RESPONSE IN MACROMOLECULAR SYNTHESES OF MOUSE L CELLS TO BLEOMYCIN, WITH SPECIAL REFERENCE TO CELL-ANTIBIOTIC INTERACTION

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The inhibitory effect of bleomycin on DNA, RNA and protein syntheses of mouse L cells was determined. DNA synthesis was most affected. Protein and RNA syntheses were less affected in this order. Inhibition of DNA synthesis exhibited an upward-concave curve as a function of time of exposure to bleomycin. Analysis of this particular curvature revealed a characteristic interaction between the antibiotic and mammalian cells. Results obtained from the experiments of two pulsed drug treatments showed that (1) when bleomycin was introduced into culture, cell resistance developed with time and was complete within 60 minutes, and (2) the resistance so induced disappeared in approximately 2 hours upon removal of bleomycin. The nature of this resistance was not elucidated. Either an enzyme which inactivates bleomycin or DNA-repair enzymes may be responsible for resistance. The above interaction may have some relevance to bleomycin chemotherapy.

Bleomycin isolated by UMEZAWA *et al.*[1,2,3] has been found to affect cellular and isolated DNA molecules, either producing strand breaks[4,5] or decreasing the melting temperature[6,7]. In the course of our studies on the cytocidal effect of bleomycin, the inhibitory effect of the antibiotic on macromolecular syntheses in cultured mammalian cells was examined. The results led us to a new concept of the mode of action of the antibiotic. This concept is not only useful for the interpretation of various types of response of mammalian cells to bleomycin but may also assist in the design of bleomycin therapy.

Materials and Methods

Preparation of Culture: A clonal derivative of mouse L cells (B929-L2J), designated as L5 cells, was used throughout. The cells were normally grown in FlOHI medium[8] supplemented with 5 % calf serum. The median generation time was about 22 hours. The growth properties of this strain were described previously[8]. Cultures were prepared by dispersing monolayers with 0.1 % trypsin (Difco, 1 : 250)-saline D2[10] and inoculating an appropriate number of dispersed cells into plastic petri dishes (35×10 mm, Falcon Plastics, Los Angeles) after hemocytometer or Coulter counting. Dishes were incubated in CO_2-chambers at 37°C. Cells were attached to the bottom of the dish 2 to 3 hours after incubation and ready to divide at 5 hours. For synchronizing cells, the harvesting procedure consisted of selection of mitotic cells from a randomly growing population[9]. Cultures

were initiated with 2×10^6 cells per 180-ml glass bottle. After incubating bottles for 36~48 hours, the first medium renewal was made. The fragile cells and debris together with mitotic cells were detached by shaking the bottle rather vigorously and discarded. The mitotic frequency of the culture returned to a normal level in 5~6 hours of incubation with fresh medium. At this time, the second medium renewal was made with an 8~10 ml portion of FlO medium. The mitotic cells were then harvested by 1-minute agitation of the bottles on a shaking apparatus. Yields of harvested cells usually amounted to about 1×10^5 cells. Most of the harvested cells normally divided within 1 hour after plating into dishes. Working at 37°C was a basic requirement for consistent synchronous growth as well as for labeling studies with radioisotopes.

Determination of DNA-synthetic Rate: Trypsinized cells were suspended in FlO mixture from which thymidine and hypoxanthine were omitted (deficient FlO mixture) and then 1×10^5 cell aliquots were dispensed into plastic dishes. After 4 hours' incubation when a depletion of thymidine pool would be expected, the medium was discarded and cultures were treated with bleomycin for desired periods of time. In the earlier experiments, thymidine-^{14}C (51 mCi/m mole, Radiochemical Centre, Amersham) was added at 0.05~0.1 μCi per dish for DNA labeling; later, the labeled thymidine dissolved in deficient FlO mixture was introduced after removal of the antibiotic and subsequent rinsing of the dish with deficient FlO mixture. Cultures labeled for 30 minutes were rinsed with cold phosphate buffered saline and acid-soluble material was extracted with ice-cold 5% trichloroacetic acid solution. Dishes were then rinsed with distilled water, dried and subjected to low background gas flow counting for radioactivity measurement.

Determination of RNA-synthetic Rate: Mechanically harvested mitotic cells suspended in deficient FlO mixture were introduced in plastic dishes at 4×10^4 cells per dish. The pre-DNA synthetic (G1) cells, 3 hours after incubation, were used for measuring RNA synthesis, since the incorporation of uridine into nuclear DNA can be excluded under such conditions. Uridine-^{14}C (228 mCi/m mole, Radiochemical Centre, Amersham) was added to drug-treated cultures in the amount of 0.1 μCi per dish. Other steps such as labeling, fixing and counting were identical to those carried out in the DNA synthesis measurement.

