NIRS Publication M-25(1978)



Collected NIRS Papers on

# CELLULAR BASIS OF TUMOR THERAPY

Edited by

TOYOZO TERASIMA, M.D.
National Institute of Radiological Sciences, Chiba

Science and Technology Agency, Japan

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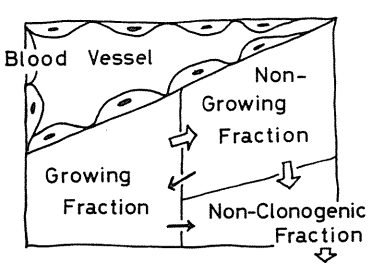


Science and Technology Agency, Japan

PREFACE

Since 1965, the year I founded the cell culture research section in the Division of Physiology and Pathology, NIRS, the research subjects of the laboratory have been focused on cell-cycle dependence of radiation effects and on cellular repair after irradiation. Additional effort has also been made for the development of a method for synchrony of cultured mammalian cells by Professor Y. Fujiwara (Kobe University), Drs. H. Ohara, T. Miyamoto (NIRS), and myself. In 1971, the laboratory has initiated a new line of studies on the antitumor antibiotic, bleomycin, when Drs. T. Katsumata, Y. Takabe and M. Watanabe of Chiba University School of Medicine came to work together in my laboratory. As the research developed, a cellular basis of tumor therapy has become one of our great concerns and, lately, the studies are being extended to a specific area of clinical field.

Studies on bleomycin action could be traced back to experiments carried out by myself in 1968 upon the request of Professor H. Umezawa (discoverer of bleomycin, Institute of Microbial Chemistry, Tokyo), who had sought the possibility of combined effect of bleomycin and radiation. Having studied with only 16 mg of bleomycin A5 compound which was then on the way of development, I was barely able to find the cell cycle dependence of survival response and an upward-concave nature of the dose-response relationship with a few strains of cultured mammalian cells. Its report was accordingly brief and the interpretation was made only in an unsatisfactory manner. Such incompleteness of my

initial work (J. Antibiotics, 1970) might have acted as a trigger for subsequent studies by my colleagues. Since then, the unusual kinetics in growth and survival of bleomycin-treated cells has been elucidated one after another, and, luckily, opportunities have been given repeatedly by Professor Umezawa to present our data in international meetings, e.g., *Internationale Arbeitstagung über Chemo- und Immunotherapie der Leukosen und Malignen Lymphome*, Vienna, 1972 (Prof. A. Stacher); *Symposium on Biological Basis of Clinical Effects of Bleomycin*, Venezia, 1974 (Prof. A. Caputo); *The Bleomycin - Current Status and New Developments*, Oakland, 1977 (Prof. S. K. Carter), and several U.S.- Japan seminars.

This volume contains our papers regarding the action of bleomycin and of a few other chemotherapeutic agents which are relevant to the title of this book. It is my pleasure that Professors Y. Fujiwara and M. Urano (Massachusetts General Hospital), whose research groups had been in close contact with us, have willingly joined to contribute their papers of bleomycin studies to this issue. It should be noted that most of studies have been supported by the special project grant for Development of New Bleomycins from the Science and Technology Agency and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture.

I am very much indebted to all the workers who have been involved in researches collected in this volume and to our technical staff, Miss M. Yasukawa, Mrs. M. Furuya and Mrs. M. Kimura. My sincere thanks are particularly due to Prof. H. Umezawa for his continuing encouragement, to Drs. K. Misono (Director, NIRS), K. Okuda (Professor, Department of Medicine, Chiba Uni-

versity), A. Kurisu (Head, Hospital, NIRS), and Y. Umegaki (Head, Division of Clinical Research, NIRS) for their generous management and cooperation in pursuing our studies, and to Dr. S. Ito, Dr. A. Matsuda, and Mr. E. Yugeta (Nippon Kayaku Co. Ltd., Tokyo) for their keen interests in our results and kind supply of the materials used in our work. Lastly, I wish to thank the publishers who kindly granted us permission for reproduction of our papers.

December 1977

Toyozo Terasima, M.D.

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# LETHAL EFFECT OF BLEOMYCIN ON CULTURED MAMMALIAN CELLS

TOYOZO TERASIMA* and HAMAO UMEZAWA**

Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, Chiba-shi*
and The Institute of Microbial Chemistry, Kamiosaki-3, Shinagawa-ku, Tokyo, Japan**

(Received for publication May 4, 1970)

By using cultured mammalian cells lethal effect of bleomycin was measured in terms of mean lethal dose (Do); 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 experimental 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 0.05% 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 same 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 in fresh culture vesseles. All the procedure was carried out in

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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 ($D_0$), the doses needed for giving 37 % ($e^{-1}$) survival in an exponential portion of dose-response curve, was approximately 12 $\mu$g/ml.

The lower part of Fig. 1 shows the inactivation of cells as a function of time of treatment with 10 $\mu$g/ml bleomycin A2. The inactivation occurred 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 $\mu$g/ml. Arrows connecting curves indicate corresponding scales.

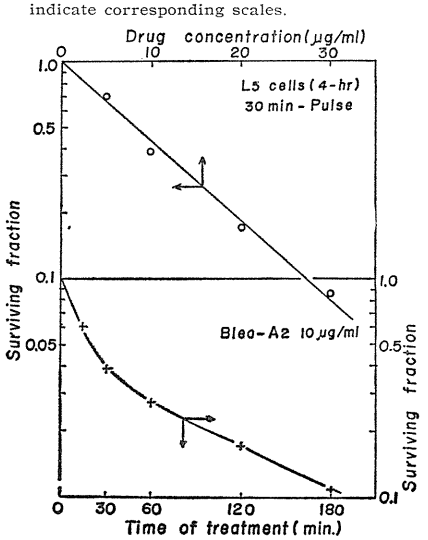
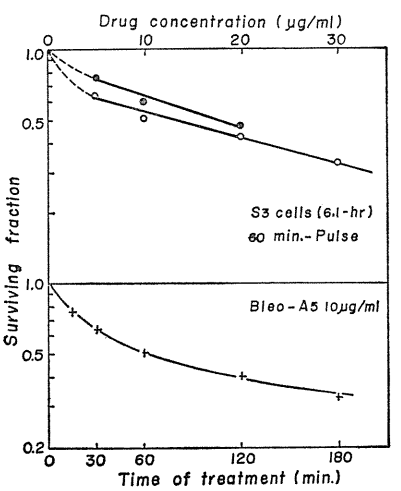



Fig. 2. Lethal effect of bleomycin A5 on S3 cells.

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 $\mu$g/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 Do dose for S3 cells was measured to be about 45 $\mu$g/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.

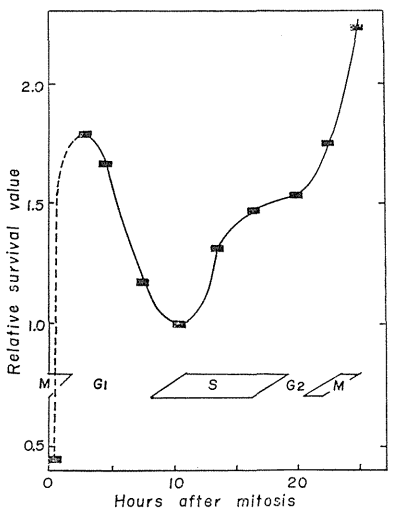
### Fluctuation of Drug-Response during the Cell Cycle

Two-cell colonies of synchronously growing S3 cells were treated with 60 minutes-pulse at 20 $\mu$g/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 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.

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.




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 shown 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 Do dose of roughly 0.5 $\mu$g/ml for the same HeLa S3 cells[6]. Therefore, bleomycin is less cytotoxic than mitomycin C by a factor of 90. Incidentally, Do doses of actinomycin D and sulfur mustard on Chinese hamster cells were reported to be 1.1 $\mu$g/ml for 30-minute-treatment[7], 0.05 $\mu$g/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 Do dose yields the value of 7.5 $\mu$g/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 exponential. This does not necessarily mean the inactivation of a single component, since it is possible that a composite curve of several kinds of sigmoidal inactivation is of a single-hit nature[4]. Apparently, the dose-response curve of S3 cells (Fig. 2) exhibited an upward concavity, suggesting the mixture 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]. It implies that bleomycin affects cell's reproductive machinary during the cell cycle in the same manner as in 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 for 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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[GANN, 61, 513~516; October, 1970]

UDC 615.33 :576.343

# BREAKS AND REJOINING OF DNA IN CULTURED MAMMALIAN CELLS TREATED WITH BLEOMYCIN

Toyozo TERASIMA,*1 Mieko YASUKAWA,*1 and Hamao UMEZAWA*2
(Division of Physiology and Pathology, National Institute of Radiological Sciences,*1 and Institute of Microbial Chemistry*2)

Action of Bleomycin on mammalian cells has been investigated rather extensively in recent years.[3,4,5,8,13] Regarding its effect on DNA the remarkable change in the melting temperature was found by Nagai *et al.*[7] In the course of our studies concerning radiation effect on mammalian DNA, we found another notable action of the antibiotic on DNA *in vivo* which will be presented in this communication.

## MATERIALS AND METHODS

A clonal derivative of the mouse L cells, designated L5 strain, was grown in F10HI medium[2] supplemented with 5% calf serum. The growth property was described previously.[10]

For labeling DNA of the cells, the culture was incubated with $^3$H-thymidine (5 Ci/m*M*, Radiochemical Centre, Amersham) at a concentration of 1.5 $\mu$Ci/ml for 15~20 hr. Labeled cells dispersed by trypsin treatment were suspended in F10 medium and then dispensed at an appropriate concentration into plastic petri dishes (35 × 10 mm, Falcon Plastics, U.S.A.) which were Silicone-greased at the bottom previously.

Cells in plastic dishes were incubated in a $CO_2$-chamber at 37° and treated with Bleomycin at a given concentration for 30 min. After treatment the culture was repeatedly rinsed with a cold fresh medium to remove the antibiotic, followed either by harvesting the cells or reincubation. For harvesting, the cells were scraped with a fine-tipped glass rod and the cell suspension thus prepared was kept in an ice-bath until the time of isolation of DNA.

To isolate near-intact DNA from mammalian cells, we previously developed a method in which 2% sodium dodecyl sulfate solution (SDS) at pH 7.2 was used for lysing mammalian cells.[11] At the top of 5 to 20% sucrose gradient, either at pH 6.7 or pH 12.5, 0.2 ml of 2% SDS was layered, followed by the introduction of 1~4 × $10^4$ cells suspended in 0.05 ml of F10 medium. After standing for 30 min at 20°, the gradients were centrifuged at the same temperature in Hitachi 40P ultracentrifuge with the swinging bucket rotor, RPS40. Fractionation of sucrose gradients, extraction of acid-precipitable material from each fraction, and liquid-scintillation counting of radioactivity of the precipitates were described in a previous report.[11] The sedimentation pattern of DNA was obtained by determining the distribution of radioactivity of precipitates in fractions.

L5 cell-DNA recovered by this method sedimented normally as a single distinct peak in sucrose gradients. The sedimentation constants were estimated to be 330S for neutral sucrose solution and roughly 400S for alkaline sucrose solution. The best assumption that can be made on sedimenting radioactive materials at present is an aggregate, since the shape of DNA profile changes depending on the amount of cells loaded. The determination of sedimentation behavior of mammalian DNA by this method permitted a sensitive detection of strand breaks and of the rejoining.[11,12,14]

*1 Anagawa 4-9-1, Chiba 280 (寺島東洋三, 安川美恵子).
*2 Kamiosaki 3-14-23, Shinagawa-ku, Tokyo 141 (梅沢浜夫).



GANN 61, 513-516, 1970

Bleomycin A2 (copper-free, Lot #24) was used throughout the experiments. The antibiotic dissolved in water was sterilized through a Millipore filter, type GS.

## Results and Discussion

The sedimentation pattern of double-stranded DNA derived from treated cells was obtained after centrifugation of neutral sucrose gradients at 20,000 rpm for 60 min. As shown in Fig. 1, the pattern of untreated DNA exhibited a main peak at the 12th fraction from the bottom which was followed by irregular, accessory peaks in the upper fractions. Treatment with 10 $\mu$g/ml of Bleomycin induced partial decay of the main peak and a compensatory increase of radioactivity in upper fractions. At more than 20 $\mu$g/ml of Bleomycin, double strand breaks were found much more severely as shown by the highest radioactivity left at the meniscus.

Fig. 2 illustrates the antibiotic-induced breakdown of single strands of DNA which was centrifuged in alkaline sucrose gradients at 20,000 rpm for 50 min. In this experiment, an accessory peak was found abnormally high at the 17th fraction. It may come from a disaggregation due to the loading of fewer cells. Although the effect of 0.1 $\mu$g/ml of Bleomycin was, if any, slight, the obvious change in profile was produced after treatment with 1 $\mu$g/ml. Therefore, single strand breaks of DNA seem to occur roughly 10 times more efficient than double strands. Qualitatively similar results were obtained by Suzuki *et al.* with HeLa cells.[9] Incidentally, the related antibiotic, Phleomycin,[6] was found to induce single strand breaks in more than 60 min of treatment at a concentration of 20 $\mu$g/ml.[14]

The repair capability of L5 cells for the antibiotic-induced single strand breaks was

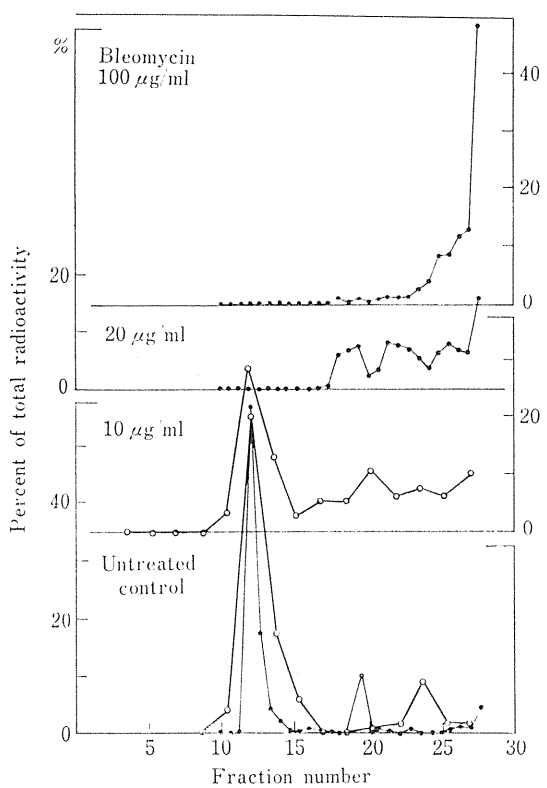


Fig. 1. Sedimentation pattern of double strands of DNA derived from L5 cells treated with Bleomycin

Fractions collected were numbered from bottom to top of the centrifuge tube. Different symbols denote separate experiments.

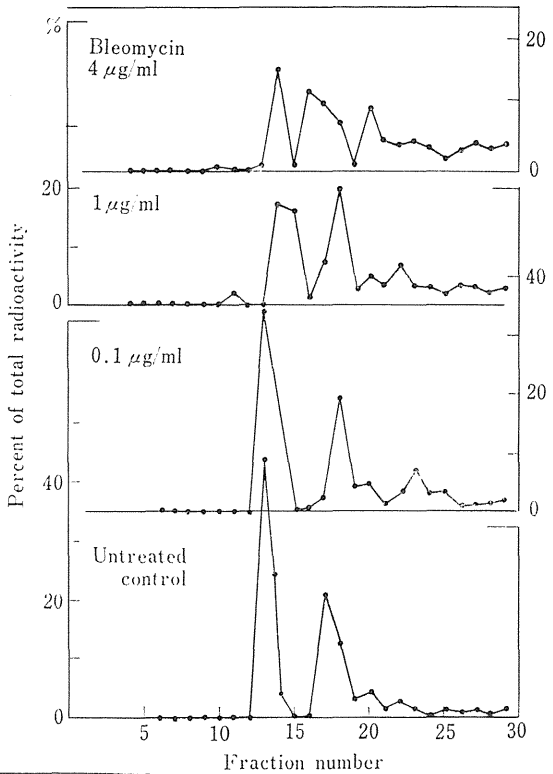


Fig. 2. Sedimentation pattern of single strands of DNA derived from L5 cells treated with Bleomycin

Fractions collected were numbered from bottom to top of the gradients.

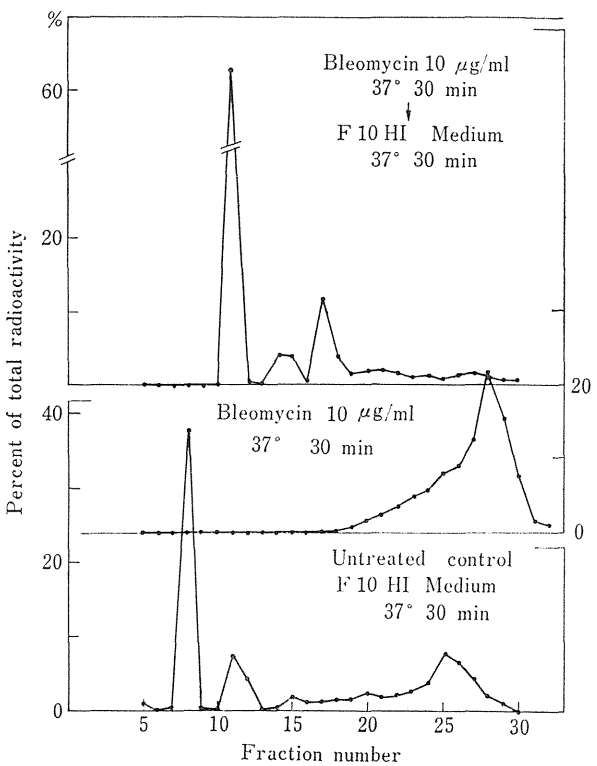


Fig. 3. Change in sedimentation pattern of single strands of DNA after incubation of Bleomycin-treated L5 cells

Fractions collected were numbered from bottom to top of the gradients.

examined by incubating the cells treated with 10 $\mu$g/ml of Bleomycin for 30 min. Fig. 3 represents the experimental result obtained after centrifugation of treated cells in alkaline sucrose gradients at 20,000 rpm for 60 min. The DNA profile, which was derived from untreated cells (Fig. 3, bottom), showed the main peak at the 8th fraction, whereas the profile obtained from treated cells revealed a notable shift of the peak toward the meniscus (Fig. 3, middle). However, when the treated culture was reincubated with a fresh medium for 30 min after removing the antibiotic, the prominent peak reappeared at the 12th fraction (Fig. 3, top). Although we are not certain whether a full return of the profile to the original level be expected after a longer incubation period, it indicates obviously the rejoining of single strand breaks which occurs rapidly just as found in the cells irradiated by X-rays.[14] In contrast, methyl methanesulfonate-induced single strand breaks are repaired only after incubation of 5 hr or more.[1] Such difference between repair processes may be due to the chemical nature of damages induced by these chemicals.

It is possible to assume that Bleomycin produces lesions which are converted to single strand breaks through the lysing procedure. It has been demonstrated that the *in vitro* reaction between the antibiotic and DNA results in the lowering of $T_m$ (melting temperature) of DNA in the presence of a sulfhydryl compound.[7] Hence, it is largely possible that the antibiotic makes intracellular DNA fragile or even broken with the cooperation of nucleases.

