S phase was slight, whereas the G2 phase showed a considerable delay. Bleomycin also affected the entry of asynchronous cells into mitosis by arresting them at the early half of the G2 phase. Interference did not occur with transition of mitosis to the G1 phase.

The observed prolongation of DNA-synthetic phase in the presence of bleomycin might be due to the inhibition of DNA synthesis or the damage to DNA molecules by the drug.

INTRODUCTION

Bleomycin is a glycopeptide antitumor antibiotic that has been isolated from the culture filtrates of Streptomyces verticillus (19, 20). In clinical studies, the drug has demonstrated certain characteristics different from those of other chemotherapeutic agents. Bleomycin shows selective activity on squamous cell carcinoma (2, 12) and malignant lymphomas (5, 22); moreover, it is rarely accompanied by hematopoietic disturbance (2, 5, 12, 22), a major side effect of other cytotoxic antitumor agents.

Studies on the mechanism of action of bleomycin at the cellular level revealed that the drug affects most strongly the synthesis of DNA and, to a lesser extent, protein and RNA syntheses (11, 21). The drug also inhibits the proliferation of various mammalian cells in culture (1, 16). The particular biphasic survival curve of mouse L-cells was reported previously and the implication was discussed by us (13, 15). On the other hand, little information is available regarding the effect of bleomycin on the cell cycle. Accordingly, the present studies on bleomycin action were focused on the progression of mouse L-cells. The findings obtained would be useful for the rational designing of cancer chemotherapy.

Received September 7, 1973; accepted January 4, 1974.

MATERIALS AND METHODS

Cell Line. A clonal derivative of mouse L-cells (B929-L2J), designated as the L5 strain, was used throughout the experiments. The cells were grown as a monolayer in F10HI medium (3) with 5% calf serum added. The median generation time was about 18 hr with a 5-hr G1 period, 9-hr S period, and 3-hr G2 period.

Synchronous Culture of L-Cells. Synchronous cells were obtained by a selection of mitotic cells from randomly growing L-cells (14). The culture was initiated with $1 \times 10^8$ cells/180-ml square culture bottle. After 36 hr of incubation, the medium was discarded to remove fragile and dying cells. The culture, fed with the prewarmed fresh growth medium, was incubated for another 5 to 6 hr. Then, the mitoses that appeared were readily shaken off by manual agitation of the bottle. The mitotic frequency of harvested cells was usually over 95%. The harvested cells were all attached to the bottom of plastic petri dishes (60 x 15 mm; Falcon Plastics, Los Angeles, Calif.) within 2 hr of incubation in a CO2 chamber at 37°C. The synchrony procedure was carried out at 37°C.

Determination of Mitotic Frequency. The cells growing in plastic dishes were dispersed with 0.1% trypsin (1:250; Difco, Detroit, Mich.): saline D2. The cells were then centrifuged, fixed in acetic acid:ethanol (1:3), and stained with acetic orcein. The mitotic frequency for each sample was determined by scoring 1000 cells.

Autoradiography of Cells. Mitotic cells were inoculated into plastic dishes in which coverslips were placed, and incubated in a CO2 chamber at 37°C. At 3 hr, when cells were attached on coverslips and the whole population of synchronous cells was in G1 phase of the cell cycle, bleomycin was added to cultures to a final concentration of 100 μg/ml. Thereafter, coverslips were taken from dishes at intervals of 2 hr and placed into medium containing thymidine-3H, 0.5 μCi/ml (5 Ci/m mole, Radiochemical Centre, Amersham, England) for 20 min. Then, cells were fixed in acetic acid:ethanol (1:3), dried, and the autoradiographs were prepared. For autoradiography, the nuclear emulsion (NRM-2, Konishiroku Photoindusty Co., Tokyo, Japan) was applied and photographic processes were followed. The fraction of labeled cells was determined by scoring 1000 cells for each sample.