Determination of Protein-synthetic Rate: Asynchronous cultures were prepared in leucine-deficient FlO mixture. Except for the use of leucine-^{14}C (231 mCi/m mole, New England Nuclear Corp., Massachusetts) in the amount of 0.05 μCi per dish, the steps in the determination of DNA synthesis were followed.

Autoradiography of Cells: Cells grown on coverslips were incubated with 0.5 μCi/ml of thymidine-^{3}H (5 Ci/m mole, Radiochemical Centre, Amersham) for 30 minutes after varying periods of bleomycin treatment. The labeled cells were then fixed in acetic acid-ethanol (1 : 3) and dried rapidly in air. For autoradiography the nuclear emulsion (NRM-2, Konishiroku Photoindust. Co., Tokyo) was applied and photographic processes were followed.

Bleomycin: Bleomycin A5 (#702 and 6), copper free sulfate, was supplied by Nipponkayaku Co., Ltd. The compound was dissolved in FlO mixture from which thymidine, hypoxanthine or leucine were omitted depending on experiments.

Results

The dose-response curves of macromolecular syntheses of L cells and bleomycin are shown in Fig. 1. DNA synthesis was most affected at every concentration tested, whereas RNA synthesis appeared to be least sensitive. A characteristic feature of the dose-response was an upward concavity in the curve shape. In other words, a small increment of concentration in the low concentration range produced greater reduction in synthetic rate than did the same increment in the high concentration range.

The time-inhibition relationship was examined for DNA and protein syntheses at a fixed concentration of bleomycin. As shown in Fig. 2, the inhibition curves of DNA and protein

Fig. 1. Dose-response curve of macromolecular syntheses of L5 cells

Identical cultures treated with bleomycin at various concentrations for 60 minutes, were labeled by adding thymidine-, uridine- and leucine-^{14}C. After 30-minute incubation, cultures were washed once with phsphate buffered saline, immersed in cold 5% trichloroacetic acid solution to remove acid-soluble fraction, washed twice with distilled water and dried. Then, dishes were placed directly into the low background counter for radioactivity measurement (counter background: 0.8 cpm, efficiency of counting: 40%). The rate of DNA (circles), RNA (triangles) and protein (squares) syntheses was determined on per culture basis, and shown as a percent rate in each untreated control culture (actual radioactivity: 98.7 cpm for DNA, 37.8 cpm for RNA and 417.1 cpm for protein synthesis). Each point represents the average of duplicate determinations. Samples were counted for at least 1,000 counts.

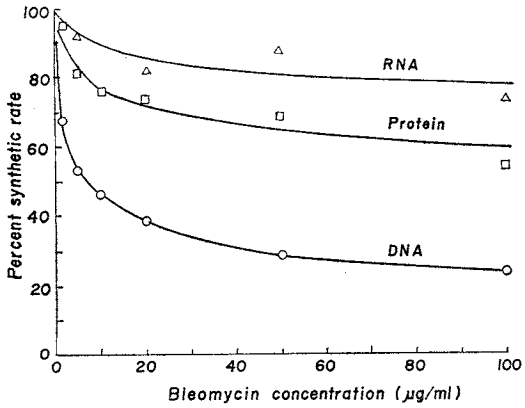
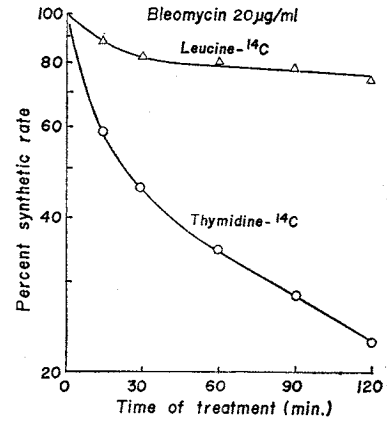


Fig. 2. Time-inhibition curve of DNA and protein syntheses of L5 cells

The cultures were treated with 20 μg/ml bleomycin. At times indicated, cultures were labeled for 30 minutes with thymidine-^{14}C (circles) and leucine-^{14}C (triangles) in the presence of the antibiotic. The extraction of an acid-soluble fraction and the counting of radioactivity incorporated were carried out as described in Fig. 1. Each point represents the average of duplicate determinations in a percentage of the untreated control value (actual radioactivity: 67.1 cpm for DNA and 519.0 cpm for protein synthesis).



syntheses (circles and triangles, respectively) showed upward-concave curvature, having inflexion points at about 30 minutes of treatment.