In previous studies regarding the cytocidal effect of Bleomycin,[13] we found that the change in sensitivity of HeLa cells during the cell cycle is very much similar to that for X-rays; namely, cells are most sensitive in the late G1 (pre-DNA-synthetic), less sensitive in the late S (DNA-synthetic), and least sensitive in the early G1 stage. The similarity of cell cycle-dependent sensitivity patterns may suggest that damages in DNA caused by Bleomycin and their repair are more or less qualitatively alike to those by X-rays, if the latter were to be responsible for the cyclic sensitivity change.

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UDC 615.33[Mitomycin-C] :576.35

# LETHAL EFFECT OF MITOMYCIN-C ON CULTURED MAMMALIAN CELLS

Hiroshi OHARA and Toyozo TERASIMA

(Division of Physiology and Pathology, National Institute of Radiological Sciences*)

## Synopsis

The lethal effect of Mitomycin on cultured mammalian cells was studied by using mitotically synchronous and asynchronous HeLa cells. The cultures were treated pulsewise with the antibiotic for 1 hr, and survivals were determined by the colony formation technique. Asynchronous cells revealed a composite type dose-response curve and the use of synchronous cells suggested that two types of responses, exponential and sigmoidal, can be assigned to different stages of the cell cycle. The cyclic change in sensitivity was also observed during the cell cycle. The G1 and G2 periods were moderately sensitive, while the latter part of the S period was the least.

Effect of the antibiotic on DNA synthesis was also studied using DNA precursors. The maximum depression of DNA synthesis was found in the cells treated in G1 period. Behavior of the antibiotic-treated cells in the post-treatment generations was observed by time-lapse cinematography. The incidence of cell death mainly occurred after the first post-treatment cell division. These findings were useful for possible interpretation of the cell cycle dependence in cell sensitivity to Mitomycin.

## INTRODUCTION

Many observations have been made in recent years that certain chemical agents have a different lethal effect with respect to the stages in the cell cycle. These were mainly found by using well-developed synchrony methods[14] for cultured mammalian cells. Among them, studies on lethal action of drugs like Mitomycin, which exerts selective inhibition of DNA synthesis and antitumor activity, might have a particular importance to cancer chemotherapy and cell biology as well.

The present investigation concerns the lethal responses of synchronous and asynchronous HeLa cells to Mitomycin-C. Further, observations were extended to the effect on DNA synthesis and to the behavior of the antibiotic-treated cells in the post-treatment generations.

## MATERIALS AND METHODS

HeLa S3 cells, which have been kept in our laboratory during several years, were grown in F10 medium[3] supplemented with 2% human serum, 6% calf serum, and 0.05% heart infusion broth (Difco). The cells were dispersed with 0.1% trypsin (Difco, 1:250) solution, counted by using a standard hemocytometer, and plated into petri dishes (Falcon Plastics). The cultures were incubated in a humidified atomosphere of 5% $CO_2$-air mixture at 37°.

* Anagawa 4-9-1, Chiba 280 (大原 弘, 寺島東洋三).



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For synchronizing the cells, the dispersed cells were inoculated into large dishes (100 × 25 mm) at the concentration of $5 \times 10^5$ cells per dish and allowed to grow for 48 hr. Then the cultures were harvested for collecting mitotic cells.[14] The harvested cells were plated into dishes (60 × 15 mm) after counting the number of cells with Tatai eosinophil counter (Kayagaki Works, Tokyo). All of the processes for harvesting were carried out in a warm room of 37°. The growth of synchronous cells was monitored by repeated scoring of cell number in specified microscopic fields. The median doubling time of the cells in these cultural conditions was 22~23 hr.

The treatment of synchronous and asynchronous cells with the antibiotic was carried out either by adding a small amount of a drug solution into cultures or by replacing the growth medium with the one containing the antibiotic. After 1-hr incubation with the antibiotic, the cultures including the untreated controls were rinsed twice carefully with warmed F10 medium and then fed with fresh and pre-warmed medium for further incubation.

Cell survival was determined by assaying a colony-forming ability of the antibiotic-treated cells. The cells were considered to be alive if it gave rise to a colony consisting of more than 50 healthy cells after 14 to 16 days of incubation. The plating efficiency of the untreated control cultures varied from 70 to 80% under these cultural conditions.

The precursor incorporation was measured by pulse-labeling of the cells with $^3$H-thymidine (0.5 $\mu$Ci/ml; specific activity, 5.0 Ci/m$M$) (Radiochemical Centre, Amersham) or with $^{14}$C-thymidine (0.05 $\mu$Ci/ml; specific activity, 35.9 mCi/m$M$) (Radiochemical Centre, Amersham) dissolved in the thymidine-deficient F10 medium. After 20 min of incubation with each radioactive precursor, the dishes or coverslips on which the cells were grown were rinsed and fixed with acetic acid-ethanol (1:3) for 20 min and dried rapidly. Subsequently, the cells were treated with cold 5% trichloroacetic acid solution for 15 min to remove the acid-soluble fraction. Autoradiography was carried out by applying the autoradiographic emulsion (NRM-1, Konishiroku Photo Industry Co., Tokyo) to the fixed cells on coverslips. The autoradiographic slides prepared were kept in a dark box at 4°, developed after 1 week of exposure, and examined for a fraction of labeled cells. Radioactivity of the cells labeled with $^{14}$C-thymidine was measured by placing the dishes directly into the low-background gas-flow counter. The rate of incorporation of radioactivity into the antibiotic-treated and the untreated control cultures was expressed as a percentage of the maximum activity in the control cultures which was usually found 14 hr after mitosis.

For cinematographic observation of synchronous cells, a drop of harvested mitotic cell suspension was placed in a small petri dish (35 × 10 mm) and pre-gassed medium was replenished after 2 hr of incubation during which time the attachment of cells was completed. Then, the cultures were exposed to the antibiotic in a $CO_2$-chamber at specified times after mitosis. After the treatment it was replaced in an air-tight glass dish to which 5% $CO_2$-air mixture was constantly introduced, and then the glass dish was fixed on the stage of an inverted microscope for cinematography. The photographs were taken at 4-min intervals during 4 to 5 days in a warm room of 37°. The recorded films were analysed for cell division, generation time, and the time of cell death in each generation. Death of cells was diagnosed by the observation of a disappearance of cell movement and subsequent degradation.

Mitomycin-C (Kyowa Hakko Kogyo Co. Ltd., Tokyo) was dissolved in distilled water for each experiment and the solution was then diluted to the desired concentrations with the culture medium just before use.

## Results

### Dose-response Curve of Mitomycin for Synchronous and Asynchronous HeLa Cells

Fig. 1 illustrates the result of experiments with asynchronous cells. All of the survival curves obtained from many independent experiments revealed the particular structure composed of early exponential and late sigmoidal regions. The shoulder of the latter appeared over the range of 0.1 to 0.5 $\mu$g/ml and its width varied considerably among experiments. It seems that these two portions of the curve are attributed to a heterogeneity of lethality in the asynchronous population, since the different killing effect of Mitomycin during the cell cycle has already been found.[1] In order to test this possibility, the survival curves of the cells located at different stages in the cell cycle were examined. As shown in Fig. 2, the replicate synchronous cultures were exposed to the antibiotic of various concentrations at 4, 14, and 18 hr after mitosis. Although 4- and 14-hr cells can be assigned to Gl and S period, respectively, 18-hr cells seemed to be a mixture of S, G2, M, and Gl cells in the next cell cycle (see Fig. 3). This result revealed that the particular structure found in the dose-response curve of asynchronous cells was recognized at least among cells at all stages examined. However, it can be noted that a decrease in survival at the initial portion of the curve was more rapid in 4- and 18-hr cells than in 14-hr cells, whereas the manifestation of a shoulder was more pronounced in 14-hr cells than in the others. Accordingly, it is most likely that the shoulder-type response is assigned specifically to the S period, while cells in the remainder of the cell cycle are rather inactivated in an exponential manner. The other finding is that the sensitivity, as judged by the shape of the curves, seemed to fluctuate through the cell cycle. This was studied at closer intervals in the next experiment.

### Change in Sensitivity of HeLa Cells during the Cell Cycle

The replicate synchronous cultures were exposed to the single concentration of 0.5 $\mu$g/ml of Mitomycin for 1 hr at different time after mitosis. This treatment gives about 20% survival for the asynchronous cells. Survival rates obtained were plotted as a function of time after mitosis in the upper part of Fig. 3. In the lower part, a change in the fraction of DNA-synthesizing cells and the relative increase in the number of cells are shown together. From these two curves, the location and duration of DNA-synthetic period and the generation time of synchronous cells were determined. The duration of Gl, S, and G2 plus M periods was about 9, 10, and 3$\sim$4 hr, respectively, and one-half of the population doubled in 22$\sim$23 hr after mitosis. These data were also confirmed by the time-lapse cinematography on synchronous cells.

As seen in Fig. 3, the cells were sensitive through the Gl period, particularly the most sensitive around 8 hr after mitosis (Gl/S transition phase) and then became less sensitive as the cells progressed through the DNA-synthetic period. The resistant peak was found in 14 to 16 hr when most of the cells were entering the latter half of the S period. From this time onward, the cells began to increase in sensitivity and finally became as much sensitive as Gl cells near the end of the cell cycle. However, sensitivity of the

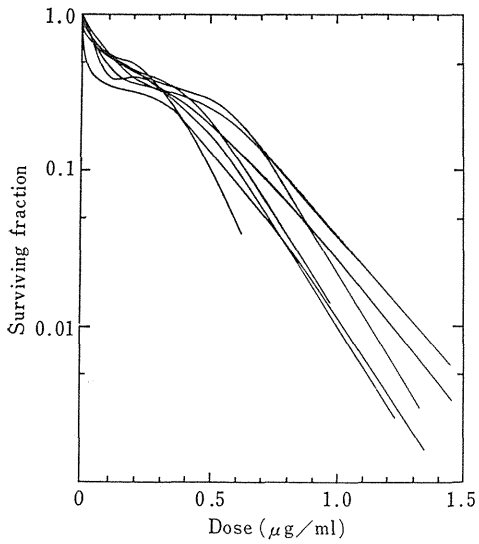


Fig. 1. Dose-response curves of asynchronous HeLa S3

The cells were treated with Mitomycin for 1 hr. The curves indicate different experiments.

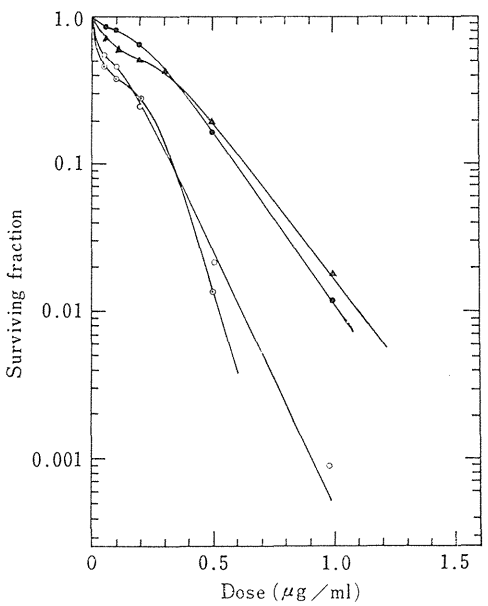


Fig. 2. Dose-response curves of synchronous HeLa S3 cells at different stages of the cell cycle

The time indicates the age of synchronous cells after mitosis. ○ Synchronous 4-hr cells, ● synchronous 14-hr cells, ◉ synchronous 18-hr cells. ▲ Typical dose-response curve of asynchronous cell population.

cells at 18 to 20 hr could not be determined accurately because of the breakdown of synchrony (see the lower part of Fig. 3). It is noted that the observed change in survival roughly followed the change in labeled fractions. It seems that the sensitivity is somewhat related to the DNA-synthetic activity. This point will be discussed later.

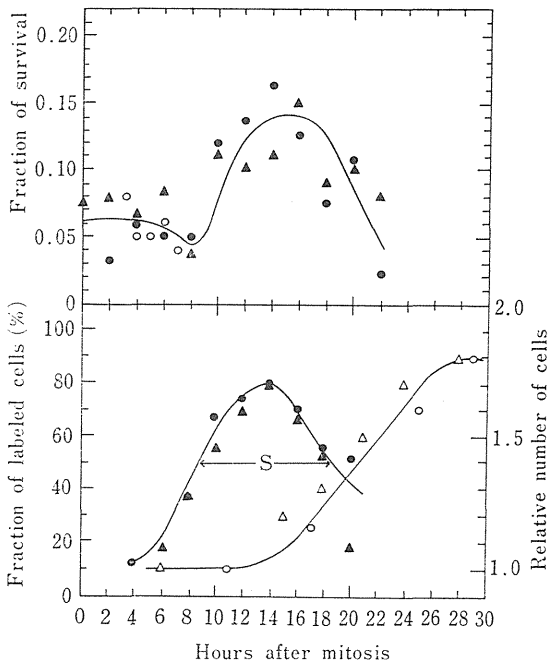


Fig. 3. Cyclic change in sensitivity to 0.5 $\mu$g/ml Mitomycin and in the fraction of labeled cells through the cell cycle

Different symbols indicate separate experiments. In the lower part, relative increase of cell number (○△) is plotted through the cell cycle.

**Effect of Mitomycin on DNA Synthesis of HeLa Cells**

As shown in Fig. 4, the synchronous cells pulse-treated at 3, 7, and 11 hr after mitosis, i.e., early Gl, late Gl, and early S periods, were examined for the rate of $^{14}$C-thymidine incorporation at different stages after removal of the antibiotic. In either case the incorporation activity of the antibiotic-treated cells was reduced through the remainder of the S period to less than 50% of the untreated control peak, although a 2-hr lag preceded such reduction when the DNA synthesis was under way. Such action of the antibiotic seems to be irreversible, since the recovery of incorporation activity was never seen throughout the post-treatment period observed. The other finding is that the beginning of DNA synthesis in the Gl-treated cells seems quite normal to occur in the time course of the cell cycle. However, it was not certain if the antibiotic had no effect on the entry of the cells into the S phase, because the incorporation was measured on a per culture basis. This point was studied by the following autoradiographic experiments.

Synchronous cells treated pulsewise 6 hr after mitosis (Gl phase) were labeled with $^{3}$H-thymidine and the fraction of labeled cells was determined at intervals. These values were plotted together with those for the untreated control and shown in Fig. 5. The labeled fraction of the antibiotic-treated cells was seen to follow the increase of the untreated control exactly until 12 hr. Then, it exceeded the level of the untreated control and stayed around 60% level even at 22 hr. This indicates that the cell progress slowed down through the S period. The result of this experiment obviously revealed that the entry of Gl cells into S period was not prevented by the antibiotic treatment. In addition to the prolongation of the S period, the delay of the post-treatment divi-

sion, which was estimated to be 16 hr or more in Fig. 5, would also indicate the appreciable prolongation of G2 period. This division delay will be dealt again in the next section.

Another autoradiographic experiments are illustrated in Fig. 6, which represents the fraction of labeled cells (A) and the rate of DNA synthesis (B) after Mitomycin treatment at various intervals in the Gl period. The rate of DNA synthesis in the untreated cells, as determined by counting the number of grains per cell, was not constant

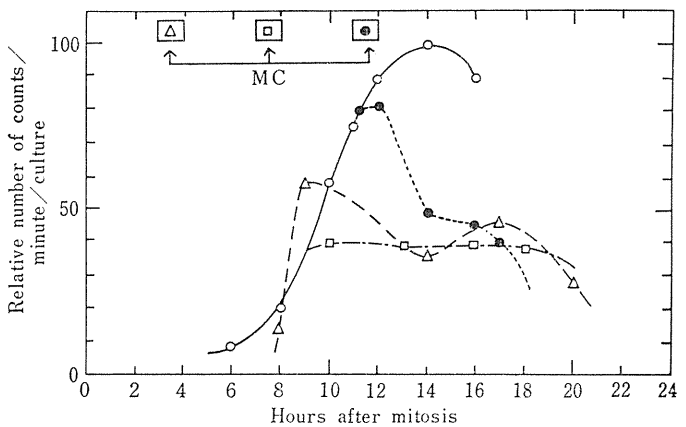


Fig. 4. DNA synthesis of synchronous HeLa S3 cells treated with 0.5 $\mu$g/ml Mitomycin (1 hr) at various stages from Gl to S period

The Mitomycin treatment is indicated by squares with corresponding symbols and the rate of synthesis for the untreated control is shown by open circles.

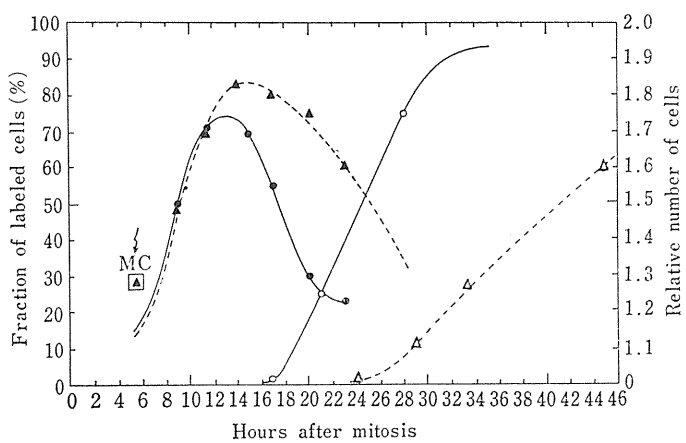


Fig. 5. S-prolongation and division delay induced by Mitomycin treatment at Gl stage of synchronous HeLa S3 cells

The treatment (0.5 $\mu$g/ml, 1 hr) is indicated by an arrow. ● Fraction of labeled cells of the untreated control, ○ relative cell number of the untreated control. ▲ Fraction of labeled cells in the Mitomycin-treated group, △ relative cell number in the Mitomycin-treated group.

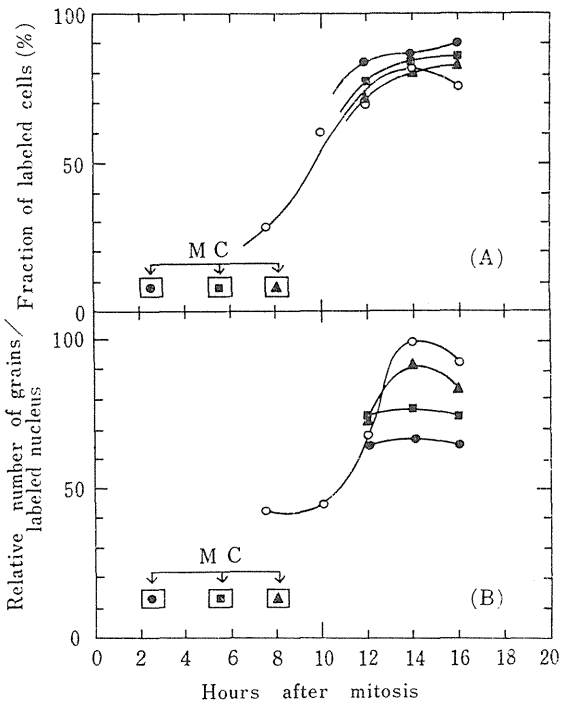


Fig. 6. DNA synthesis of synchronous HeLa S3 cells treated with Mitomycin at various periods from Gl to S period

The treatment (0.5 μg/ml, 1 hr) is indicated by squares with corresponding symbols. ○ Untreated controls. ● Cells treated at 2 hr, ■ cells treated at 5 hr, ▲ cells treated at 7.5 hr after mitosis.

through the S period; namely, relatively low at 8 to 10 hr, intermediate at 12 hr, and the highest at 14 hr, the middle of S period. In the antibiotic-treated cells, however, the increase in the rate was partially prevented; the earlier the treatment of cells in the Gl period, the greater the depression in the rate of DNA synthesis. Therefore, together with the foregoing data here and elsewhere,[16] Mitomycin is found to depress the rate of DNA synthesis without affecting any processes preceding the onset of the synthesis. Secondly, the stage-dependent reduction of DNA-synthetic rate may be partly due to the composition of synchronous population treated, since synchronous population contains more and more DNA-synthesizing cells with the progress through the Gl period, as seen in Fig. 3. Nevertheless, the underlying fact would be that the Gl phase is more sensitive than the S phase in terms of the rate of DNA synthesis.

