LETHAL EFFECT OF BLEOMYCIN ON CULTURED MAMMALIAN CELLS

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(Received for publication May 4, 1970)

By using cultured mammalian cells lethal effect of bleomycin was measured in terms of mean lethal dose (D_{37}); the cytotoxicity on proliferative system was moderate as compared with those of other anti-tumor agents; the dose-response revealed marked difference between two mammalian cells used. The pattern of drug response during the cell cycle was demonstrated by virtue of the synchrony method. The similarity to the pattern of X-ray response strongly suggested the involvement of the same mechanism in lethal action of bleomycin.

Determination of a cell-killing effect constitutes one of cardinal evaluations of anti-tumor agents. This is effected quantitatively by using clonally grown mammalian cells in culture. The basic findings of bleomycin action presented in this communication will provide us some knowledge regarding its tumoricidal effect as well as the cytotoxicity on normal proliferative system and will be also useful to search for the mechanism of cell lethality.

Materials and Methods

Cultured cells used in the present experiments were HeLa S3-9IV, kindly provided by Dr. T. T. PUCK (University of Colorado, Denver, U.S.A.) and L5, a derivative of the mouse L cells (B929-L2J). The former was grown in F10 medium[1] supplemented with 0.5 % heart infusion broth (Difco) and 10 % calf serum (Chiba Serum Institute), with the generation time of 22 hours. The DNA cycle parameter was estimated to be 8.5 hours for G1 (pre DNA-synthetic) period, 8.5 hours for S (DNA-synthetic) period, 4 hours for G2 (post DNA-synthetic) period and 1 hour for M (mitotic) period. The L5 cells were grown in the identical medium except that calf serum was added in 5 %. The growth properties of this strain were described previously[2].

For survival assay cells were dispersed by the treatment of randomly growing culture with trypsin solution. Appropriate number of cells were seeded into plastic Petri dishes (60×15 mm, Falcon Plastics) and incubated in a CO_2-chamber kept at 37°C into which a humidified 5 % CO_2-air mixture was constantly gassed. After 2-hour incubation most cells were found attached to the bottom and spread, and remained to be single by 5~6 hours. Culture dishes treated with the drug at 4~6 hours after seeding, were subjected to further incubation for colony development. After 14~16 days' incubation the cells were fixed and stained for counting number of surviving colonies.

For synchronizing HeLa cells mitotic cells were collected from randomly growing population and seeded on fresh culture vesseles. All the procedure was carried out in

the warm room at 37°C. The mitotic cells were attached and started to divide within 1~2 hours after incubation. Two-cell colonies thus formed grew synchronously and entered next division 22 hours later. The detail of harvesting method for synchrony was described previously.[2,3]

Bleomycin A2 (copper-free, Lot #24) and A5 (copper-free, Lot #29~32) were used. Drugs dissolved in water, then diluted in F10 medium were sterilized through Millipore filter type GS.

Treatment of cells was initiated by introducing the drug solution into cultures in 1/20 amount of the culture medium. After a given period of incubation, dishes were rinsed twice with F10 medium, followed by replacement with fresh growth medium.

Results

Dose-response and Time-inactivation Curves of Randomly Growing Mammalian Cells

L5 cells were treated with various concentrations of bleomycin A2 for 30 minutes. As illustrated in the upper portion of Fig. 1, the surviving fraction was reduced with increasing doses of the drug, showing the curve of simple exponential type. The mean lethal dose (D_{37}), the doses needed for giving 37 % (e^{-1}) survival in an exponential portion of dose-response curve, was approximately 12 μg/ml.

The lower part of Fig. 1 shows the inactivation of cells as a function of time of treatment with 10 μg/ml bleomycin A2. The inactivation proceeded at a faster rate until 30 minutes than the rate found at later times. However, the determination of such two-component curve was not carried out at varying concentrations of the drug.