To test the possibility that either metabolic or thermal breakdown of the antibiotic occurs as a function of time of the treatment, the experiment shown in Fig. 3 was carried out as follows. Cultures were treated for various lengths of time with bleomycin at 20 μg/ml. The curve obtained (closed circles) was essentially similar to those in Fig. 2. At 60 minutes of incubation the medium of a portion of cultures was replaced with fresh bleomycin-FlO mixture of the same concentration. Further incubation of the cultures for 30 minutes did not show any significant difference in response (open circle) from that obtained after the continuous treatment for 90 minutes (closed circle). Therefore, the possibility of thermal or metabolic inactivation of the antibiotic in the medium can be excluded. The stability of the resistant cell fraction was examined by removing the antibiotic from the medium. In the experiment shown in Fig. 3, the cultures were incubated in a deficient FlO mixture for certain periods after the first 60 minutes of treatment with drug. These intervals were succeeded by another 30-minute treatment with the antibiotic to see whether the response changes during the absence of bleomycin. As shown in the figure, the second 30-minute treatment given at 60- and 120-minute intervals revealed apparently lower synthetic rates than that obtained after the treatment given without interval. This suggests that the sensitivity of the resistant fraction to the antibiotic increased during the interval between the two exposures.

Fig. 3. Inhibition of DNA synthesis by single and two pulsed treatments with bleomycin
The time-inhibition curve was obtained by treating cells with bleomycin, 20 μg/ml, for various lengths of time and by subsequent labeling in the absence of antibiotic and counting (closed circles). At 60 minutes of bleomycin treatment, the antibiotic was removed from a portion of cultures. After rinsing dishes with F10 mixture fresh antibiotic-containing medium was introduced either immediately (open circles) or after incubation for 60 minutes (open triangle) and 120 minutes (open squares) in a deficient F10 mixture. The DNA-synthetic rate was determined after the second bleomycin treatment of 30 minutes. Actual radioactivity of untreated control was 55.1 cpm.

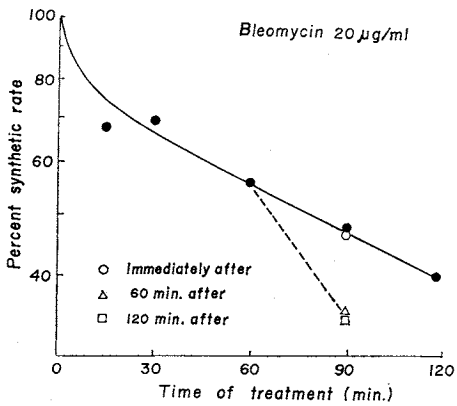
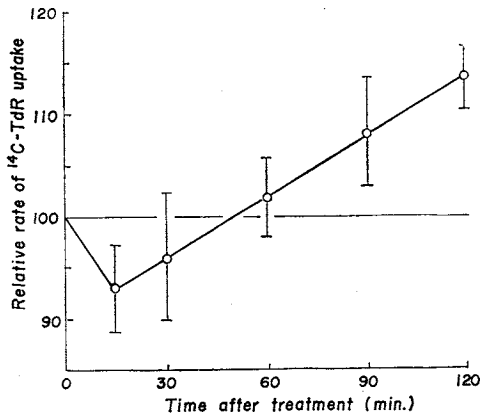


Fig. 4. Change in DNA-synthetic rate following bleomycin treatment
The cultures were treated with bleomycin 20 μg/ml for 60 minutes, then rinsed to remove the antibiotic and incubated again with F10 medium. The DNA-synthetic rate was measured at specified times. The observed rates were shown as a percentage of the 0-minute value (actual radioactivity: 52.7 cpm). Each point with bar represents the average and the standard deviation obtained from four independent experiments.



It must be noted that the synthetic rates obtained after the second drug treatments were corrected for the change in synthetic rate of resistant cells which would have occurred during the interval. Such a change is illustrated in Fig. 4, where the medium of cultures treated with the antibiotic for 60 minutes was replaced with a deficient FlO mixture and, thereafter, the synthetic rate of the culture was determined at intervals. The relative synthetic rate revealed an early depression followed by a steady increase. The depression may be explained by the residual effect of the antibiotic since the rate during the first 15 minutes appears to correspond roughly to the inhibition caused by the single treatment of 75 minutes. The gradual increase found after 15 minutes may either represent the repair of damage in the DNA-synthetic system produced by bleomycin or come from the entry of cells into the DNA-synthetic phase.