**Effect of Mitomycin on Cell Proliferation**

The synchronous cells treated with Mitomycin were followed until the third post-treatment generation by means of the time-lapse cinematography. Fig. 7 illustrates observations on the first post-treatment cell division of the synchronous cells. The cells were treated pulsewise at various stages in the first cycle and its result indicated that practically all of the cells could complete the first generation by successful divisions, no matter when the antibiotic treatment was applied in the cell cycle but the extent

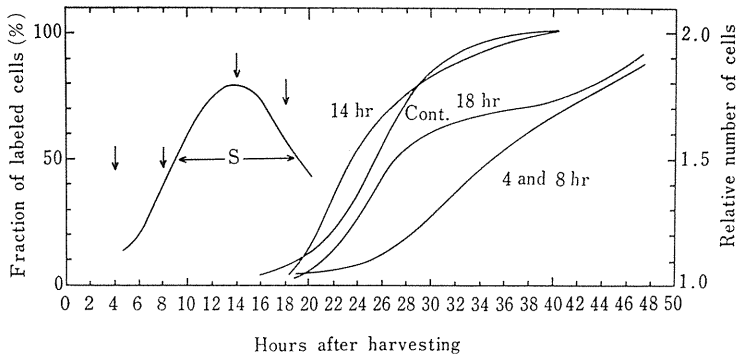


Fig. 7. Cell division of Mitomycin-treated synchronous HeLa S3 cells

Divisions were examined for the first post-treatment division by using time-lapse cinematography. Solid line designated by S represents the fraction of labeled cells copied from Fig. 3. Arrows indicate the time of Mitomycin treatment (0.5 $\mu$g/ml, 1 hr).

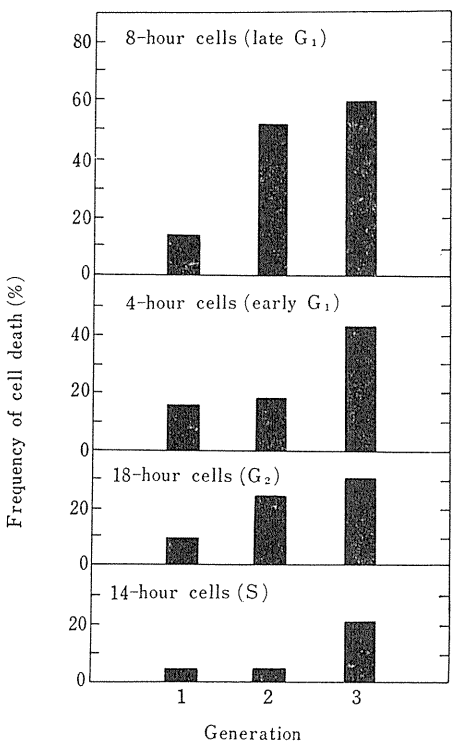


Fig. 8. Frequency of cell death in successive generations of Mitomycin-treated synchronous HeLa S3 cells

The treatment was made at different period of the first generation. The frequency was obtained by dividing the number of cells that died by the number of cells which divided and were able to follow during a given generation. The term "generation" is defined as the duration from the beginning of Gl to the end of the next mitosis.

of division delay varied with different stages. The delay of cell division was pronounced in Gl and G2 cells, while S cells could divide quite normally. The presence of 18-hr cells seemed to be rather complicated by an involvement of two different fractions, i.e., the one approximately 60% of the population which could divide normally, and

the other which began to divide slowly when the relative cell number reached around 1.6. It would be reasonable to assume that the former would represent the S-cell fraction, since roughly 60% of the cells were labeled at 18 hr. The other corresponds to G2 cells or possibly a mixture of G2, M, and G1 cells in the next cell cycle. Therefore, G2 cells may be assumed to be as much sensitive as G1 cells. As far as the first post-treatment divisions are concerned, the sensitivity pattern during the cycle was almost similar to that for the killing effect shown in Fig. 3.

Another analysis was done to see the incidence of cell death after the antibiotic treatment. As seen in Fig. 8, the frequency of cell death was minimum at the first generation in which the antibiotic was applied. However, it generally increased with successive generations of the cells treated at four different stages. This suggests that the damage given by Mitomycin is transmissible through generations and becomes lethal with time, presumably by cell division. The least sensitive was 14-hr cells, and the general sensitivity pattern shown in Fig. 3 was also confirmed by this analysis.

## Discussion

The exposure of asynchronous cells to varying doses of Mitomycin revealed a particular shape of survival curve. Further experiments carried out with synchronous cells permitted us to recognize two different types of elementary responses. Hence, it is hypothesized that two different mechanisms operate alternately at different stages in the cell cycle. As already suggested above, let us assume that the cells in the S period show a sigmoidal response with a broad shoulder, while the cells in other stages simply follow an exponential inactivation, and that the parameters are assigned to both components as follows:

| | $n$ | $D_0(\mu g/ml)$ |
|---|---|---|
| Exponential component (curve A) | 1.0 | 0.05 |
| Sigmoidal component (cuvre B) | 4.5 | 0.20 |

where $n$ is the extrapolation number and $D_0$ is the mean lethal dose. A composite survival curve could be constructed like curve C in Fig. 9, which is for asynchronous population containing approximately 50% of S cells as normally found. Similarly, curves D and E in Fig. 9 represent 14- and 4-hr synchronous cells, respectively. Thus, the particular curves we have observed may be interpreted under the foregoing presumption, although appreciable difference is seen between curve E and the survival curve of 4-hr cells. This might be solved by introducing some other parameters.

With respect to the primary site of the antibiotic action, many of the past studies have shown that the antibiotic causes severe fragmentation,[9] depolymerization,[10,11] and irreversible cross-linking of DNA in the cells.[5,19,20] Accordingly, the selective inhibition of cellular DNA synthesis by this antibiotic[7,12] may be interpreted as a secondary effect which is derived from molecular lesions induced in DNA. In the present study, we are particularly concerned with the effect of the anitibiotic treatment given at various stages on DNA synthesis. The important finding is that the damage inflicted at G1 phase induces more inhibitory effect on DNA synthesis than the one inflicted at S phase. This implies that a primary molecular damage responsible for the synthesis inhibition can develop as the rest of G1 phase progresses. On the other hand, the cinematographic analysis indicated that the damage responsible for cell death was

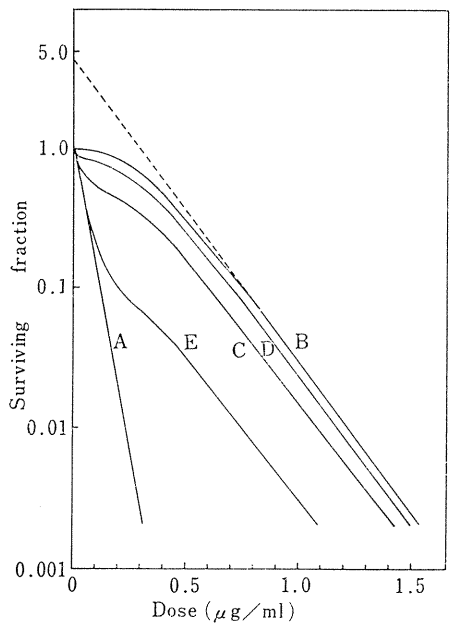


Fig. 9. Survival curves assumed by population heterogeneity hypothesis for synchronous and asynchronous population of HeLa S3 cells

A: Sensitive part in the cell cycle, B: resistant part in the cell cycle, C: asynchronous cells and synchronous 18-hr cells, D: synchronous 14-hr cells, E: synchronous 4-hr cells.

transmissible through the post-treatment generations. This strongly suggests that the damage induced by the antibiotic has a close association with genetic elements of the cells. Thus, the molecular lesions inflicted in DNA would be most suspected as the main reason for cell death. It would be highly probable that a depression of DNA synthesis or its consequence finally results in the loss of a proliferative capacity of the cell.[4,17] In this context, sensitivity change dependent on the cell cycle will be discussed as follows. The pattern of survival response to Mitomycin during the cell cycle is not entirely consistent with that for other antibiotics[2,6,18] and for X-rays.[13,15] The specific feature of the present pattern is the loss of a resistant peak in the G1 period. The possible interpretation of the pattern will be mentioned below.

1) The DNA synthesis is important for the survival, since the repair of induced damage may require DNA synthesis. Thus, the rough coincidence found between the survival peak and the peak of DNA-synthetic activity can be understood. This interpretation would be valid if it is assumed that a damage fixed in G1 phase or chromosome type of genetic damage is not repairable. At this moment, one can recall that the damage given at the G1 phase is greater than that at the S phase with respect to DNA synthesis (Fig. 6). If the injury or abnormality in DNA synthesis caused by Mitomycin is somehow correlated with loss of proliferative capacity, the observed pattern would be successfully explained.

2) The amount of DNA per cell is related to the survival. The amount remains constant during the G1 period and then increases until the cell acquires its genetic twoness at some time in the latter portion of the S period. On this basis, a higher level of survival should be expected as the DNA synthesis proceeds. However, as a matter of fact, the survivals found after 18 hr are reduced. Thus, to explain this, one should make the

assumption that the G2 phase is intrinsically more sensitive than the S phase. At present, a simple hypothesis is not possible.

Based on the above hypothesis, DNA molecule may be duly regarded as one of the most probable targets for the lethal effect of Mitomycin. However, a possible involvement of damages that occur in other cellular structure, e.g., mitotic apparatus or cellular membrane,[8] should not be neglected. The complete understanding of the cell cycle dependence of sensitivity to exogenous agents obviously requires further extensive investigations. The accumulations of this kind of informations would be particularly useful for studies on the inactivation of proliferative capacity of cells.

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# — COMMUNICATION —

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UDC 615.33[Bleomycin] :578.085.23

## LETHAL EFFECT OF BLEOMYCIN ON CULTURED MOUSE L CELLS: COMPARISON BETWEEN FRACTIONATED AND CONTINUOUS TREATMENT

Recently, the effect of Bleomycin-$A_5$ on the proliferative capacity of cultured mammalian cells was studied extensively by Terasima and his colleagues.[2,3] Their results have provided us a new aspect concerning its clinical application.

Experiments were carried out with mouse L cells in F10 medium supplemented with 5% calf serum and 0.05% heart infusion broth (Difco, U.S.A.). Plating for colony counts was carried out with 60-mm plastic petri dishes (Falcon Plastics, U.S.A.). Control and Bleomycin-treated plates prepared from trypsinized single cells were incubated in a $CO_2$-chamber at 37° for 12 to 14 days. Survival was determined by counting the number of colonies that developed in triplicate dishes.

L cells were exposed to 5 $\mu$g/ml Bleomycin as a function of time. The time–inactivation curve obtained exhibited an upward concavity[1,2] (closed circles in Fig. 1). The curve consisted of the initial sensitive and the final resistant components, the inflexion point being at 15 min after introduction of the antibiotic. As shown in other papers,[2,4] the analysis of this biphasic survival response revealed the following: (1) Bleomycin kills the cells exponentially and then induces resistance as the duration of exposure to the antibiotic increases, and (2) the induced resistance disappeared within 4 hr after the antibiotic was removed. Based on these findings, it would be expected that the adequately fractionated treatment is much more efficient to sterilize the cells than a continuous treatment by resulting in a simple exponential survival curve.

Then, L cell cultures were treated repeatedly with 60-min pulse of 5 $\mu$g/ml of Bleomycin at intervals of 5 hr. Ten fractionations yielded a simple one-hit type of inactivation (open circles in Fig. 1). This result was in good agreement with the one expected from the analysis.

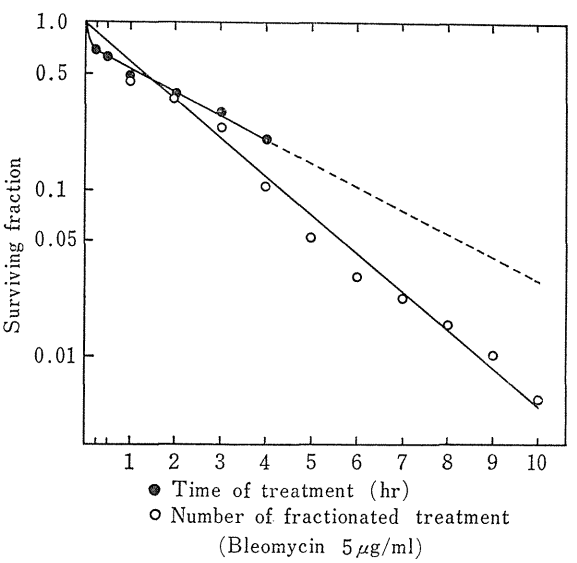


Fig. 1. Comparison of surviving fraction between fractionated and continuous treatment



GANN 63, 645-646, 1972

The model treatment shown here may provide some useful idea for clinical application of Bleomycin.

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*First Department of Medicine,*
*Chiba University School of Medicine,*
*Inohana, Chiba 280*

Yosinobu TAKABE (高部吉庸)
Takesi KATSUMATA (勝俣剛志)
Michinori WATANABE (渡辺道典)

*Division of Physiology and Pathology,*
*National Institute of Radiological Sciences,*
*Anagawa, Chiba 280*

Toyozo TERASIMA (寺島東洋三)

# Effect of Bleomycin on Mammalian Cell Survival[1,2]

T. Terasima, Y. Takabe,[3] T. Katsumata,[3] M. Watanabe,[3] *and* H. Umezawa,[4,5] *Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, Chiba, Japan*

SUMMARY—The survival response of cultured mammalian cells treated with bleomycin was studied. The dose-response curves of 4 cell lines of different origin exhibited an upward concavity. The true sensitivity of cells, shown by the initial slope, appeared to be slightly different among cell lines, whereas the final slope did not differ appreciably. The fraction of the terminal portion ranged from 30–60% of the population, depending on the cell lines. The inactivation of the mouse L cells, determined as a function of time of the treatment, was biphasic, with the inflection point during the first 60 minutes. The inactivation constant of the terminal resistant portion increased with greater concentration of bleomycin, though not directly proportional to the increase. The interpretation of the biphasic survival response was that bleomycin inactivated cells exponentially and then induced the resistance of cells as the time of incubation elapsed. This was evidenced by a two-dose fractionation experiment in which the induced resistance disappeared within 2–4 hours after the antibiotic was removed. It was concluded that bleomycin not only exerts a lethal effect on but also induces resistance in mammalian cells. From these findings, a chemotherapeutic scheme of bleomycin was proposed.—J Natl Cancer Inst 49: 1093–1100, 1972.

SINCE BLEOMYCIN was discovered by Umezawa et al. (*1*, *2*), actions of this antibiotic on various properties of mammalian cells have been studied extensively (*3–7*).

The quantitative determination of survival response in cultured mammalian cells should provide essential chemotherapeutic information.

Umezawa and colleagues (*8*, *9*) reported the selective distribution of this compound in the body; i.e., higher concentrations were found in the skin and lung than in bone marrow, liver, intestine, and other organs. Furthermore, better clinical results were obtained with squamous cell carcinomas in the skin, lung, and mucous mem-

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[2] Work supported by special project grant for development of bleomycin from the Science and Technology Agency.

[3] First Department of Medicine, Chiba University School of Medicine, Inohana, Chiba.

[4] Institute of Microbial Chemistry, Kamiosaki 3–14, Shinagawa-ku, Tokyo.

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brane (*10*, *11*). These particular findings might be attributed to a differential distribution of a bleomycin-inactivating enzyme among tissues (*9*). Accordingly, cells of different origin may have different sensitivity to the antibiotic.

This communication concerns the sensitivity measurement of several cultured mammalian cells and a kinetic study of survival response. From these results, some principles of bleomycin chemotherapy are outlined

## MATERIALS AND METHODS

*Cell lines and their growth properties.*—A clone of the mouse L cells (B929-L2J), designated "L5 strain," was used in most experiments. The cells were grown in F10HI medium (*12*) supplemented with 5% calf serum. On plastic culture dishes (Falcon Plastics, Los Angeles, Calif.) the plating efficiency was normally 60–100%. The growth properties of asynchronous and synchronous populations were described in (*13*). Melanoma cells, B16-XI strain, were isolated by Terasima and Tanaka from the transplantable mouse melanoma B16, provided by Dr. T. Kasuga of this Institute. The culture produced slightly brown pigment and normally grew with a median generation time of 23 hours. The plating efficiency was 30–80%. P388 mouse leukemia cells were provided by Dr. H. Ohara of this Laboratory. The cells attached weakly to the surface of plastic culture dishes, and the doubling time was about 30 hours. Vero cells were isolated by Dr. Y. Yasumura, Chiba University, from a green monkey kidney. A near-diploid number of chromosomes has been maintained up to the present (H. Ohara, personal communication). The plating efficiency was 40–80%. The growth rate was not determined. The growth medium of these cell lines, except for the L5 strain, was F10 medium supplemented with 10% calf serum.

*Preparation of synchronous culture.*—The method, including the harvest of mitoses by shaking, was detailed previously (*13*, *14*). In the present studies, only L5 cultures were subjected to this procedure.

*Preparation of cultures.*—The cells growing actively in a monolayer were dispersed with 0.1% trypsin (1:250, Difco, Detroit, Mich.)-saline D2 (*15*). The suspended cells were plated out in plastic dishes (60×15 mm), after they were counted with a hemocytometer, and diluted appropriately with growth medium. Dishes were then incubated at 37° C in a $CO_2$ chamber constantly supplied with a humidified 5% $CO_2$-air mixture. At 4–5 hours after incubation, individual cells were attached firmly to the bottom of the dish and a few were ready to divide. Cultures at this stage were treated with bleomycin.

*Treatment of cells with bleomycin.*—At treatment, the growth medium in the dishes was replaced with medium containing bleomycin that had been prewarmed in a $CO_2$ chamber. Dishes were then incubated for periods ranging from 15 minutes to 4 hours. All procedures were done in a room maintained at 37° C. When the treatment terminated, the cultures were removed from the chamber, rinsed 3 times with F10 mixture (*12*), and fed the growth medium. The rinsing step was performed in a sterile room, at 20° C, and precautions were taken to prevent contamination. In the two-dose fractionation experiment, however, cultures were rinsed at 37° C only after the first treatment, to keep them at a constant temperature during the interval between fractionated treatments. This was necessary for exact determination of a change in the response of cells after the first treatment.

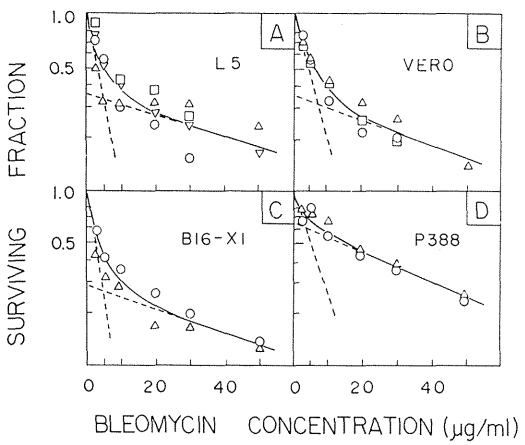
*Assay of survival.*—Cultures from which bleomycin was removed were incubated in a $CO_2$ chamber for 12–14 days to develop survivors. Colonies developing in triplicate dishes were counted to determine survival.