The identical experiments with HeLa S3 cells were shown in Fig. 2. Circles of

Fig. 1. Lethal effect of bleomycin A2 on L5 cells.

The upper figure represents the dose-response curve as determined by 30-minute treatment of cells. The lower figure shows the time-inactivation curve obtained from the treatment of cells at the concentration of 10 μg/ml. Arrows connecting curves indicate corresponding scales.

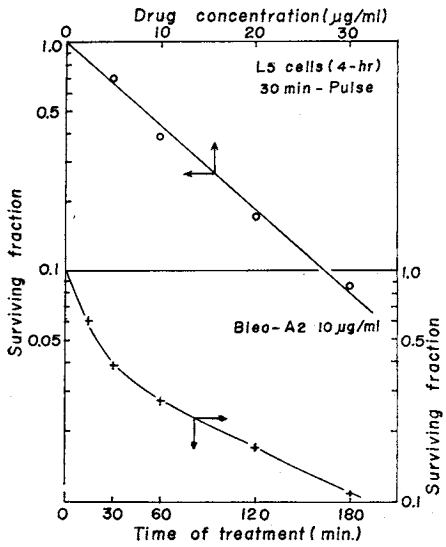
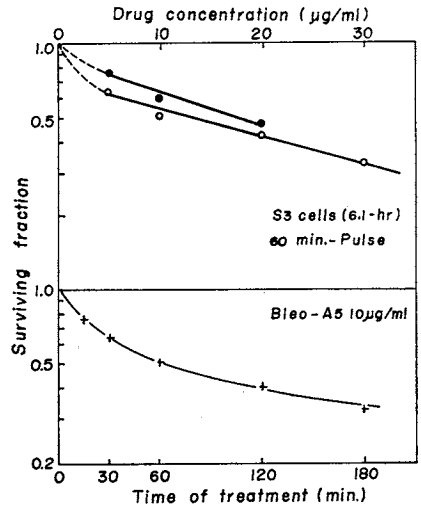


Fig. 2. Lethal effect of bleomycin A5 on S3 cells.

The upper picture indicates dose-response curves resulted from 60-minute treatment of cells. Circles, A5 compound; black spots, A2 compound. The lower picture shows the time-inactivation curve by the treatment with 10 μg/ml A5 compound.

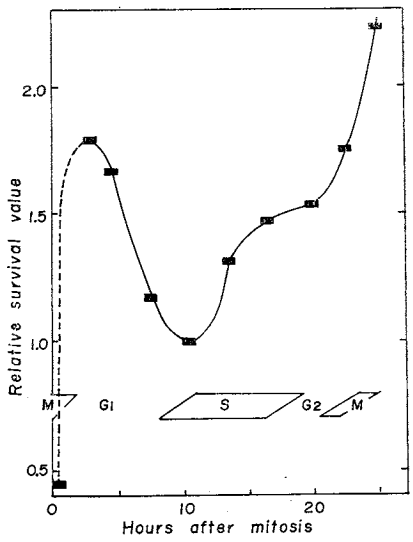


the upper part represent the dose-response curve of S3 cells which were treated with various amounts of bleomycin A5 for 60 minutes, whereas black spots were obtained from cells which were treated similarly with bleomycin A2. The D_{37} dose for S3 cells was measured to be about 45 μg/ml.

The lower part again shows the inactivation of cells with increasing duration of treatment. Either results demonstrated less lethal effect on S3 cells than on L5 cells.

Fig. 3. Change in drug-response during the cell cycle of S3 cells.

Bars represent survival levels together with the time and duration of treatment. Survival values obtained were normalized to the value at 10 hours after mitosis. Length of different stages during the cell cycle was properly allotted along the time scale.



Fluctuation of Drug-Response during the Cell Cycle

Two-cell colonies of synchronously growing S3 cells were treated with 60 minutes-pulse at 20 μg/ml bleomycin A5, and relative survival values were determined at different stages of the cell cycle.