The next experiment was designed to follow the change in response to bleomycin during the second 30-minute treatment. After the first 60-minute treatment with bleomycin, the cultures were washed and incubated in FlO medium. Then, duplicate cultures were subjected to the second bleomycin treatment at different intervals. After the second treatment for 30 minutes (or 15 minutes in some case), the DNA synthetic rate was measured. The result of a typical experiment is plotted in Fig. 5 in which actual synthetic rates obtained after the second treatment were again corrected for the change in synthetic activity found during the interval between two pulsed drug treatments (Fig. 4). The change in response to the second treatment is indicated in the inset (Fig. 5). The slight but significant increase of sensitivity was recognized as early as 15 minutes after removal of

Fig. 5. Two pulsed drug treatments of L5 cells
After the first 60-minute treatment, the cultures were washed and incubated in F10 mixture. At intervals duplicate cultures were subjected to the second bleomycin treatment. After the second treatment of 30 minutes (or 15 minutes in some cases), the DNA-synthetic rate was measured and plotted in the inset scale. The result was shown by open symbols after the correction based on data in Fig. 4, *i.e.*, circle for 15 minute-, triangle for 30 minute-, inverted triangles for 60 minute- and square for 90 minute-intervals between two treatments. Inhibition of DNA synthesis after single treatments was shown by closed circles with standard deviation (actual radioactivity of untreated control: 93.4 cpm). Response of the original population is indicated by the broken line in the inset scale.

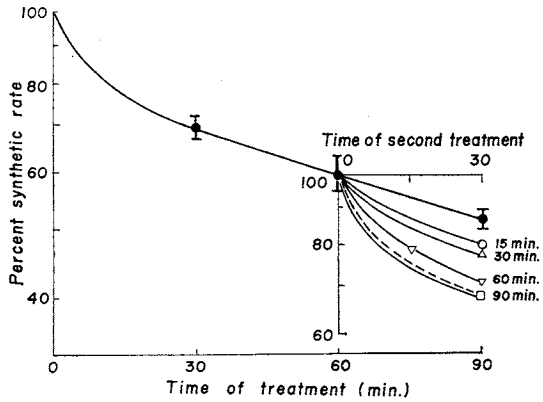
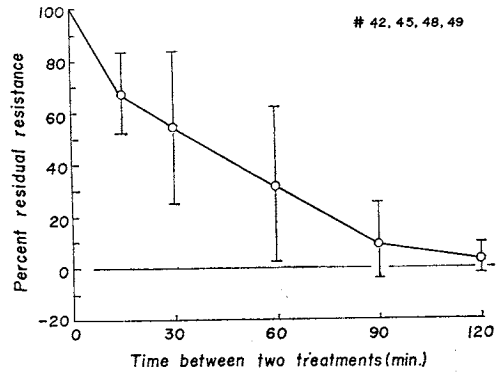


Fig. 6. Decay of bleomycin-induced resistance
The DNA-synthetic rate obtained after the second treatment of 30 minutes (as found in the inset scale of Fig. 5) was taken as a measure of resistance. The difference between the value of 0-minute interval (closed circle at 30 minutes in the same figure) and the value that would be expected if the response returned to the original state (the level of broken line at 30 minutes in the same figure) was assumed to be 100 % resistance. The residual resistance was plotted against the interval between two pulsed drug treatments. Each point with bar is the average and standard deviation derived from four independent experiments.



the drug and the extent of sensitization seemed to be proportional to that time. The inhibition curve examined after the 60-minute interval exhibited roughly the same shape as that of the original untreated population. Finally, the DNA synthetic rate found after 90-minute interval was reduced to 68 % in the inset scale. The value was approximately the one expected from the response of the original cell population as shown by the broken line. The result clearly indicates that resistance decays with time after removal of the antibiotic.

The same type of experiment as shown in Fig. 5 was repeated and the results expressed by plotting the residual resistance against the interval between the first and the second drug treatments, as illustrated in Fig. 6. It is seen that resistance decayed rather rapidly at early times, slowly after 90 minutes and finally the original sensitivity was reached approximately 2 hours after the first treatment. The reproducibility of the induction and subsequent decay of resistance to bleomycin was not tested with reverted cells.

Discussion

The inhibitory effect of the antibiolic bleomycin on macromolecular syntheses of HeLa cells has been demonstrated by KUNIMOTO *et al.*[18], and confirmed in the present studies. In view of the interaction of bleomycin with DNA[4,5,6,7], it can be assumed that inhibition of protein and RNA syntheses is brought about through the primary damage of DNA produced by the antibiotic.