*Antibiotic.*—Bleomycin A5, a copper-free compound (Lots 00702 and 6), was kindly supplied by Nippon Kayaku Co., Ltd. The compound was dissolved in F10 mixture and refrigerated until use.

## RESULTS

### Survival Response of Various Mammalian Cells to Bleomycin

Four mammalian cell lines of different origin were treated with a 60-minute pulse of varying concentrations of the antibiotic, and survival fractions were measured as a function of the drug concentration (text-fig. 1). All these dose-response curves demonstrated the exponentially inactivating, initial portion followed by the less sensitive final portion. Therefore, an upward concavity was




TEXT-FIGURE 1.—Dose-response curve of mammalian cell lines. Survivals were assayed by exposure of single cells to bleomycin at varying concentrations for 60 minutes and by their incubation for clonal growth. Symbols denote different experiments.

manifested with the inflection point at the region of 5–10 μg/ml. To compare the response of cell lines, the curves were resolved into 2 broken lines, shown in text-figure 1, which were approximated by eye to the initial and final slopes; the sensitivity was determined in terms of the mean lethal dose ($D_0$), defined as the concentration needed to yield 37% survival in the exponential region of survival curve. The values obtained are shown in table 1. The initial slope appeared to differ, depending on the cell line, though the exactness was extremely limited because of the paucity of points on which broken lines were based; B16-XI and L5 cells proved more sensitive than the other 2 cell lines. In contrast, the final slopes were approximately similar. The values were within the range of 50–60 μg/ml. Notable was a fraction of tail portion, as measured by extrapolation of the final slope to the ordinate. The rough estimates obtained were 30% for B16-XI, L5, and vero cells and 60% for P388 cells. These findings appear to indicate that the population is composed of fractions of different sensitivity.

With L5 cells, the inactivation at several different concentrations of the drug was determined as a function of time (*see* text-fig. 2, the time-inactivation curve). Here again, the 2 components were demonstrated in all the inactivation curves. The final slopes appeared within 60 minutes after any concentration of bleomycin treatment and manifested different rates of inactivation, depending on the concentration of the antibiotic.

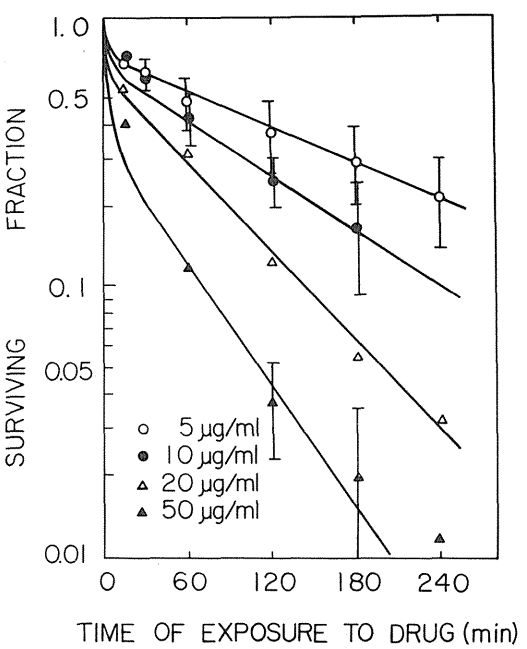
## Survival Response After Fractionated Treatment

To interpret the upward concavity of the time-inactivation curve, the possibility of thermal or metabolic breakdown of the antibiotic was con-

TABLE 1.—*Bleomycin-survival parameters of 4 mammalian cell lines*

| Cell lines | $D_0$ dose* (μg/ml) | |
|---|---|---|
| | Initial slope | Final slope |
| L5 | 4. 5 | 63 |
| B16-XI | 3. 7 | 62 |
| Vero | 6. 5 | 56 |
| P388 | 7. 5 | 50 |

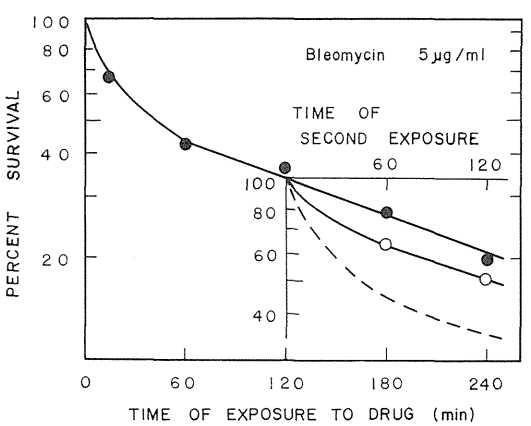
*Defined in text.




TEXT-FIGURE 2.—Time-inactivation curve of L5 cells. Survivals were determined as a function of time of the treatment with 4 different concentrations of bleomycin. Limit shown is the standard deviation of the mean obtained from repeated experiments.

sidered, but was rejected because the terminal slope for L5 cells remained unchanged, even when the bleomycin-containing medium was replaced with fresh medium after the first 60-minute treatment. Therefore, the curve strongly suggests that cells become less sensitive to bleomycin as the time of treatment elapses. Thus we hypothesized that the terminal slope resulted from an induction of partial resistance by bleomycin, which disappeared when bleomycin was removed. This was studied in the two-dose fractionation experiment, showing that the first treatment induced resistance, but the second treatment, given at intervals, only fixed the extent of resistance, which then gradually disappeared without bleomycin.

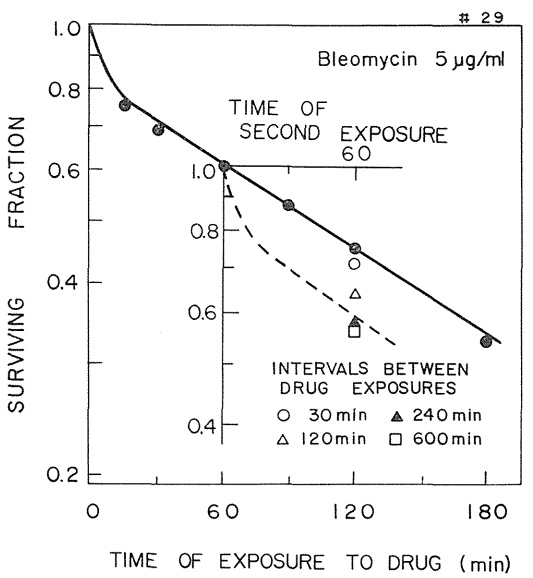
Text-figure 3 shows one result of the fractionation experiment, in which L5 cells were treated with 5 μg/ml bleomycin. As in text-figure 2, the rapid initial inactivation was followed by a gradual development of less sensitivity, which finally resulted in a slow terminal slope. At 120 minutes of treatment, the antibiotic was removed from the portion of the cultures and the incubation was continued with ordinary growth medium for another 60 minutes. The cultures were then examined for survival response to a second treatment of 60 and 120 minutes at the same concentration. The inset demonstrates the reduction of survival level. It indicates that the resistance induced by the first treatment disappeared when the antibiotic was removed. However, the disappearance was not complete after 60-minute incubation, since reversion to the original sensitivity requires the reduction of survival level to the broken line.



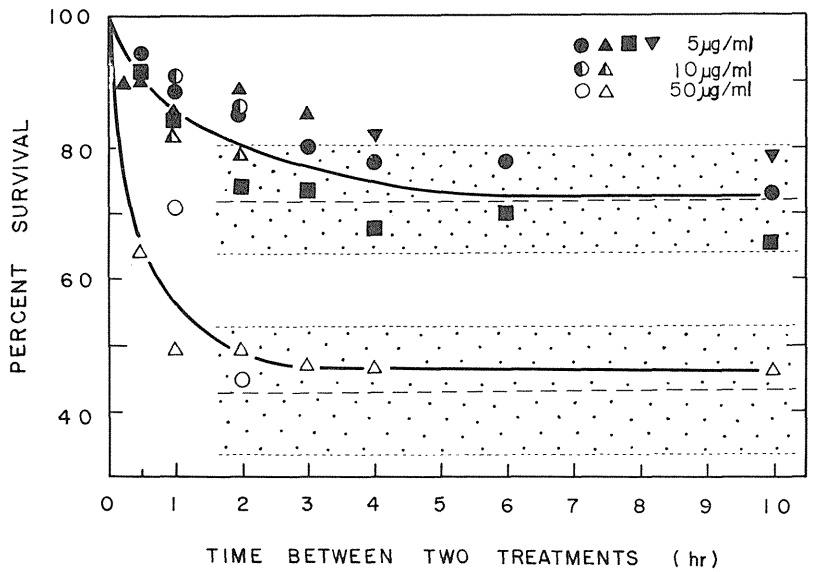

TEXT-FIGURE 3.—Change in survival response after removal of bleomycin. *Closed symbols* represent the time-inactivation curve of L5 cells at 5 μg/ml bleomycin. *Open symbols* represent the time-inactivation curve obtained with cultures exposed to bleomycin for 120 minutes, rinsed to remove the antibiotic, and then incubated for 60 minutes in ordinary growth medium. *Broken line* in *inset* illustrates the survival response of the original culture.

Text-figure 4 shows survivals obtained after two 60-minute fractionated treatments with 5 μg/ml bleomycin given at intervals of 30 minutes to 10 hours. As a whole, the loss of resistance appeared to be related to length of the interval between treatments. Survivals obtained after an interval of 4 hours or more stayed practically at the level of a full reversion.

To determine the temporal change and the extent of loss of resistance, the fractionation experiment with two 60-minute treatments (*see* text-fig. 4) was repeated with 3 different concentrations of bleomycin. Survival values obtained from these experiments are shown (text-fig. 5) in terms of per-




TEXT-FIGURE 4.—Survival change in the two-dose fractionation experiment. *Closed circles* represent the time-inactivation curve of L5 cells treated with 5 μg/ml bleomycin. Symbols show survivals after two 60-minute treatments given at various intervals.




TEXT-FIGURE 5.—Loss of resistance after removal of bleomycin. Survivals of L5 cells were determined after two 60-minute treatments given at various intervals and normalized to the survival obtained after the straight treatment of 120 minutes (i.e., 0-minute interval). Symbols denote different experiments. *Broken line with shaded area* represents the mean survival value and its standard deviation expected when the resistance induced by the first dose disappeared entirely.

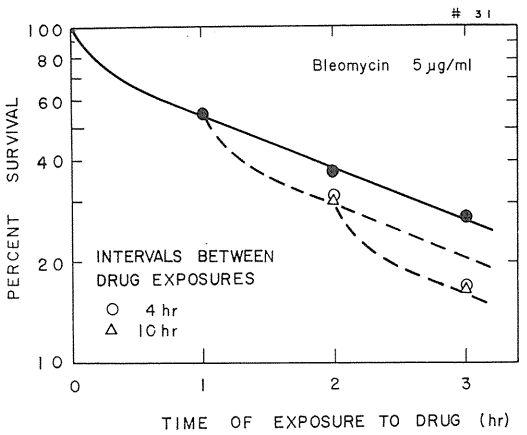
centage of the survival, which was obtained after the straight treatment for 120 minutes, or at 0-minute interval, in each experiment. Data from 2 experiments at 10 μg/ml concentration were combined with those obtained at 5 μg/ml, since they did not differ appreciably. Text-figure 5 indicates that the relative survival was reduced rapidly soon after bleomycin was removed, leveled off at 2 hours for 50 μg/ml bleomycin, at 4 hours for 5 μg/ml, and, ultimately, stayed constant approximately at the level of the original sensitivity. It was concluded that the reversion completed at 2–4 hours after bleomycin was removed. Whether the observed difference in the loss of resistance time between 2 different concentrations was significant could not be definitely determined.

### Repeatability of Induction and Loss of Resistance

To ascertain whether the induction and the loss of resistance to bleomycin were repeatable, the fractionated treatment with bleomycin of 60 minutes each at 5 μg/ml was given 3 times successively at 4- and 10-hour intervals. Survivals obtained after these treatments are shown in text-figure 6. They were found approximately at the levels which would be expected when a full reversion in sensitivity occurred during the intervals. Therefore, it was confirmed that the cellular response to bleomycin was repeatable at appropriate intervals. This finding might be of great importance in relation to the therapeutic design of bleomycin, as discussed later.

## DISCUSSION

Recently the biphasic inactivation of mammalian cells by bleomycin was reported by Barranco and Humphrey (*7*). Their findings relating to the survival response of Chinese hamster cells were essentially like ours, though an exact correlation cannot be made because of differences in




TEXT-FIGURE 6.—Multiple-dose fractionation experiment. *Closed symbols* denote the time-inactivation curve of L5 cells at 5 μg/ml bleomycin. *Open symbols* show survivals obtained after repeated 60-minute treatments given at 4- and 10-hour intervals.

experimental conditions. Their studies were directed mainly to effects of the antibiotic on the cell cycle events, whereas our studies especially concerned the kinetics of the lethal effect.

The upward concavity of the dose-response curve cannot readily be interpreted. In view of the extremely high frequency of resistant cells, generally true among cell lines, it is unlikely that the resistance is genetically determined. Incidentally, the same shape of curve was obtained from survival response of a clonal L5 culture isolated from a survival on the 50 μg/ml dish. A remaining possibility is the involvement of differential sensitivity during the cell cycle. However, the survival curve obtained with the synchronous G1 population (at 3.5 hours after mitosis) of L5 cells also exhibited the same upward-concave shape, not permitting a simple allotment of the two components to specific cell stages. Additional supportive evidence is that any appreciable inflection was not recognized when the composite dose-response curve was calculated by sensitivities measured at each stage of the cell cycle (unpublished). Hence the assumption that the heterogeneity of sensitivity in L5 culture is predetermined either genetically or physiologically was not justifiable. From analyses of the time-inactivation curve, the resistant terminal portion of the dose-response curve is attributable to the rapid induction of resistance by the antibiotic (*see* text-fig. 2).

For the present, the induction hypothesis fits most of the kinetic data here. Based on the hypothesis, our findings were: 1) When bleomycin was added to the culture, cells were killed rather rapidly. In the meantime, the resistance of cells was induced gradually and was established at 30–60 minutes. They remained resistant while the antibiotic was in the growth medium. Data presented in text-figure 2 indicate that the resistance was not absolute and cells in this state were inactivated at a slow and definite rate, depending on the concentration of the antibiotic. 2) As soon as the antibiotic was removed, loss of sensitivity occurred quickly and then slowed down; finally, the original sensitivity, together with the capability of cells to develop resistance, was acquired again within 2–4 hours.

The sensitization after the removal of the antibiotic may be due to a partial synchrony developed after a selective killing of the population. However, this seems unlikely, since no oscillatory change in survival was found during a 10-hour incubation period (*see* text-fig. 5).

No material basis has yet been found for the nature of resistance induced. In our studies regarding the effect of bleomycin on macromolecular syntheses of L5 cells, which will be reported elsewhere, the inactivation of DNA and protein syntheses by bleomycin also showed an upward-concave curve. This type of response led us to assume that some sort of barrier in the cell against bleomycin is formed by its addition to the medium.

Umezawa and colleagues demonstrated the bleomycin-inactivating enzyme in various tissues of mice (*9*). Accordingly, different cultured mammalian cells might have this inactivating activity. Development of the resistance would be understood if the inactivating activity were produced or released in the presence of bleomycin.

If we assume that most biologic effects of bleomycin are derived from the primary damage in cellular DNA, the resistance probably is based on the action of DNA polymerase which can repair DNA damage. In particular, single strand breaks of DNA produced by the antibiotic were quickly repaired (*5*).

That bleomycin induces an alteration in permeability of cells is unlikely. Such evidence will be reported in another communication.

Terasima and Umezawa (*6*) made some quantitative determinations of the lethal effect of bleomycin on HeLa S3 and mouse L cells. The rough estimation of $D_0$ dose, made collectively on the initial and terminal portions of the dose-response curve, was 45 and 12 μg/ml, being fairly large as compared to those obtained for other antitumor agents. In view of cancer chemotherapy based on a sterilization of tumor cells, the effect seemed moderate or rather weak. However, the present results pointed out that the true lethal effect is shown exclusively by the early portion of the curve, though the exact measurement cannot be made because of the development of resistance. As to the initial portion of the curves, two points were noted. 1) The $D_0$ values measured with 4 cell lines seemed slightly different from each other, ranging from 4–8 μg/ml (*see* table 1). Cells derived from blood and kidney may be somewhat less sensitive than melanoma cells. However, much more information is necessary regarding the sensitivity of cells from different origins before any definite conclusion can be reached. 2) Taking advantage of the early portion of the response would be most effective for treatment with bleomycin, and such emphasis will be realized only by a fractionation scheme of treatment. This is discussed more fully.

The present results provide much information with which some cellular basis of bleomycin chemotherapy can be constructed. To the first approximation, the inactivation curve (text-fig. 2) would be described by the following exponential function:

$$S=\Sigma Ci \cdot e^{-ki \cdot t} \qquad [1]$$

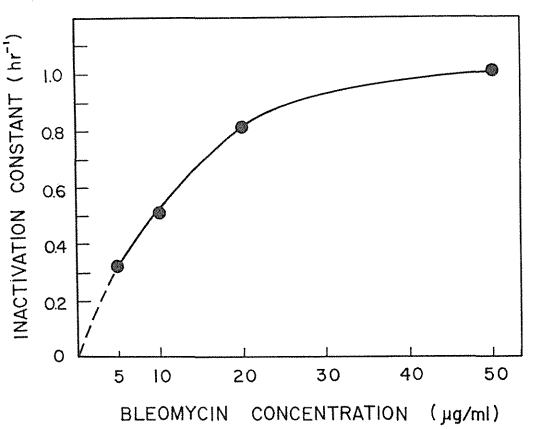
where $Ci$ is a fraction of initial and terminal components. The fraction of the latter is given by extrapolation of the terminal slope to the ordinate. Respective inactivation constant of components is denoted by $ki$.

Let us assume that the level of the antibiotic in the tumor can be kept constant for 60 minutes after a single administration. Then, with continuous treatment by consecutive administrations of the antibiotic every 60 minutes, a total amount of antibiotic which assures 90% cure of tumor can be estimated by equation [1]. As far as continuous treatment is concerned, the final slope is more important than the initial slope in determining the total amount. Text-figure 7 illustrates the inactivation constant of final slopes against the concentration of bleomycin—obtained from data in text-figure 2. Since the relation is not linear, lower concentrations would result in smaller total doses to effect the same result; if a tumor contains $10^8$ cells, the continuous treatment with the tumor dose of 50 μg/ml for a single administration needs 18.1 hours or a total of 910 μg, whereas the tumor dose of 5 μg/ml can sterilize tumor cells with 317 μg.

Secondly, we understand immediately from the time-inactivation curve that the continuous treatment is not profitable in relation to the total amount required, simply because the resistance once induced would never have a chance to disappear. If the fractionation scheme is followed at the interval which permits a complete loss of resistance, the survival will be given by

$$S_{\text{fraction}}=(S_{\text{t=60 minutes}})^{\nu} \qquad [2]$$

where $\nu$ is a number of fractions of treatment. Again, when the same tumor is treated with the tumor dose of 5 μg/ml on the above fractionation scheme, the total amount calculated from equation [2] is 168 μg; i.e., approximately 50% of the dose required for the continuous treatment. Encouraging




TEXT-FIGURE 7.—Relationship between the terminal slope in the time-inactivation curve of L5 cells and bleomycin concentration. The values were obtained from data in text-figure 2.

evidence was recently available from the experimental therapy of transplantable mammary carcinoma of C3H mice. The result demonstrated that growth of the tumor was much more affected in fractions than in a single administration of the same total dose (M. Urano, NIRS, personal communication). The clinical results thus far obtained suggest that the use of more than 300 mg tends to produce certain side effects (*10*). Therefore, the foregoing arguments for reducing a total amount may be useful for tumor therapy of bleomycin.