Colcemid Treatment of Cells. An exponentially growing
culture of L-cells was treated continuously with Colcemid, 0.1 µg/ml, and bleomycin, 100 µg/ml. In control culture, medium contained Colcemid but no bleomycin. After the addition of drugs, samples were taken at 30-min intervals over a 5-hr period. The entry of cells into mitosis was examined by scoring the number of cells in mitosis in each sample.

Bleomycin. Bleomycin A5 (Lots 00702 and 6), a copper-free sulfate, was obtained from Nippon Kayaku Co. Ltd., Tokyo, Japan. The drug was dissolved in F10 medium and kept in a refrigerator until used.

RESULTS

The effect of bleomycin on the progression of mitotic cells into G1 phase is shown in Chart 1. For treatment, cells in mitosis were suspended in medium containing bleomycin, 100 µg/ml, immediately after harvest and were kept incubating at 37°C. Samples were taken at 30-min intervals, and cells were dispersed with 0.1% trypsin. In samples taken during the 1st 2 hr of incubation, the cells still floating in culture medium were collected and put together with trypsinized cells. Then, cells were centrifuged, fixed, and stained for determination of mitotic frequency. The harvested cells exposed continuously to bleomycin, 100 µg/ml, underwent the same rate of decrease in mitotic frequency as did untreated control cells. The values of control and drug-treated cells dropped to 0% by 1 hr of incubation, indicating that bleomycin did not affect the transition from mitosis to G1 phase.

In order to examine the effect of the drug on the DNA-synthetic phase, autoradiographic studies were performed on synchronous L-cells. Three hr after harvest of mitotic cells, bleomycin was added to culture medium to a final concentration of 100 µg/ml, and thereafter the fraction of DNA-synthetic cells was determined by pulse-labeling cells with thymidine-3H at 2-hr intervals (Chart 2). The beginning of DNA-synthetic phase was not affected in cells treated with bleomycin, since the labeled fraction of both control and drug-treated cells increased at the same rate from 5 hr onward and finally reached 90% at 9 hr after mitosis (middle of S phase). In contrast, the completion of DNA-synthetic phase was blocked by the drug. The labeled fraction of the control culture dropped to 30% at 15 hr, while 70% of drug-treated cells still remained in S phase even at 17 hr. This indicates that bleomycin prevented the passage of cells from S to G2 phase. The duration of S phase measured at the 50% level was, therefore, prolonged 5 hr by the drug treatment. Incidentally, the number of grains per labeled cells was apparently lower in bleomycin-treated cells than in untreated control cells.

Chart 3 shows the effect of pulse treatment on the cell cycle progression. Three hr after mitosis, synchronous G1 populations were treated for 1 hr with bleomycin, 100 µg/ml. Then cultures were rinsed twice with F10 medium and fed with fresh growth medium for further incubation. The progression of G1-treated cells through the following cell cycle phases was examined by pulse-labeling cells with thymidine-3H at intervals and prepared for autoradiography (circles). The division of synchronous cells was determined by repeated scoring of cell number in specified microscopic fields (triangles).
thymidine-$^3$H at various times. After the pulse treatment of $G_1$ cells, cells were counted at intervals under a microscope. The change of percentage labeled cells in the $G_1$-treated population was almost like that of the untreated control through 17 hr after mitosis, except for slightly higher values found at 13 and 15 hr. This result indicates that bleomycin did not significantly affect the progression of $G_1$-treated cells through $S$ phase, although the exit of cells from $S$ phase was slightly delayed (less than 1 hr). However, cell division of $G_1$-treated cells was inhibited up to 68 hr after inoculation of mitotic cells, and the 2nd wave of labeled fraction was not observed. The results indicate that cells exposed to bleomycin in $G_1$ phase progressed through $S$ into $G_2$ but were blocked at $G_2$ phase with a considerable delay.

The $G_2$ block was also observed when the asynchronous population of L-cells was treated with bleomycin and Colcemid (Chart 4). The accumulation of mitotic cells was shown in terms of the collection function (10). The value obtained for untreated control increased linearly with time after a short lag period which may correspond to the time needed for Colcemid to reach its active site. The value for bleomycin-treated cells closely followed the control curve during the 1st 90 min of exposure, then practically leveled off, suggesting that cells were arrested in $G_2$ phase (about 90 min before mitosis) and failed to enter mitosis.