At present, however, a direct action of the antibiotic on protein and RNA syntheses can not be excluded.

The upward concavity found in the time-inhibition curve may represent differential bleomycin sensitivities in the DNA-synthesizing cells. To examine this possibility individual bleomycin-treated cells were examined autoradiographically. The result revealed that the fraction of labeled cells stayed constant at 43% over the 2 hours treatment period. Therefore, it is obvious that the time-inhibition curve shown in Fig. 2 represents the reduction of average synthetic rate per cell. If a fraction of cells was more sensitive than the remainder of the population, the grain number distribution would vary. There was no evidence for such a sensitive fraction from the temporal change in the distribution of number of grains per cell. Thus, the autoradiographic results did not provide any definite information on whether or not DNA-synthesizing cells are heterogeneous in relation to bleomycin sensitivity.

At this time, it may be important to note that upward concavity was also found in the time-inhibition curve for protein synthesis and even in the time-inactivation curve for the proliferative capacity of cells[11,14]. Such a general occurrence suggests that the whole population, not DNA-synthesizing cells alone, is concerned with heterogeneity in sensitivity. It is unlikely that the heterogeneity is due to a genetic difference since the cells failed to give any different response on cloning. Therefore, the most likely explanation involves the induction of resistance by the antibiotic, since the results of two pulsed drug treatments could best be explained by such an assumption. Experimental support for this hypothesis is summarized as follows: (1) Inhibition of DNA synthesis occurs quickly after the addition of bleomycin. In the meantime, the resistance develops with time of exposure and is complete within 30~60 minutes. (2) The resistance is not complete, since the cells represented by the terminal portion in the time-inhibition curve are less sensitive than these in the initial portion at least by a factor of 6 at a concentration of 20 μg/ml. Conversion of the antibiotic to a less potent form may be possible. (3) Upon removal of the antibiotic the resistance decays with time and, finally, the original sensitivity as well as the capability of cells to develop resistance seem to be acquired again in approximately 2 hours' time. Such reversion in response is not simply due to an entry of G1 cells into the DNA-synthetic period becauce 2 hours' progression of cells during the cell cycle can replace only 20% of DNA-synthesizing population.

The shape of the dose-response curve is not easily explaned, it could occur if the inactivation constant of the time-inhibition curve was not proportional to the drug concentration. However, the situation is complicated by inducible resistance to the antibiotic. To answer the problem it seems most essential to establish means which can measure inactivation and the induction separately.

In general, a quantitative tracer study has disadvantages when the size of the metabolic pool changes appreciably in an experimental system, because the availability of radioactivity to be incorporated largely depends on the size of precursor pool which may dilute exogenous radioactive precursors. If we assume that the DNA of cells was degraded after bleomycin treatment[4,5], thereby increasing the size of the precursor pool, an extreme case would be that the observed terminal slope of the time-inhibition curve is a reflection of a temporal increase in the precursor pool size. After removal of the antibiotic, the precursor pool would probably tend to restore its original size and the synthetic rate of untreated cells would be regained. Neverthless, the result shown in Fig. 4 revealed only a 10% increase of the incorporation rate 2 hours after the durg removal when a nearly complete reversion to sensitivity was found by the second drug treatment. Therefore, the above possibility is not considered likely, and the inhibition curve of DNA synthesis would undergo only a slight alteration, if any, by a change in the precursor pool size.

A mechanism for induced resistance to bleomycin has not yet been found. As mentioned previously, upward concavity was found in the time-inhibition curve for protein synthesis and in the time-inactivation curve for poliferative capacity of cells. These findings led us to assume that the same mechanism operates for the induction of resistance, whatever the inactivating entity. Enzymes which inactivate bleomycin[12] or repair DNA damage may possibly be responsible for resistance. Also, the possibility of an induced change in permeability should not be dismissed.

The demenstration that bleomycin not only inhibits cellular activities, but also induces

resistance to itself in cultured mammalian cells, has relevance to cancer chemotherapy. Recently, we have obtained evidence for bleomycin-induced resistance with respect to the inactivation of proliferative capacity of cultured mammalian cells[14]. If bleomycin resistance is induced in tumor cells, the lethal effect of single exposure or continuous treatment with the antibiotic would be limited. The results presented here suggest that the most efficient use of bleomycin will be made only when a tumor was treated repeatedly at appropriate intervals. The discussion pertinent to this scheme of chemotherapy, is presented in another report[14].

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