Finally, the elucidation of the nature of the resistance is important for a successful clinical application of the antibiotic, since a control of the induction process might offer an advantage to a differential sterilization between normal and malignant tissues.

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# Basic Findings in Bleomycin Action

By *T. Terasima*

In 1962, *Umezawa* and his colleagues successfully isolated an antibiotic, designated as bleomycin, from the culture filtrates of Streptomyces verticillus [1]. The antibiotic was differentiated from a previous antibiotic phleomycin, in the lack of renal toxicity. It was separated into bleomycins A1-A6, $A'_2$-a, $A'_2$-b, B1-B6. Among them bleomycin A2, which is the major component of the bleomycin clinically employed, and A5 have been studied most in detail.

The results of studies on toxicity and distribution by *Umezawa's* group revealed the preferential localization of bleomycin in skin and lung tissues and its more rapid inactivation in other tissues [2]. This might explain the rather late toxic effects observed in experimental animals and men, such as depilation, sclerosis of skin and fibrosis of lung. Recent data obtained with mice bearing squamous cell carcinoma or sarcoma, induced by 20-methylcholanthrene, demonstrated that bleomycin in the active form was localized in squamous cell carcinoma as well as in skin in higher concentration than in sarcoma.

These experimental results were in a good agreement with clinical results thus far observed. *Ichikawa et al.* [3] found that squamous cell carcinomas developed in skin, lung and genital regions were sensitive. Approximately similar results were reported by the Clinical Screening Group of EORTC [4]. There are some promising reports that *Hodgkin*'s disease responds to bleomycin. The mechanism of this selective effect on *Hodgkin*'s disease may be different from that against squamous cell carcinoma.

## Actions of Bleomycin on Mammalian Cells

The effect of bleomycin on macromolecular syntheses of cultured mammalian cells was first studied by *Kunimoto et al.* [2]. Extensive determinations of the effect in the mouse L cells were carried out by *Watanabe et al.* (in preparation). The results revealed that the rate of DNA synthesis was most strongly affected whereas those of protein and RNA syntheses were less affected in this order. The shape of these dose-response curves was quite notable, indicating that the reduction of synthetic rates per unit increment of the concentration was much greater in the low concentration range (less than 10 $\mu$g/ml) than in the high concentration range. The interpretation will be given when the survival response is discussed later. Such overall inhibition of syntheses may be attributed to the remarkable effect in the DNA molecule.

*Nagai et al.* [5] reported that the melting temperature of calf thymus DNA was lowered appreciably when DNA molecules were incubated with bleomycin in the presence of SH compounds. In relation to this effect, the prominent molecular change in DNA induced by bleomycin was disclosed by ultracentrifugation studies. The sedi-



LEUKAEMIEN UND MALIGNE LYMPHOME (Edited by A. Stacher) pp. 29-33,
Urban & Schwarzenberg, 1972

mentation profile of DNA released from the mouse L cells by the SDS method clearly indicated that bleomycin broke cellular DNA into short pieces of polynucleotide chains [2, 6]. Recently, *Yamaki et al.* [7] reported that actions of exo-type DNase and DNA-polymerase were apparently stimulated by A2 compound. As they emphasize, it is very likely that the inhibition of DNA synthesis results from a breakdown of template DNA which is caused by the endonucleolytic action of the antibiotic and by subsequent lysis with DNase.

Regarding the effects on the growth of mammalian cells, several studies have been reported [2, 8, 9]. The most extensive work on the proliferation of cultured mammalian cells has been carried out by *Terasima* and his colleagues. A few aspects would be appropriate to mention in this communication.

When the fate of bleomycin-treated mouse L cells was followed by time-lapse cinematography, the incidence of cell death at the first post-treatment generation was low to moderate although dependent on the concentration of bleomycin. Contrarily, cells were killed most frequently at the second (or probably later) post-treatment generation (s). Therefore, it appears that the potential damage produced at the first generation must be transmitted through mitosis before it becomes lethal.

The lethal effect on the proliferative capacity of mammalian cells was determined by counting the number of survival colonies developed in culture dishes. Fig. 1 shows the dose-survival relation of four cultured mammalian cells. The shape of the survival curves was all upward-concave, as found in the case of macromolecular syntheses, and has never been observed for other antibiotics. Such inactivation curves would not immediately give us any promissing idea about a sterilization of cancer cells. This point will be solved in the next section.

The time-survival relation obtained with several fixed concentrations of bleomycin A5 against mouse L cells was illustrated in Fig. 2. The curve again showed the upward-concavity which was formed with the steep initial and the slow terminal portions. We examined a few possibilities for the observed heterogeneity in sensitivity, and finally reached the conclusion that bleomycin inactivates cells exponentially and, then, induces a partial resistance of mammalian cells as shown by the terminal slope. The evidence was provided by the two-dose fractionation experiments. Cultures ex-

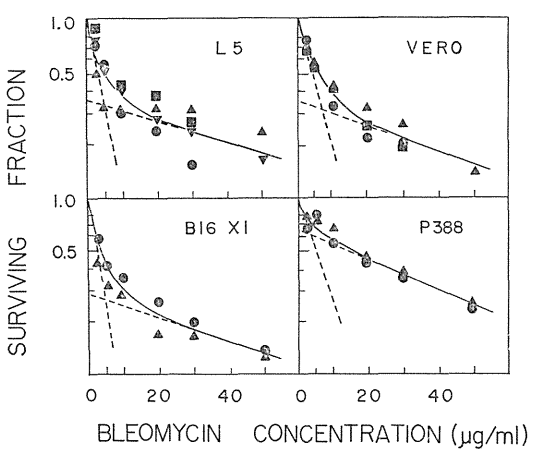


Fig. 1. Dose survival curve of cultured mammalian cells. Survivals were determined by counting number of colonies developed in culture dishes after 60 min-pulse treatment of single cells and subsequent incubation. L5: a clone of mouse L cells (fibroblastic origin). B16-XI: a clone of melanoma cells derived from the transplantable mouse melanoma B16. Vero: a diploid culture derived from a green monkey kidney. P388: a culture of mouse lymphatic leukemia.

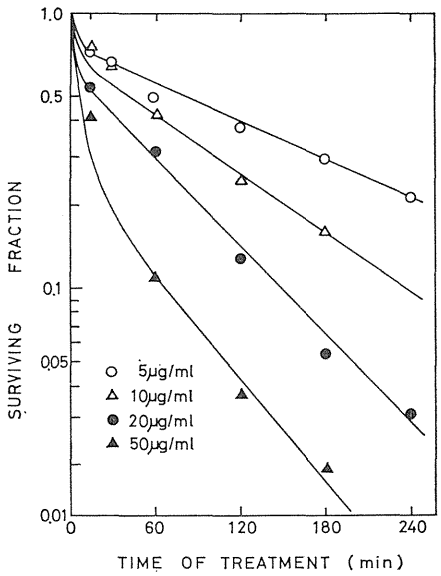


Fig. 2. Time-survival curve of L5 cells.

posed to the first dose were rinsed to remove the antibiotic at the time when the terminal slope had appeared, and incubated further with the ordinary growth medium. At intervals the time-survival curve was examined by re-introducing bleomycin and incubating for desired periods. From repeated experiments of this type, it was found that the resistance induced by the first dose decays with time after the removal of the antibiotic and the survival response ultimately returns to the original level. The decay of the induced resistance was determined as a function of time between two treatments, as shown in Fig. 3. It demonstrated that the original sensitivity was attained about 2 to 4 hr after the removal of bleomycin. Further experiments provided the evidence that the induction of resistance and subsequent decay was repeatable at appropriate intervals.

No material basis for the resistance has yet been found. At this moment, it might be pertinent to mention that the same conclusion was drawn from the kinetic analysis

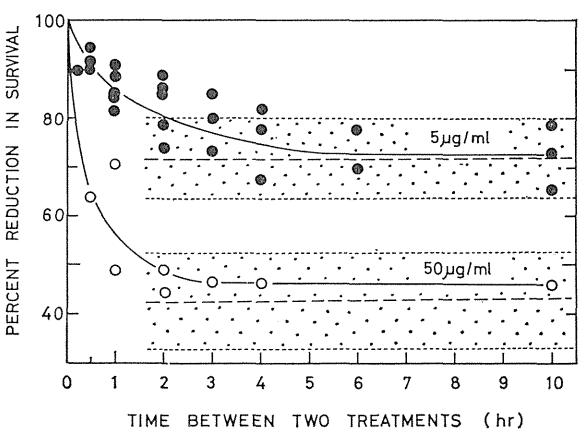


Fig. 3. Decay of resistance after removal of bleomycin. Survivals were determined after two 60-min treatments given at various time intervals and normalized to the value obtained after the straight 120-min treatment (0 min interval). Broken line with shaded band represents the survival value and its standard deviation expected when the resistance induced by the first dose disappeared entirely.

of the bleomycin inactivation of DNA synthesis of L cells *(Watanabe et al.)*. In view of such generality of the response, the involvement of bleomycin-inactivating enzyme seems to be likely. *Umezawa's* group has shown recently that mammalian tissues have the enzyme which can inactivate bleomycin [2]. Accordingly, it is largely possible that such enzyme can be either induced or activated in cultured mammalian cells by bleomycin.

## Cellular Basis of Bleomycin Chemotherapy

The present results might have some relevance to the tumor chemotherapy. To evaluate some therapeutic schemes on the basis of data in Fig. 2, some basic assumptions must be made; 1.) as found in Fig. 2, the tumor cell killing obeys the equation,

$$S \text{ (survival)} = \sum Ci.e^{-ki.t}$$

where Ci is a fraction of the initial and terminal components, ki is each inactivation constant, and these are derived from Fig. 2, 2.) the level of the antibiotic in a tumor is kept constant for 60 min after a single administration. Then, the continuous treatment is effected by consecutive administrations of the antibiotic every 60 min. If a tumor contains $10^8$ cells, a total amount of bleomycin which is needed for 90% cure of tumor, can be calculated. As far as continuous treatment is concerned, a tumor dose of 50 $\mu$g/ml needs 18 hr or a total of 900 $\mu$g to sterilize the tumor, whereas 5 $\mu$g/ml can kill the same tumor with 300 $\mu$g. This difference is due to the disproportionality between the terminal slope and the antibiotic concentration.

It would obviously be profitable to consider the fractionation scheme by which the disadvantage brought about by a development of resistance will be avoided. If the interval of fractionated treatments is chosen to allow a complete decay of resistance, the survival would be given by $(St = 60 \text{ min})^n$, where n is a number of fractionated treatments. Therefore, the total amount required to cure the same tumor with the dose of 5 $\mu$g/ml will be 160 $\mu$g, approximately 50% of the amount needed for the continuous treatment. The supporting evidence for these considerations are now becoming available from the experimental therapy of transplantable mammary carcinoma of mice (*Urano,* NIRS).

Finally, it should be emphasized that the elucidation of resistance induction is of particular importance in bleomycin chemotherapy, since the control of the induction process may contribute to the strategy for a differential sterilization between normal and malignant tissues.

Present studies carried out in collaboration with Drs. *Y. Takabe, T. Katsumata, M. Watanabe* (Chiba Univ. School of Medicine) and *H. Umezawa* (Inst. Microb. Chem.), were supported by the project grant for Development of Bleomycin from the Science and Technology Agency and the kind gift of Nippon Kayaku Co. Tokyo.

### Summary

Studies on bleomycin actions with mammalian cells revealed several findings. (1) Remarkable action was the strand scission of DNA molecule which possibly underlay the depression of DNA synthesis caused by the antibiotic. (2) The unique survival response of cultured mammalian cells to the antibiotic was observed. The kinetic analysis of the response by the

two-dose fractionation experiment disclosed the interaction between mammalian cells and bleomycin; namely, bleomycin not only inactivates the proliferative capacity of cells but also induces the resistance against bleomycin.

On the basis of the latter finding, some principles were pointed out in relation to the bleomycin chemotherapy.

**Zusammenfassung**

Die Untersuchungen über die Wirkung von Bleomycin auf die Säugetierzellen ergaben verschiedene Befunde: 1.) Eine bemerkenswerte Wirkung war der Strangbruch des DNS-Moleküls, der möglicherweise für die Antibiotikum-bedingte Unterdrückung der DNS-Synthese verantwortlich war. 2.) Es wurden Überlebenskurven von kultivierten Säugetierzellen unter Einfluß des Antibiotikums erstellt. Die kinetischen Analysen der Reaktion beim Zwei-Dosis-Fraktionierungsexperiment ergaben eine Interaktion zwischen den tierischen Zellen und Bleomycin. Bleomycin inaktivierte nicht nur die Proliferationsfähigkeit der Zellen, sondern induzierte auch eine Resistenz gegen sich selbst. Aufgrund des letzteren Befundes wird auf einige Grundsätze im Zusammenhang mit der Bleomycin-Chemotherapie eingegangen.

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[GANN, 64, 71～77; February, 1973]

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# CINEMICROGRAPHIC ANALYSIS OF DEATH IN SYNCHRONOUSLY GROWING MOUSE L CELLS AFTER EXPOSURE TO BLEOMYCIN*1

(Plate VIII)

Takesi KATSUMATA,*2 Michinori WATANABE,*2 Yosinobu TAKABE,*2
Toyozo TERASIMA,*3 and Hamao UMEZAWA*4

(First Department of Medicine, Chiba University School of Medicine,*2 Division of Physiology and Pathology, National Institute of Radiological Sciences,*3 and Institute of Microbial Chemistry*4)

## Synopsis

By using time-lapse cinemicrography, generation time and mode of death in synchronously growing mouse L cells were analyzed after a pulse-treatment with Bleomycin at the $G_1$～S transition phase. Approximately 90% and slightly more than 50% of the cells were able to pass through the first cycle after the treatment with 20 and 100 $\mu$g/ml, respectively. At either concentration, fraction of the cells killed at the first cycle was low although it depended slightly on the concentration of the antibiotic, whereas a large fraction of the cells showed death of their offsprings in the second cycle. This fact indicates that Bleomycin gives the cells a lethal damage most of which becomes apparent only after mitosis.

On the basis of a cumulative frequency distribution of the cell division, two types of damage were considered to be responsible for the prolongation of the first cycle. Greater prolongation was found especially when the cells were killed after mitosis.

Morphological change in the cells observed during the prolonged first cycle was the increase of cross-sectional area and the remarkable granule formation around the nucleus.

## INTRODUCTION

Bleomycin isolated by Umezawa *et al.*[12,13] is an antibiotic which has a significant antitumor activity against squamous cell carcinomas and Hodgkin's disease. Much investigations have been made on the action of this antibiotic on DNA molecules and on macromolecular synthesis.[4,5,7,8,15] On the other hand, only a few studies have been carried out recently on cell killing effect of the antibiotic,[1,9,10] and the fate of Bleomycin-treated individual cells has not been analyzed yet. This report deals with cinematographic analysis of the effect of Bleomycin on the clonal growth of mouse L cells.

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*2 313 Inohana, Chiba 280 (勝俣剛志, 渡辺道典, 高部吉庸).

*3 Anagawa 4-9-1, Chiba 280 (寺島東洋三).

*4 Kamiôsaki 3-14-23, Shinagawa-ku, Tokyo 141 (梅沢浜夫).



GANN 64, 71-77, 1973

## Materials and Methods

**Cells** L5 cells, a clonal derivative of the mouse L cells (B929-L2J), were grown in F10HI medium[2] supplemented with 5% calf serum. The cultures were incubated in a $CO_2$-chamber placed in a room of 37°. The chamber was supplied with a constant flow of humidified 5% $CO_2$-air mixture. The median generation time of the cells was 22 hr and the cell cycle parameters were described previously.[11]

**Preparation of Culture** Synchronous cultures were prepared by the harvesting method which consisted of a selective collection of mitotic cells by mechanical shaking of a bottle in which the cells were grown randomly. Harvested mitotic cells were diluted appropriately with a growth medium and placed in several drops in plastic culture dishes (35 × 10 mm, Falcon Plastics, Calif.). Individual cells attached to the bottom of the dish and were found normally in pairs within a few hours of incubation. After incubation for 4 hr, the growth medium was replenished for further synchronous growth.

**Bleomycin Treatment** Treatment of synchronous cells was carried out 8 hr after mitosis ($G_1 \sim S$ transition phase) when the cells were found to be most sensitive to Bleomycin.[9] At the time of treatment, growth medium of the synchronous culture was replaced with the one containing Bleomycin in a concentration of 20 or 100 $\mu$g/ml. After incubation for 60 min, the dishes were rinsed with F10 medium, followed by reintroduction of the growth medium. Above treatment normally gives about 30 and 10% survival of randomly growing population.[10]

**Time-lapse Cinemicrography** Cinematographic apparatus (Bolex, B16) connected with an inverted phase-contrast microscope (Nikon, type MD) was set in a room of 37°. The air-tight glass dish (60 × 15 mm) with a tubing for circulating 5% $CO_2$-air mixture was fixed on the stage of the microscope. In the glass dish, the culture dish which had undergone Bleomycin treatment, was placed without the cover. A microscopic field containing 30 to 40 cells was selected and photographs were taken at 2-min intervals until 140 hr of incubation of the cells.

**Photographic Observation** To observe morphological change in Bleomycin-treated cells, specimens were prepared by the following procedure. Drops of mitotic cell suspension were placed on coverslips in a plastic dish. After an 8-hr incubation, treatment with Bleomycin was carried out as described above. At specified periods, the coverslips were taken out and fixed on the round window made in the center of a slide glass. The other side of the window was sealed with another coverslip after filling the window space with the growth medium. Photographs were taken under phase-contrast optics (Nikon MD) on a 35-mm film.

**Bleomycin** Bleomycin A5 (Lot #702 and #6), copper-free sulfate, was supplied by Nippon Kayaku Co. Ltd. The compound was dissolved in F10 medium[2] and kept in a refrigerator until use.

## Results

**Prolongation of Generation Time** On the basis of cinematographic record, division of synchronous L5 cells was scored after the antibiotic treatment and the cumulative frequency of division was determined as a function of time after mitosis, i.e., time after harvesting.

Thus, the cumulative frequency curves, or division curves, shown in Fig. 1 represent the duration of the first cell cycle for untreated and treated cells. The median generation time for untreated control cells was 22 hr, while those for cells treated with 20 and 100 $\mu$g/ml of Bleomycin were 32 and 95 hr, respectively. The division curve obtained after treatment with 20 $\mu$g/ml apparently indicates that there are two fractions differing in the cumulative frequency distribution of division, as shown by steep and less steep slopes. The former fraction was more than 50% of the population at 20 $\mu$g/ml while the latter fraction predominated after treatment with 100 $\mu$g/ml. This point will be discussed later.

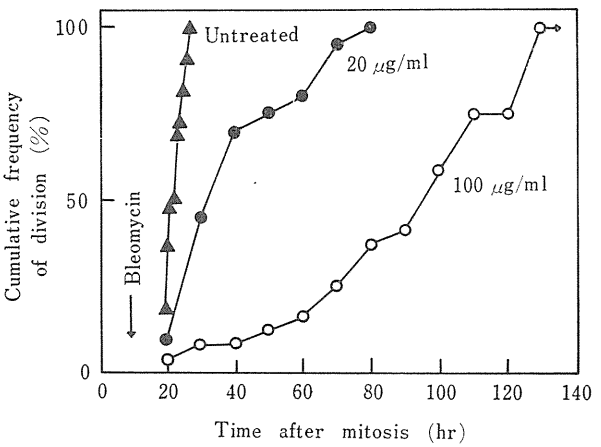


Fig. 1. Effect of bleomycin on the duration of first cell cycle

Synchronous cells were treated with 60-min pulse of Bleomycin in 20 or 100 $\mu$g/ml, 8 hr after mitosis ($G_1$~S transition phase). Then, clonal growth was recorded by time-lapse cinemicrography. Cumulative frequency of division determined as a function of time represents division delay induced by Bleomycin.