When the drug was introduced at 0 hour, mitotic cells were still in suspended state and divided cells poorly attached at the end of treatment had a great possibility to be rinsed off. Therefore, the striking reduction in survival value found may be partly artifactual, although such an extreme sensitiveness to the drug was obvious.

Once cells moved into the early half of G1 period, marked resistance developped and, thereafter, the sensitivity increased with time toward the late G1 and the early S period, attaining the peak at 10 hours after mitosis. Finally cells reached the intermediate resistance at the middle and late S period. The rapid increase in survival observed from 22 hours onward coincided with the increase of number of cells per colony by the division. A trough in survival that would be expected from the high sensitivity of mitotic cells was not revealed at the second division, simply because of the decay of synchrony.

Discussion

The lethal effect as measured by dose-response curve seems to be moderate, although it largely depends upon cell strains used. Mitomycin C, one of other anti-tumor agents, showed D_{37} dose of roughly 0.5 μg/ml for the same HeLa S3 cells[6]. Therefore, bleomycin is less cytotoxic than mitomycin C by a factor of 90. Incidentally, D_{37} doses of actinomycin D and sulfur mustard on Chinese hamster cells were reported to be 1.1 μg/ml for 30-minute-treatment[7], 0.05 μg/ml for 8-minute treatment[8], respectively.

Difference in dose-response between L5 and S3 cells is notable. Assuming an exponential response of L5 cells after 60 minutes-pulse treatment with A2 compound, the estimation of D_{37} dose yields the value of 7.5 μg/ml. Therefore, it appears reasonable to conclude that S3 cells are at least 6 times as less sensitive as L5 cells, since the lethal effect of A5 compound is roughly comparable to or even slightly stronger than that of A2. Such strain difference in drug response may suggest the possibility that the drug acts

differently or selectively on cells in an organism. In this regard the investigation with cells of various origins will be useful.

The shape of dose-response curve of L5 cells, unlike those found with other antibiotics or deleterious agents, is sigmoidal. This does not necessarily mean the inactivation of a single component, since it is possible that composite curve of several different kinds of inactivation is of a quasi-sigmoidal nature[4]. Apparently, the dose-response curve of S3 cells (Fig. 2) exhibited an upward concavity, suggesting the involvement of sensitive and less sensitive fractions in a randomly growing population. As shown in Fig. 3, cells in the late G1 and early S periods were found relatively sensitive as compared to cells at the rest of stages. On the basis of stage-distribution of randomly growing cells[5], fraction of S3 cells in the sensitive portion, *i.e.*, 8~12 hours after mitosis, is estimated to be about 20 % of total population. Accordingly, the sensitive fraction may possibly explain the observed concavity. However, a successful reconstruction based on dose-response data of individual fractions would be necessary before the conclusion will be reached.

The pattern of sensitivity change during the cell cycle is quite similar to that found for X-rays[9,10] and chromomycin A_3[11]. In other words, the lethal action of bleomycin is correlated with cell's machinery during the cycle in the same manner as the case of X-ray. Therefore, a common mechanism of lethal action should be expected between these agents. Such similarity of the mechanism will be strongly supported by evidences that bleomycin not only exerts an apparent inhibitory action on DNA synthesis[12,13] but also breaks strikingly intracellular DNA strands.[14,15]

The pattern of sensitivity to mitomycin C is partly different, being defective of a resistant peak in the G1 period. Nevertheless, the pattern in the remainder of the cycle, namely, the peak sensitivity at the late G1 to the early S stages, followed by the development of resistance toward the latter half of S period, seems consistent among all these agents. In view of DNA as a promissing target to be affected lethally by these agents, above finding appears to agree with the hypothesis that the genetic material which replicates at the early S period is essential for an indefinite proliferation of cells[11].

Acknowledgement

This investigation was supported by a special project grant for Development of Bleomycin from the Science and Technology Agency. The authors wish to thank Miss M. YASUKAWA for her excellent technical assistance.

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