**DISCUSSION**

In the presence of bleomycin, $G_1$ cells entered the DNA-synthetic phase at the same rate as the untreated control cells, but the progression of cells from $S$ to $G_2$ phase was delayed considerably (Chart 2). In contrast to our results, it was reported that bleomycin did not affect the DNA-synthetic phase of Chinese hamster cells (1, 18). These results may be specific for the Chinese hamster cell line, since macromolecular syntheses were less inhibited in Chinese hamster cells (18) than in other mammalian cells (11, 21).

In L-cells, the prolongation of DNA-synthetic phase could be correlated with the remarkable action of bleomycin on the DNA synthesis and DNA molecules of cells. Suzuki et al. (11) found that the DNA synthesis of HeLa cells was moderately inhibited by the antibiotic, while protein synthesis was less affected. This finding was confirmed again by our study with mouse L-cells (21) in which bleomycin inhibited DNA synthesis most strongly and inhibited protein and RNA syntheses, in that order, to a lesser extent. Bleomycin also affected cellular and isolated DNA molecules, producing single- and double-strand scissions in DNA (17), decreasing the melting temperature (8), or liberating thymine base from DNA (6). In view of these actions of bleomycin on DNA, the results presented in Chart 2 can be explained as follows. The beginning of DNA-synthetic phase was not affected by drug-caused strand scission of, or any other possible damage in, DNA molecules. However, the processes of genome replication would be delayed either by the degradation of DNA molecules and subsequent repair in cells (17) or by the possible inhibition of enzymes related to DNA synthesis (7). Thus, the completion of the DNA-synthetic phase in drug-treated cells was delayed.

Another site of bleomycin action on the cell cycle traverse of L-cells is at the $G_2$ phase. Other investigators (1, 18) have reported that bleomycin interfered with cell progression during $G_2$ phase. In our observation, more than 90 min before mitosis, the cells failed to reach mitosis in the presence of bleomycin (Chart 4). This finding would indicate that the early part of $G_2$ phase is intrinsically sensitive to bleomycin. In L-cells, the $G_2$ block may be related to the inhibition of protein or RNA synthesis required for cell division, since gross reduction of these macromolecular syntheses was shown (21).

It is particularly noted that the great prolongation in $G_2$ phase was also observed when cells were exposed pulse-wise to bleomycin at $G_1$ phase, as shown in Chart 3. This result is in agreement with our cinematographic observations on bleomycin-treated cells (4), in which synchronous culture of L-cells pulse treated with bleomycin at the $G_1$-$S$ transition phase showed a remarkable cell division delay. Incidentally, similar division delay has been observed in cells treated with a DNA-attacking agent during the $G_1$ phase of the cell cycle. Ohara and Terasima (9) reported that damages produced by mitomycin in the $G_1$ phase of HeLa cells brought about the marked prolongation of the following $S$ and $G_2$ phases. This was assumed to be due to irreversible damage to DNA of $G_1$ cells. In this study, however, it is less likely that damage to DNA is primarily responsible for the observed $G_2$ prolongation, since the DNA-synthetic phase was minimally affected in $G_1$-treated cells. Another possible explanation involves the damages in mitotic apparatus. For example, damage to centriole, spindles, or nuclear membranes may have a relation to the division delay in $G_1$-treated cells. More extensive knowledge of the mechanism of cell division, especially of the division-essential syntheses occurring through the cell cycle, might aid in elucidating the specific action of bleomycin on $G_2$ phase of cells.
ACKNOWLEDGMENTS

The authors thank Nippon Kayaku Co. Ltd., for the kind supply of bleomycin throughout this work. We are also grateful to Professor K. Okuda, Chiba University School of Medicine, for his encouragement. The skillful technical assistance of M. Yasukawa is acknowledged.

REFERENCES

Effects of Bleomycin on Progression through the Cell Cycle of Mouse L-Cells

Michinori Watanabe, Yosinobu Takabe, Takesi Katsumata, et al.