Fig. 2 compares the duration of the first cycle between cells which completed the second cycle and cells which were disintegrated during the second cycle. Generally, the generation time of the latter cells showed a remarkable prolongation together with a slightly wide distribution of values. The mean generation time and their coefficient of variation were 22 hr and 12% for untreated control, and 50 hr and 42% for 20 $\mu$g/ml and 92 hr and 14% for 100 $\mu$g/ml treatment. On the other hand, the duration of the first cycle of cells that could traverse the second cycle was about 27 hr irrespective of the concentration of the antibiotic.

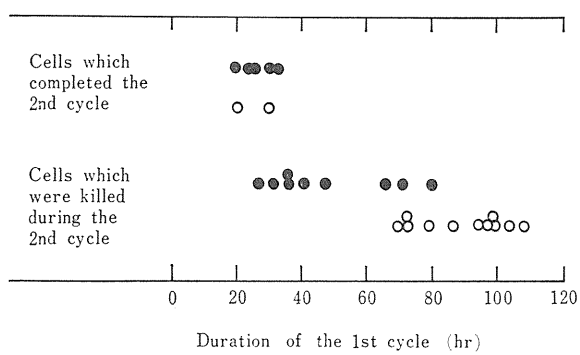


Fig. 2. Comparison of the duration of first cycle between cells which completed the second cycle and those killed during the second cycle

Cells were treated and observed as described in Fig. 1.
● 20 $\mu$g/ml of Bleomycin
○ 100 $\mu$g/ml of Bleomycin

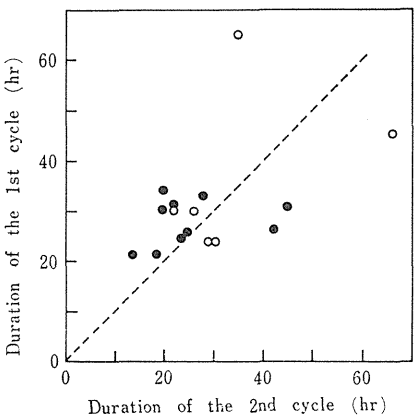


Fig. 3. Correlation of the duration between the first and second cycle of cells which completed the second cycle

Experiments and symbols are the same as in Fig. 1.

The correlation between durations of the first and second cycle of the cells which completed their second cycle is shown in Fig. 3. Distribution of the points, although scattered, along the broken line implies that both cycle times were rather comparable with the duration of 20 to 35 hr and the difference between the two, if any, was not consistent.

**Cell Death** The clonal growth of Bleomycin-treated cells was classified into five different patterns as shown in Table I. Fraction of various types of clonal growth was determined for two different levels of treatment. In pattern A, the cells were killed without dividing. Mean duration of mitosis to death was 68 and 81 hr, respectively, for 20 and 100 $\mu$g/ml treatment. In pattern B, the cells were neither killed nor divided until the termination of the film. This represents an extraordinary delay of division in cells treated with 100 $\mu$g/ml, as shown in Fig. 1. The cells in patten C completed the first cell cycle and both sister cells disintegrated during the second cycle. In pattern D, one of sister cells was killed during the second cycle and the other survived. In E, the cells went on dividing without any death among the progeny. A total of 22 untreated control cells all exhibited pattern E.

Table I. Pattern of Clonal Growth of Bleomycin-treated Cells

| Pattern of clonal growth | Fraction of clones (%) Untreated control | Bleomycin concn. ($\mu$g/ml) 20 | 100 |
|---|---|---|---|
| A——× | 0 | 7 | 21 |
| B——○ | 0 | 0 | 25 |
| C —┐× <br> —┘× | 0 | 60 | 45 |
| D —┐ <br> —┘× | 0 | 13 | 0 |
| E —┐ <br> —┘ | 100 | 20 | 8 |
| No. of cells examined | 22 | 15 | 24 |

Crosses represent death of individual cell. In pattern B, the cells were neither killed nor divided within 140 hr of observation.

We were especially interested in patterns A and C, where a clonal development of treated cells was obstructed; 7% of the cells were killed before mitosis by treatment with 20 $\mu$g/ml of Bleomycin and 21% with 100 $\mu$g/ml (pattern A). Amount of this fraction seemed to depend on the concentration of the antibiotic. In pattern C, 60% and 45% of the cells were killed during the second cycle by treatment with 20 and 100 $\mu$g/ml, respectively. A comparison between these patterns indicates that the cells were killed more frequently in the second cycle than in the first cycle during which the treatment was made. In other words, the potential damage given at the first generation must be tarnsmitted through mitosis before it becomes lethal.

In pattern E, 20% and 8% of cells were not disintegrated after 20 and 100 $\mu$g/ml treatment, and went on dividing with a slight prolongation of cell cycle time, as mentioned previously (Figs. 2 and 3). However, the fraction decreased as the Bleomycin concentration increased. This finding was consistent with the dose–response curve determined in terms of proliferative capacity of cells.[10]

Unequal occurrence of death between two sister cells was found exclusively in the cells treated with 20 $\mu$g/ml of Bleomycin (pattern D). It may imply that a damage given by the antibiotic in a lower concentration was not great enough to be distributed equally into sister cells.

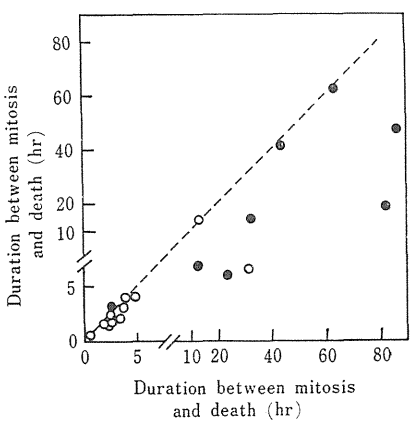


Fig. 4. Correlation between sister cells in duration of time from mitosis to death

Data were obtained from the cells of pattern C in Table I. Symbols are the same as in Fig. 2.

Analysis shown in Fig. 4 is on the cells classified in pattern C of Table I. It represents the correlation between two sister cells in durations of time from mitosis to death. After treatment with 20 $\mu$g/ml, the two sister cells showed little correlation as seen by scattered dots in Fig. 4. Contrarily, the treatment with 100 $\mu$g/ml gave results shown by open circles in Fig. 4 which were exclusively located along the broken line. This fact indicates that there was a positive correlation in the time of cell death between the sisters and most of the cell death occurred within 5 hr after mitosis.

Morphologically, most of the cells with prolonged generation time were about 5 times larger in cross-section than the untreated controls and showed many granules around the nucleus (Photo 1). Average volume of treated and then suspended cells, as measured by the Coulter counter (electronic cell-size analyzer), was about 3 times as much as that of untreated control. This increase in cell volume may be due to inhibition of mitosis as related to the partial inhibition of macromolecular syntheses by Bleomycin.[3,7,14]

## Discussion

According to the Bleomycin–survival experiments with HeLa cells reported by Terasima *et al.*,[9] the $G_1 \sim S$ transition phase is the most sensitive to the antibiotic during the cell cycle. From experiments with L5 cells, essentially the same result was obtained, although a variation in sensitivity during the cell cycle was less pronounced than that found in HeLa cells (unpublished). Based on this finding, treatment with the antibiotic in the present studies was confined to the $G_1 \sim S$ transition phase of synchronous L5 cells.

The cells treated with 60-min pulse of Bleomycin at $G_1 \sim S$ transition phase showed apparent delay of division (Fig. 1). This indicates that the S and/or $G_2$ period was prolonged by the antibiotic. As suggested by two different slopes in the cumulative frequency curve of division, the cells showed two different kinds of damage as related to the division delay; namely, the damage responsible for moderate prolongation of generation time, the distribution of which is slightly greater than in untreated control, and the other damage leading to severe prolongation as well as much greater distribution of generation time. The target of the former damage seems to be more sensitive than the latter, since the slope of the division curve representing the former damage disappeared after treatment with 100 $\mu$g/ml. It has been known from our results (unpublished) and other reports[1] that a cell progression was arrested at the late $G_2$ stage when the antibiotic was present in the culture. Thus, it is possible that one of the damages under consideration produces the $G_2$ prolongation. Also, a possibility must be considered that these damages resulted in a prolongation or a block of some other stages. Experiments are now under way along this line.

From data shown in Fig. 2 it is immediately apparent that there is some correlation between prolongation of the first cycle and cell death at the second cycle. However, it cannot be determined whether damage leading to the cycle prologation and that to ultimate death of cells are identical or whether such a correlation is only a fortuitous overlapping of two types of damage in a cell. We are rather inclined to the latter idea since a fraction of the cells killed at the second cycle after 20 $\mu$g/ml treatment showed the generation time of less than 30 hr which fell within the range of those of the cells completing the second cycle.

The most important finding in Table I is that the major type of cell death is not an immediate death in the first post-treatment generation (pattern A) but rather a "transmitotic death" (patterns C and D), particularly a "clonal death" (pattern C). Similar finding has been obtained from the cinematographic observation of Mitomycin-treated cells[6] and, typically, of cells treated by ionizing radiations. In view of DNA-attacking properties of these agents, e.g., a formation of DNA strand breaks and crosslinks, it is possible to assume that damages to DNA result in the production of abnormal or incomplete DNA and, therefore, progeny cells are destined to die either by abnormal synthesis or by lacking in vital synthesis. In addition to this, a possible involvement of damage in mitotic apparatus cannot be disregarded. For instance, damage in centriole, spindles, chromosomal proteins, and membranes associated with DNA may be deeply involved in abnormal or unequal allotment of genetic materials for progeny cells. All these possi-

bilities can explain the damage which leads the cells to death after mitosis without hampering cell progression of the Bleomycin-treated generation.

The authors acknowledge the kind supply of Bleomycin from Nippon Kayaku Co. Ltd. throughout this work. They also thank Prof. K. Okuda, Chiba University School of Medicine, for his encouragement, and Dr. H. Ohara for his advice on photographic procedures. The excellent technical assistance of Miss M. Yasukawa is greatly appreciated.

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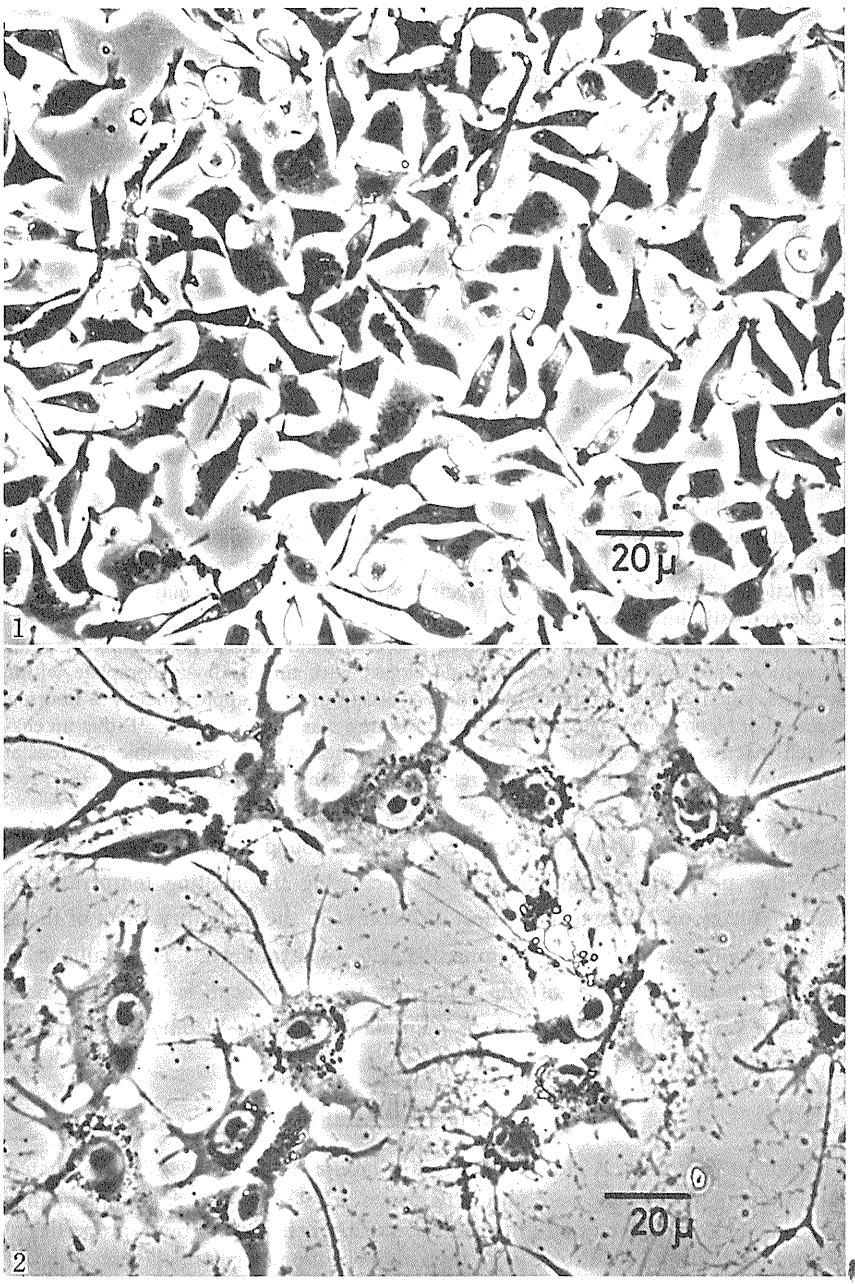
## Explanation of Plate VIII

Photo 1. Mouse L5 cells normally grown in F10HI medium. Phase-contrast. ×100.

Photo 2. Appearance of L5 cells 36 hr after treatment with 100 μg/ml of Bleomycin for 60 min. Phase-contrast. ×100.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# RESPONSE IN MACROMOLECULAR SYNTHESES OF MOUSE L CELLS TO BLEOMYCIN, WITH SPECIAL REFERENCE TO CELL-ANTIBIOTIC INTERACTION

MICHINORI WATANABE, YOSINOBU TAKABE and TAKESI KATSUMATA

First Department of Medicine, Chiba University School of Medicine, Inohana, Chiba-si, Japan

TOYOZO TERASIMA

Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa, Chiba-si, Japan

HAMAO UMEZAWA

Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo, Japan

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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[9]. 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

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were initiated with $2\times10^6$ 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 $1\times10^5$ 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 $1\times10^5$ 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 $\mu$Ci 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 $4\times10^4$ 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 $\mu$Ci 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 $\mu$Ci per dish, the steps in the determination of DNA synthesis were followed.

Autoradiography of Cells: Cells grown on coverslips were incubated with 0.5 $\mu$Ci/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.

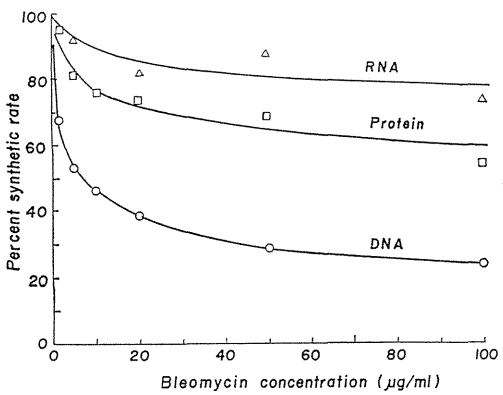
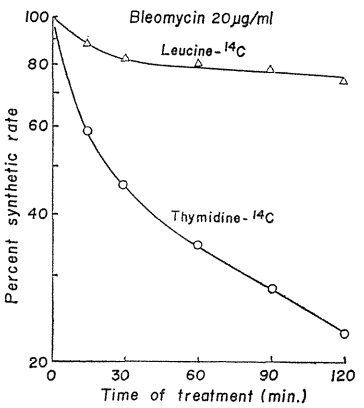



Fig. 2. Time-inhibition curve of DNA and protein syntheses of L5 cells

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.

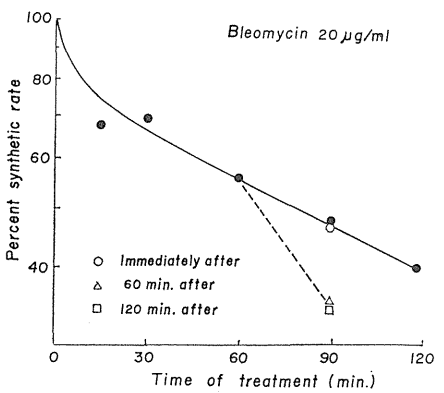
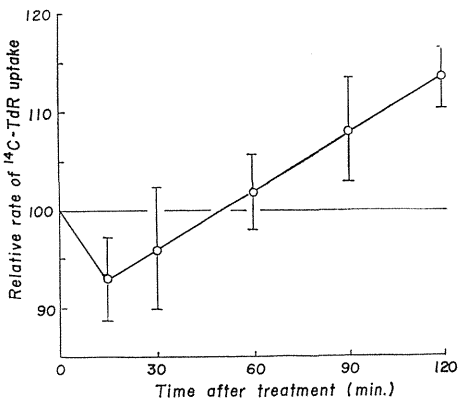



Fig. 4. Change in DNA-synthetic rate following bleomycin treatment
The cultures were treated with bleomycin 20 μg/ml for 60 minutes, then rinsed to remove the antibiotic and incubated again with F10 medium. The DNA-synthetic rate was measured at specified times. The observed rates were shown as a percentage 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.

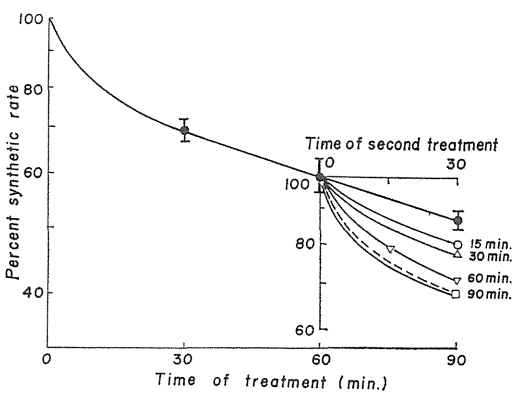
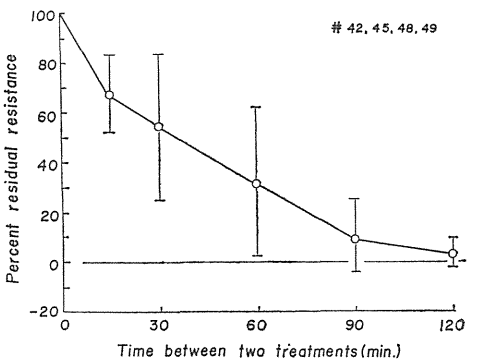



Fig. 6. Decay of bleomycin-induced resistance
The DNA-synthetic rate obtained after the second treatment of 30 minutes (as found in the inset scale of Fig. 5) was taken as a measure of resistance. The difference between the value of 0-minute interval (closed circle at 30 minutes in the same figure) and 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.*[13], 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 $\mu$g/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].

Acknowledgements

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[CANCER RESEARCH 33, 2849-2855, November 1973]

# The Effect of Bleomycin on Survival and Tumor Growth in a C3H Mouse Mammary Carcinoma[1]

**Muneyasu Urano, Nobuo Fukuda, and Sachiko Koike**

*Division of Clinical Research, National Institute of Radiological Sciences, 4-9-1, Anagawa, Chiba 280, Japan*

## SUMMARY

The response of a C3H mouse mammary carcinoma to bleomycin was studied *in vivo*. The dose survival curve exhibited upward concavity, suggesting that the initial sensitive portion was of an exponential nature and that approximately 10% of the tumor cells were in an apparently drug-resistant fraction. The tumor growth was inhibited more effectively by fractionated administrations than by a single treatment of the same total dose. This evidence was interpreted by a hypothesis that the resistant fraction was induced by the antibiotic and was reduced during each treatment interval. A mathematical model called "binding-saturation model" was proposed, by which the dose-response relationship was interpreted without assuming multicomponents in cellular sensitivity. The optimum fractionation regimens in tumor therapy by the antibiotic might be simply obtained by use of the model.

## INTRODUCTION

BLM,[2] a recently developed antibiotic (19), is now extensively used in clinical tumor therapy (5, 6, 10). The response to the agent of cultured mammalian cells is characterized by an upward concave or biphasic survival curve; these cells are sensitive at lower concentration and are less sensitive at higher concentration (1, 4, 14, 15). It is also demonstrated that the responses of cultured cells differ throughout the cell cycle (1) and that drug resistance found after 60 min of treatment with the antibiotic will be reduced if BLM-treated cells are reincubated in fresh culture medium for more than 3 hr (14).

This communication will describe that the upward concave survival curve is also characteristic in an experimental animal tumor system and that BLM resistance induced by the treatment is reduced *in vivo*. In addition, a mathematical model for cellular BLM sensitivity will be proposed.

[1] This work is partially supported by Grant 5R013 from Japan Society for the Promotion of Science.

[2] Abbreviations used are: BLM, bleomycin, $TD_{50}$, number of viable tumor cells expected to transplant a tumor in one-half of the recipients; TGD (tumor growth delay) time, the time difference between time required for nontreated tumor to reach a certain volume and that required for drug-treated tumor to reach the same volume.

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## MATERIALS AND METHODS

### Animal Tumor System

Experimental animals were 8- to 12-week-old C3H/He mice supplied by Funabashi Farm Co., Chiba, Japan. They were kept in a small animal facility in our institute where constant temperature was maintained. Animals were provided with standard Purina pellets and water *ad libitum*. Equal numbers of both sexes were used. Third-generation isotransplants of a C3H mouse mammary adenocarcinoma that arose spontaneously in a C3H/He female mouse were used in all the experiments. A method to obtain 3rd-generation tumors was already described elsewhere (20). Spontaneous and 1st-generation tumors were stored in a liquid nitrogen refrigerator and 2nd-generation tumors were proliferated in s.c. flank tissue of several C3H female mice for experimental use as needed. Therefore, all the experiments were based on a single spontaneous tumor.

### Test Agent

The test agent was BLM complex (copper free), which was kindly supplied by Nippon Kayaku Co., Tokyo Japan. The agent was dissolved in 0.9% NaCl solution, and 0.01 ml of desired concentration per g of mouse body weight was administered i.p.

### Suspension of Monodispersed Cells

Animals bearing the 2nd-generation tumors were sacrificed by cervical dislocation. Tumors were excised and necrotic portions were carefully removed. Intact tumor tissues were minced finely by scissors and kept in Hanks' medium containing 5% fetal calf serum. The mince was diluted approximately one-third by the medium and was removed to test tubes which were placed in crushed ice. After 15 min of sedimentation, the supernatant was removed carefully by a syringe and passed through a swinny filter. The filtrate was centrifuged at 1600 rpm for 5 min. The sediment was suspended in a small amount of Hanks' medium and was served for transplantation. Viable tumor cells were counted in a hemocytometer by use of the trypan blue staining method.

### Experimental Assay Method

**$TD_{50}$ Assay.** $TD_{50}$ was used as one of the assay methods. To begin with, animals were randomly arranged in groups in



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each assay. Usually, 50 animals were used for an assay. The cell suspension containing a known number of tumor cells was serially diluted in 2- to 3-fold by Hanks' medium into 6 to 8 doses. A transplantation of 10 $\mu$l of the cell suspension was made s.c. into the mouse right thigh. At 48 hr after transplantation, when the inoculum was expected to form an actively proliferating microcolony, a fixed amount of the antibiotic was injected into each animal in an assay group. This method is schematically illustrated in Chart 1. The transplanted area was palpated for a possible tumor growth once a week up to 80 days after transplantation. If a tumor measured over 10 mm in an average diameter, it was scored as a tumor take. If an animal died without any palpable tumor, it was excluded from the assay. If an animal died with a tumor that was smaller than 10 mm in diameter, the tumor take was examined by autopsy. From a tumor take frequency in 80 days, the $TD_{50}$ was calculated on the basis of logit analysis.

The surviving fraction of drug-treated tumor cells was expressed as a ratio of $TD_{50}$ (nontreated) to $TD_{50}$ (drug-treated).

**Measurement of Tumor Growth.** To test drug effect on the tumor growth, 10 $\mu$l of single cell suspension containing $10^5$ viable tumor cells were transplanted into the right flank skin. The measurement of tumor size was started when the inoculum reached ~5 mm in diameter. The volume of each tumor was calculated as that of an ellipsoid, *i.e.*, $(4/3)\ \pi abc$, where $a$, $b$, and $c$ were radii of 3 diameters. The administration of the antibiotic was started when the tumor was measured as 8 mm in average diameter or 250 cu mm, and 7 to 10 mice were used for an experimental group.

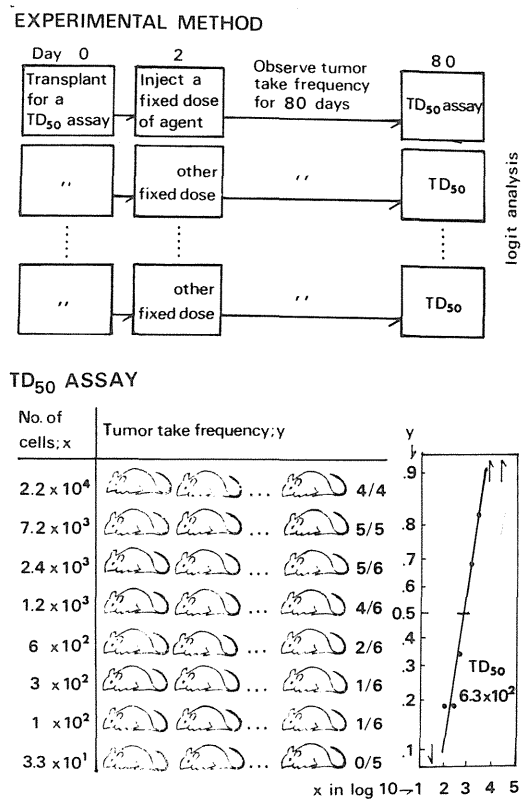


Chart 1. Schematic illustration of experimental assays. To determine surviving fractions of tumor cells treated with BLM, several concomitant $TD_{50}$ assays were performed. On Day 0, serially diluted tumor cell suspension was transplanted as shown in the *lower half*. At 48 hr after transplantation every animal in an $TD_{50}$ assay group received one of the test doses and tumor take frequency were observed for 80 days. According to the tumor take frequency in 80 days, logit analysis was fitted to calculate $TD_{50}$.

## RESULTS

The response of C3H mouse mammary carcinoma cells to BLM was examined. Two experiments, each composed of 4 concomitant $TD_{50}$ assays, were performed. Increasing doses of BLM were administered by single i.p. injection at 48 hr after transplantation. The dose survival curve was biphasic or upward concave (Chart 2). It was exponential in shape at lower dose range and any shoulder was not recognized. At doses higher than 30 mg/kg, the survival curve became less steep. The $D_0$, drug dose to reduce the

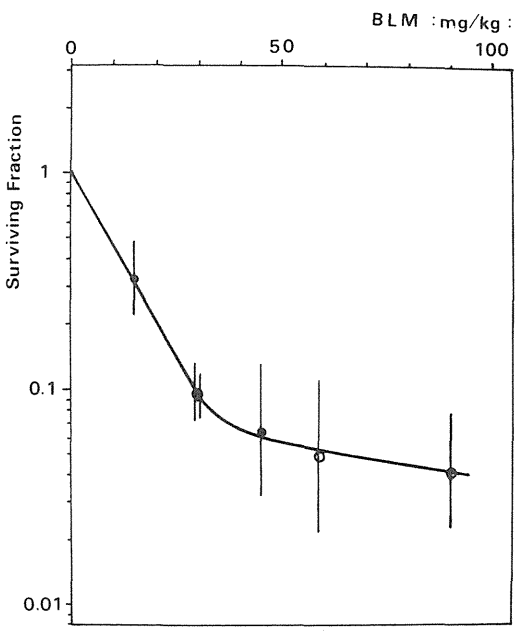


Chart 2. Survival curve of C3H mouse mammary carcinoma cells treated with various doses of BLM.

survival down to $1/e$ in the exponential portion of the survival curve, was 12.8 and 85 mg/kg in the sensitive and resistant portions, respectively. The extrapolation of the apparent resistant portion to the ordinate showed a surviving fraction of 0.09 to 0.1, suggesting that 9 to 10% of tumor cells were apparently BLM resistant.

In the 2nd series of experiments, the effect of BLM on the tumor growth was examined. Forty-five mice received a transplant and were randomized into 5 experimental groups. Animals in Groups A, B, and C received a single BLM injection of 0, 50, and 100 mg/kg, respectively, while those in Groups D and E received 4 BLM injections, each of 25 mg/kg separated by 12 and 24 hr, respectively. In Chart 3, tumor volumes are plotted as a function of time after the initiation of BLM treatment. The tumor growth was delayed by the antibiotic, and fractionated treatments were more effective than a single administration of the same total dose. TGD time, if measured at a tumor volume of 500 cu mm, was 3 or 6 to 8 days for tumors that received 100 mg/kg in a single or in fractionated administrations. The TGD time of tumors treated with a single dose of 100 mg/kg was not significantly different from that of tumors that received a single dose of 50 mg/kg. No difference was obtained between fractionated treatments at intervals of 12 hr and those at intervals of 24 hr (Table 1).

Further study was made to investigate the effect of different treatment intervals on fractionated treatments. The results are shown in Chart 4 and Table 1. The TGD time at 500 cu mm was approximately 5.5, 6.0, and 2.5 days for tumors that received 4 fractions of BLM, 25 mg/kg, at intervals of 24, 48, and 72 hr, respectively; while that for tumors that received a single dose of 25 mg/kg was 2.0 days. Tumors receiving fractionated treatments with a time interval of 72 hr were still under treatment when they reached 500 cu mm. Therefore, TGD time was measured again at a tumor volume of 1000 cu mm. As presented in Table 1, Column 6, the tumor growth was similarly

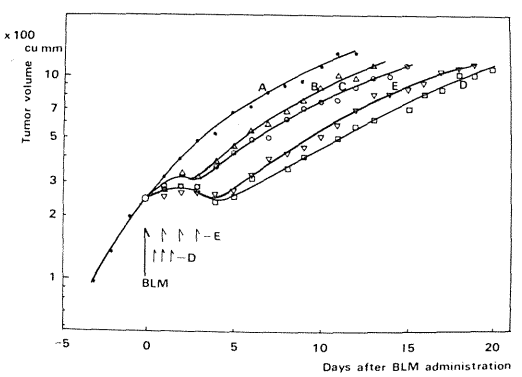


Chart 3. Effect of single or fractionated treatment by BLM on the tumor growth curve of C3H mouse mammary carcinoma. Animals in *Curves A*, *B*, and *C* received a single injection of 0, 50, and 100 mg/kg; while those in *Curves D* and *E* received 4 antibiotic treatments of 25 mg/kg each at time intervals of 12 and 24 hr, respectively.

inhibited by multiple doses given at intervals of 24 and 48 hr, while those given at a 72 hr interval were less effective than other fractionated treatments with shorter intervals. In the 72-hr interval schedule, tumors were more resistant to the drug after the 2nd administration.

Growth curves of tumors treated with fractionated treatments were less steep than those of nontreated tumors, while those of tumors that received a single dose were not different from that of control tumors.

## DISCUSSION

The dose-cell survival curve of C3H mouse mammary adenocarcinoma cells after BLM exhibited the upward concavity *in vivo* and was greatly different from that obtained after treatment with X-rays (9) or other chemotherapeutic agents (2, 21). The dose-response curve was obtained in microcolonies where tumor cells were actively proliferating and >99% of tumor cells were well oxygenated (11). Therefore, the upward concavity may not be the specific property in this tumor and could be interpreted by the same nature in cultured mammalian cells (1, 4, 14, 15).

Terasima *et al.* (14) extensively discussed the kinetics of the lethal effect of BLM. The appearance of resistant component in the survival curve could be interpreted neither by the different sensitivity throughout the cell cycle nor by the genetically determined heterogeneity in sensitivity. In the meantime, the resistant tail was also found in the time-survival curve and was found by the 2-dose fractionation experiment, to be caused by the rapid induction of resistance by the antibiotic. Therefore, they assumed that the resistant tail in the dose-survival curve might be related to the capability of the antibiotic to induce resistance.

It is still controversial regarding the primary target of BLM for cell lethality, while the DNA strand break or inhibition of mitotic activity is reported as a principal damage (3, 7, 8, 16, 18). Accordingly, to explain the upward concave dose-response curve, we propose a more generalized mathematical model called "binding-saturation model." This is based on assumptions that BLM is normally bound to a critical target (cell organelle or macromolecule) for cell lethality, that at higher concentration it will be saturated on the target, and finally that it will reach an equilibrium state and that the dose-response curve is exponential. The formula introduced was

$$K_r \left(\ln \frac{1}{S}\right)^{-1} = \frac{b}{\bar{\lambda}} + \frac{b}{\bar{\lambda}.\overline{A.U}} \frac{1}{D}$$

where the relation between surviving fraction ($S$) of treated cells and BLM dose administered ($D$) is in a form of the linear regression (see "Appendix" for other denotations and details). The kinetics in this model would be ($a$) the binding and the dissociation between BLM and target, ($b$) the production of damage by the agent and cellular repair of the damage or, ($c$) the amount of BLM incorporated in the cell and that inactivated by BLM-inactivating activity of the cell. Experimental observations such as the rejoining of DNA strand breaks in BLM-treated cells (16) or the

Table 1

*Effect of BLM on the growth of a C3H mouse mammary adenocarcinoma*

Each value of TGD time was obtained by graphic analysis of individual tumors.

| Treatment schedule | | | | TGD time (days) | |
|---|---|---|---|---|---|
| Injection dose (mg/kg) | No. of injections | Time interval (hr) | Total dose (mg/kg) | At 500 cu mm | At 1000 cu mm |
| 0 | 1 | 0 | 0 | 0 ± 0.5[a] | 0 ± 1.5 |
| 50 | 1 | 0 | 50 | 2.5 ± 1.0 | 3.0 ± 1.5 |
| 100 | 1 | 0 | 100 | 3.0 ± 1.0 | 4.0 ± 2.0 |
| 25 | 4 | 12 | 100 | 8.0 ± 2.0 | 10.0 ± 3.0 |
| 25 | 4 | 24 | 100 | 6.0 ± 2.0 | 8.0 ± 2.5 |
| 0 | 1 | 0 | 0 | 0 ± 1.0 | 0 ± 1.5 |
| 25 | 1 | 0 | 25 | 2.0 ± 0.5 | 2.5 ± 1.0 |
| 25 | 4 | 24 | 100 | 5.5 ± 1.5 | 8.0 ± 3.0 |
| 25 | 4 | 48 | 100 | 6.0 ± 1.5 | 9.0 ± 2.5 |
| 25 | 4 | 72 | 100 | 2.5 ± 1.0 | 6.0 ± 2.5 |

[a] Mean ± S.D.

Table 2

*BLM sensitivity of mammalian cells illustrated in Chart 5*

| Cell line | Initial Sensitivity[a] | Minimum surviving fraction[a] |
|---|---|---|
| L5 (mouse fibroblast) | 0.13 | 0.05 |
| B16-XI (mouse melanoma) | 0.39 | 0.10 |
| Vero (green monkey embryo) | 0.18 | 0.10 |
| P388 (mouse leukemia) | 0.12 | 0.30 |
| C3H mouse mammary adenocarcinoma | 0.09 | 0.0003 |

[a] See "Appendix" for these designations.

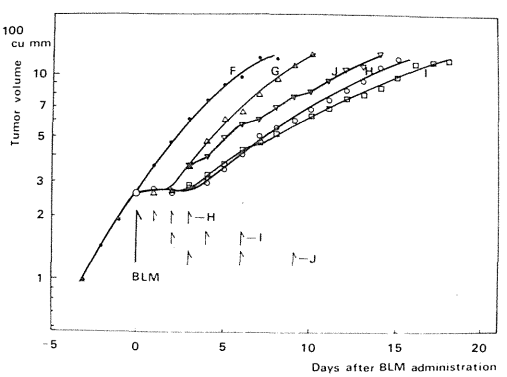


Chart 4. Effect of BLM treatments on the tumor growth of C3H mouse mammary carcinoma. Animals in *Curves F* and *G* were treated with single antibiotic doses of 0 and 25 mg/kg, while those in *Curves H, I,* and J recieved 4 injections of 25 mg/kg each at intervals of 24, 48, and 72 hr, respectively.

existence of a BLM-inactivating activity in mouse tissues (17) could support the basic assumptions in this model.

A notable feature of the model is that the dose-response curve is interpreted without assuming multicomponents. The dose-response relation shown in Chart 2 and those of 4 mammalian cell lines obtained by Terasima *et al.* (14) were analyzed by the least squares method. As shown in Chart 5 and Table 2, all the results were well fitted to the formula where the cellular sensitivity to BLM is shown by the slope of the linear regression line; namely, the less steep the slope, the more sensitive are the cells to BLM.

A present result that fractionated treatments were more effective than the single administration of the same total dose agrees with the result obtained *in vitro* (12). Accordingly, our finding may imply that anti-BLM activity is induced by the addition of BLM and disappears rapidly after the excretion of the agent. If tumor therapy by BLM is considered, this evidence might provide the important information that tumor cells are sterilized more effectively by multiple doses than by a single treatment when the same total dose is used. The surviving fraction of tumor cells expected after the fractionated administrations might be simply calculated by use of the proposed formula (see "Appendix," Formula 20). The repopulation capability of BLM-treated tumor cells during the treatment interval, which might be an important factor for an optimum fractionation scheme, is also taken into account in the formula. (The optimum fractionation scheme will be discussed in detail in a separate paper.)

Several studies were reported on the response of experimental animal tumors to BLM (13, 18), while accurate comparisons in their sensitivities might not be available because of different assay methods used. Our C3H mouse mammary carcinoma cells are surprisingly resistant to the

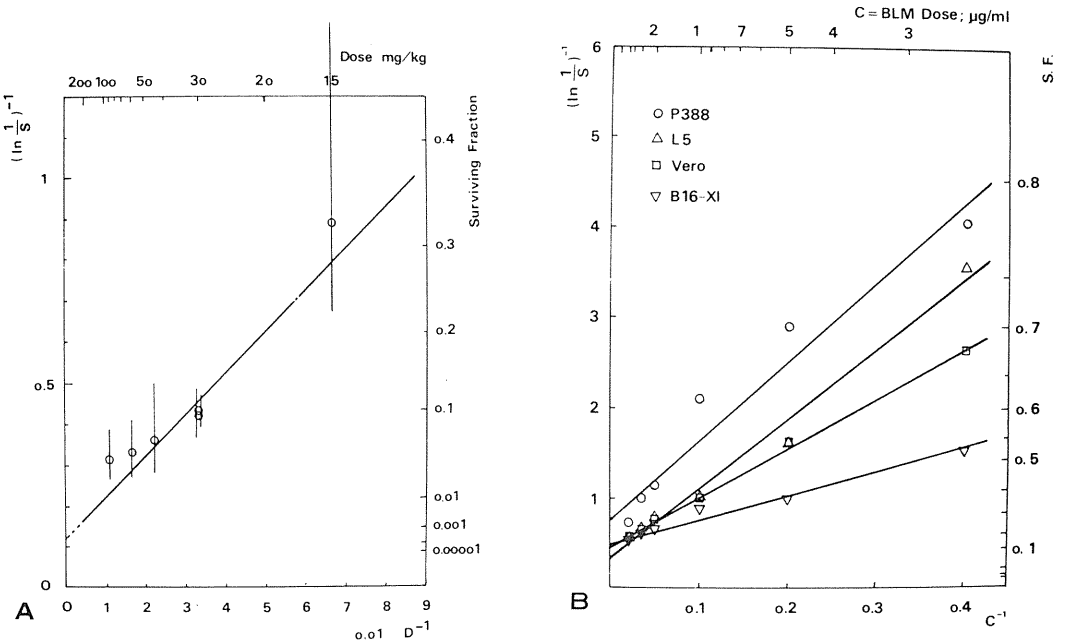


Chart 5. Dose-response relations analyzed by "binding-saturation model" of BLM-treated cells. *A*, C3H mouse mammary carcinoma cells treated *in vivo*; *B*, 4 cultured mammalian cell lines reported by Terasima *et al.* (14).

antibiotic. Recently, a dose-response curve of spontaneous mouse epidermoid carcinoma cells was obtained and demonstrated that the cells were approximately 100 times more sensitive than the present adenocarcinoma cells (K. Sakamoto, personal communication). These results coincide with clinical findings that the antibiotic is particularly effective on the epidermoid carcinoma (5) and suggest that tumors originating in different tissues may have different activities of inactivating BLM or of repairing BLM-induced damage.

## ACKNOWLEDGMENTS

We acknowledge Dr. Toyozo Terasima, Division of Physiology and Pathology, National Institute of Radiological Sciences, for his kind permission to use his data for present computation and for valuable discussion while this manuscript was in preparation.

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## APPENDIX: BINDING-SATURATION MODEL FOR THE LETHAL EFFECT OF BLEOMYCIN

***In Vitro* Treatment.** In order to explain the saturation-type behavior of the lethal effect of BLM, the following assumptions were made.

*Assumption 1.* The BLM molecule combines with the critical target in a cell and forms a target-BLM complex in a rapid reversible manner as follows:

$$\text{Target} + \text{BLM} \underset{K_d}{\overset{K_a}{\rightleftharpoons}} \text{target-BLM complex} \tag{1}$$

where $K_a$ and $K_d$ are rate constants of association and dissociation, respectively.

*Assumption 2.* Surviving fraction ($S$) of a BLM-treated cell decreases with the increase of inactivated fraction ($P_b/P$) in the following manner.

$$S = exp\left(-\lambda \frac{P_b}{P}\right) \tag{2}$$

where $P$ is total concentration of the critical target, $P_b$ is concentration of the target-BLM complex, and $\lambda$ is sensitivity coefficient of cells against the antibiotic.

If $C_i$ is intracellular BLM concentration, then Equation 1 is expressed by

$$K_a \cdot C_i \cdot (P - P_b) = K_d \cdot P_b \tag{3}$$

or

$$P_b = \frac{K_a \cdot P \cdot C_i}{K_d + K_a \cdot C_i} \tag{4}$$

Let us insert Equation 4 into Equation 2 and take the inverse of $\ln (1/S)$, Then

$$\frac{1}{\ln (1/S)} = \frac{1}{\lambda} + \frac{K_d}{\lambda \cdot K_a} \cdot \frac{1}{C_i} \tag{5}$$

If extracellular concentration of BLM is denoted by $C$,

$$C_i = \eta \cdot C \tag{6}$$

where $\eta$ is partition coefficient of the antibiotic between intra- and extracellular medium. Therefore, Equation 5 is given by

$$\frac{1}{\ln (1/S)} = \frac{1}{\lambda} + \frac{K_d}{\eta \cdot \lambda \cdot K_a} \frac{1}{C} \tag{7}$$

Initial sensitivity ($\sigma$) cited in Table 2 is given by

$$\sigma = \left(\frac{d \ln (1/S)}{dC}\right)_{C=0} = \frac{\eta \cdot \lambda \cdot K_a}{K_d} \tag{8}$$

where $K_a/K_d$ is binding affinity of the target.

Minimum survival ($S_{min}$) is defined by

$$S_{min} = \lim_{C \to \infty} S = \lim_{C \to \infty} exp\left(-\frac{\lambda \cdot K_a \cdot \eta \cdot C}{K_d + K_a \cdot \eta \cdot C}\right) = e^{-\lambda} \tag{9}$$

and

$$\lambda = -\ln S_{min} \tag{10}$$

***In Vivo* Treatment.** Let us assume that the intracellular BLM concentration increases instantaneously after the administration and then decreases exponentially. The association-dissociation equilibrium is expressed by

$$K_a(P - P_b)C_{max}\, e^{-K_r \cdot t} = K_d \cdot P_b \tag{11}$$

where, $C_{max}$ and $K_r$ stand for maximum concentration and rate constant of release of the antibiotic from tumor cells, respectively.

Hence

$$\frac{P_b}{P} = \frac{K_a \cdot C_{max}}{K_d e^{K_r \cdot t} + K_a \cdot C_{max}} \tag{12}$$

Force of mortality is assumed to be proportional to $P_b/P$, and the rate of decrease in number of proliferating tumor cells $N(t)$ at time $t$ after administration of the antibiotic is given by

$$\frac{dN(t)}{dt} = -\tilde{\lambda} \frac{K_a \cdot C_{max}}{K_d \cdot e^{K_r \cdot t} + K_a \cdot C_{max}} N(t) \tag{13}$$

which yields the following expression of surviving fraction at $t \to \infty$,

$$\ln S \equiv \lim_{t \to \infty} \ln \frac{N(t)}{N(0)} = -\frac{\tilde{\lambda}}{K_r} \{\ln (K_a \cdot C_{max} + K_d) - \ln K_d\}$$

$$= -\frac{\tilde{\lambda}}{K_r} \ln (1 + A \cdot U \cdot D) \tag{14}$$

where $A = (K_a/K_d)$ (affinity of the critical target with the antibiotic) and $U = (C_{max}/D)$ (efficiency of the drug incorporation into a tumor cell). By taking the 1st-order term of the Taylor expansion series of Equation 14, the following expression is obtained.

$$\ln S = -\frac{\tilde{\lambda}}{K_r} \cdot \frac{A \cdot U \cdot D}{1 + A \cdot U \cdot D} \tag{15}$$

Hence

$$\frac{1}{\ln (1/S)} = \frac{K_r}{\tilde{\lambda}} + \frac{K_r}{\tilde{\lambda} \cdot A \cdot U \cdot D} \tag{16}$$

**Fractionated Treatment *in Vivo*.** If tumor cells do not proliferate during $n$-fractionated treatments with time intervals of $T_i$, surviving fraction $[S(D, n)]$ after total dose ($D$) is given by

$$\ln S(D, \nu) = \nu \cdot \ln S_{D,\nu} = -\frac{\bar{\lambda}}{K_r}\frac{\nu \cdot A \cdot U \cdot D}{1 + \frac{A \cdot U \cdot D}{\nu}} = \frac{\nu \cdot \sigma \cdot D \cdot \ln S_{\min}}{\sigma D - \nu \cdot \ln S_{\min}} \tag{17}$$

where initial sensitivity ($\sigma$) and minimum survival ($S_{\min}$) in *in vivo* treatment are given by

$$\sigma = \left(\frac{d \ln (1/S)}{dD}\right)_{D=0} = \frac{\bar{\lambda}}{K_r} A U \tag{18}$$

and

$$\ln S_{\min} = -\lim_{D \to \infty} \frac{\bar{\lambda} \cdot A \cdot U \cdot D}{K_r(1 + A \cdot U \cdot D)} = -\frac{\bar{\lambda}}{K_r} \tag{19}$$

respectively.

If tumor cells are proliferating with tumor-doubling time, $T_d$ throughout the treatment period,

$$\ln S(D, \nu) = (\nu - 1)\frac{T_t \cdot \ln 2}{T_d} + \frac{\nu \cdot \sigma \cdot D \cdot \ln S_{\min}}{\sigma \cdot D - \nu \cdot \ln S_{\min}} \tag{20}$$

**Relationship between *in Vivo* and *in Vitro* Treatment.** Sensitivity coefficient, $\lambda$ in *in vitro* treatment is decomposed into a product of $\bar{\lambda}$ and the period of drug treatment of cultured cells. Effective period of drug exposure in *in vivo* treatment is given by $1/K_r$.

Hence Equation 19 corresponds to Equation 10.

Binding affinity ($A$) is given by $K_a/K_d$ in both treatments, while efficiency ($U$) of drug incorporation into a tumor cell *in vivo* corresponds to partition coefficient ($\eta$) of the antibiotic between intra- and extracellular medium *in vitro*.

Biochemical Pharmacology, Vol. 22, pp. 323–333. Pergamon Press, 1973. Printed in Great Britain.

# STRAND-SCISSION OF HeLa CELL DEOXYRIBONUCLEIC ACID BY BLEOMYCIN *IN VITRO* AND *IN VIVO*

YOSHISADA FUJIWARA and TERUO KONDO

Laboratory of Medical Biophysics, Kobe University, School of Medicine, Kusunoki-cho 7, Ikuta-ku, Kobe 650, Japan

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**Abstract**—The ability of bleomycin (BLM) to cause strand breaks of DNA *in vitro* has been confirmed by means of alkaline sucrose sedimentation. BLM-induced breakage of extracted HeLa S3 cell DNA occurs extensively after dialysis following reaction with 50 μg/ml of BLM. 2-Mercaptoethanol (0·003 M) is not obligatory for BLM-induced degradation of DNA *in vitro*, but enhances it. The presence of 0·025 M EDTA in the reaction mixture completely prevents single-strand breaks of DNA by BLM. In good agreement with the *in vitro* results, the DNA from the HeLa cell lysate prepared in a lysing medium containing 0·015 M of EDTA is fragmented a little after a 30-min treatment with 25 μg/ml of BLM, while nonspecific and extensive degradation of DNA occurs when the cells are lysed in the absence of EDTA. A 30-min treatment with BLM also provokes a small amount of unscheduled incorporation of $^3$H-thymidine into non-S phase cells, indicating that a small number of single-strand breaks induced are repair-patched. Moreover, 25 μg/ml of BLM exerts a somewhat inhibitory effect on the joining of short segments of replicating DNA after a 30-min $^3$H-thymidine pulse, but the joining ability is soon resumed. These data suggest that BLM may either hardly enter HeLa S3 cells or may be readily inactivated.

BLEOMYCIN (BLM), first discovered by Umezawa *et al.*,[1] is a peptide-containing antibiotic which has antineoplastic activity toward transplantable animal tumors[2,3] and squamous cell carcinoma in man.[4]

The mechanism of action of BLM has been extensively studied: the antibiotic inhibits the synthesis of DNA as well as protein,[5] lowers the melting temperature of DNA in the presence of sulfhydryl compounds and produces a marked breakage of DNA both *in vivo* and *in vitro*.[6–11] In addition, BLM also interferes effectively with the ligase reaction *in vitro*.[12] All these reactions are enhanced by 2-mercaptoethanol (ME).

Furthermore, the action of BLM appears to be similar to that of radiation such as X-rays in the following features: both agents provoke a similar change in sensitivity of cultured mammalian cells during their growth cycle,[13] break single strands as well as double strands of DNA of both microbial and mammalian cells,[6–11] and degrade DNA.[6,10,14] On the other hand, recent studies using various radiation-sensitive mutants such as *uvr*$^-$ (excision-defective) and *rec*$^-$ (recombinational repair-less) bacteria indicate that BLM-induced injury is different from radiation-induced damage, since these mutants are almost as resistant to BLM as wild strains in which the respective genes function normally.[14,15]



BIOCHEMICAL PHARMACOLOGY 22, 323-333, 1973
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The present study is an attempt to elucidate more details of the reaction of BLM with HeLa cell DNA *in vitro* and *in vivo* in order to learn the mechanism by which BLM fragments DNA strands.

## MATERIALS AND METHODS

### *Chemicals*

BLM mixture (Lot No. B-40, copper-free) was a gift from Nippon Kayaku Company, Tokyo, Japan, and contained the main component, BLM $A_2$, in at least 50 per cent concentration (Nippon Kayaku's data). The BLM solution was prepared by dissolving in 0·9% (w/v) NaCl solution and sterilizing through membrane filters just before use. Thymidine (TdR)-2-$^{14}$C (30 mCi/m-mole) and TdR-methyl-$^{3}$H (23·4 Ci/m-mole) were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y., and Radiochemical Centre, Amersham, England respectively. Pronase-P was obtained from Kaken Chemical Company, Tokyo, Japan, pancreatic RNase, from Worthington Biochemical Company, Freehold, N.J., and $T_1$ RNase, from Sankyo Chemical Company, Tokyo, Japan. All other reagents used were purchased from commercial sources.

### *Cell culture*

HeLa S3-9IV,[16] generously provided by Dr. T. Terasima, National Institute of Radiological Sciences, Chiba, Japan, were cultured routinely in plastic or glass culture vessels in synthetic F10 medium[17] supplemented with 0·05% (w/v) Difco heart infusion broth and 10% (v/v) calf serum, as described previously.[18] The cells were shown to be PPLO-free, using a modified Hayflick agar. Cell survival after a 30-min treatment with BLM was determined by the colony-forming ability of cells after incubation for 12 days.

### *Labeling of cellular DNA*

(a) HeLa S3 cultures in log-phase were labeled with 0·2 μCi/ml of $^{14}$C-TdR for 2 days in order to obtain the extracted radioactive DNA. (b) For analysis of the breakage of cellular DNA in alkaline sucrose gradients, exponentially growing cells were labeled with 0·5–1·0 μCi/ml of $^{3}$H-TdR for 24 hr. (c) Newly synthesized DNA in BLM-treated cells was labeled with 23·4 μCi/ml of $^{3}$H-TdR ($10^{-6}$ M) for 30 min and 2 hr. (d) To detect unscheduled DNA synthesis, cells were prelabeled with 5 μCi/ml of $^{3}$H-TdR for 2 hr for elimination of S-phase cells and treated with 0, 25 and 100 μg/ml of BLM for 30 min, followed by an incubation for 2 hr in media containing the same amount of $^{3}$H-TdR. The cells were subjected to autoradiography with an exposure for 10 days.[18]

### *Isolation of DNA*

Approximately $5 \times 10^7$ cells labeled with $^{14}$C-TdR according to the protocol (a) were trypsinized, harvested and lysed in 2·5% (w/v) sodium dodecyl sulfate (SDS)–0·015 M EDTA–1 × SSC (0·15 M NaCl + 0·015 M trisodium citrate, pH 7·4). The lysate was digested with 100 μg/ml of preheated pronase for 4 hr at 37°, and was made 1 M with respect to sodium perchlorate. After vortex-mixing, DNA was isolated after deproteinization (five-times) with chloroform–isoamyl alcohol (24:1)

according to our previous modification[19] of the method of Haut and Taylor.[20] Finally, both RNA and traces of protein were removed by further digestions with pancreatic RNase (20 $\mu$g/ml) plus $T_1$ RNase (100 units/ml) and subsequently with pronase (20 $\mu$g/ml). Thus, purified $^{14}$C-DNA was precipitated with 90% ethanol, dissolved in 1 × SSC, and kept at 4° until use. DNA concentration was assayed by the method of Burton.[21] An appropriate amount of the DNA was subjected to reaction with BLM *in vitro*.

*Reaction of DNA with BLM*

*Reaction* in vitro. Before reaction, $^{14}$C-DNA in 1 × SSC was dialyzed overnight against 1 liter of 0·0125 M Tris–HCl, pH 7·6. The sedimentation coefficient for this DNA was approximately 20S as estimated by alkaline sucrose sedimentation. The standard reaction mixture contained 5 $\mu$g $^{14}$C-DNA, 0·003 M ME, 50 $\mu$g/ml BLM and 0·025 M EDTA in a total volume of 0·2 ml of 0·0125 M Tris–HCl, pH 7·6. Variations in this mixture are given in the legend to Fig. 2. All reactions were carried out for 30 min at 37° under sterilized condition. In some experiments, the reacted material was dialyzed for 24 hr against 2 l. of 1/10 × SSC before alkalinization followed by centrifugation.

*Reaction* in vivo. HeLa S3 cells prelabeled under the protocol (b) were incubated in media containing 0 or 25 $\mu$g/ml of BLM for 30 min alone. In the case of protocol (c), the same amount of BLM had been added to log-phase cells 30 min before $^3$H-TdR pulses started, and the drug was present continuously during labeling for 30 min or 2 hr. Immediately after reaction, the cells were lysed and centrifuged in alkaline sucrose gradients.

*Lysing cells and alkaline sucrose gradient centrifugation*

The entire method has been described elsewhere.[22] Briefly, $^3$H-TdR-labeled and BLM-treated whole cells were lysed in a lysing solution of 0·25% (w/v) SDS, 0·015 M EDTA and 10% (w/v) nuclease-free sucrose in 0·15 M bicarbonate buffer, pH 8·0, and digested with 2 mg/ml of preheated pronase for 4 hr at 37°. The lysing solution sometimes lacked EDTA. After alkali was added to bring the pH up to 12·5, a 0·2-ml aliquot of the final lysate, corresponding to 5000 cells (less than 0·1 $\mu$g of denatured DNA), was layered on top of a 4·8-ml linear gradient of 5–20% (w/v) alkaline sucrose in 0·8 M NaCl, 0·2 M NaOH, 0·01 M EDTA and 0·05 M *p*-aminosalicylate, pH 12·5. For the *in vitro* reacted DNA, a total volume of 0·2 ml (5 $\mu$g of DNA) was layered following alkalinization before or after dialysis. The gradients were centrifuged in a SW39L rotor of a Spinco model L ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 35,000 rev/min at 20° for 2 hr for the *in vivo* DNA and 5 hr for the *in vitro* DNA. After centrifugation, 10 drop-fractions were collected from the bottoms of the tubes onto 2·3 cm Whatman No. 3 MM filter paper discs. The discs were dried, washed exhaustively with cold 5% (w/v) trichloroacetic acid, and placed in scintillation vials with 10 ml of 2,5-diphenyloxazole–1,4-bis-2, 5-phenyl-oxazolyl)–benzene–toluene for the measurement of radioactivity.

*Molecular weight determination*

S values and molecular weights of HeLa DNA after centrifugation were determined by use of T4D DNA for reference and calculated from the equations of Burgi and Hershey[23] and Studier.[24]

## RESULTS

*Survival of asynchronous HeLa S3 cells.* The survival of cells after a 30-min treatment with increasing doses of BLM is shown in Fig. 1. The dose–survival curve is biphasic, suggesting that a sensitive fraction (cells in mitosis, at the G1/S transition and in G2) as well as a less sensitive fraction (cells in early G1 and late S) is involved.[13,25] The sensitive half of the cell population is defined by a $D_0$ of 20 $\mu$g/ml (30 min), which is almost twice as high as the $D_0$ dose [12 $\mu$g/ml (30 min)] for mouse L5 cells by Terasima and Umezawa[13] and Chinese hamster CHO cells by Barranco and Humphrey.[25] The rest of the cell population has a $D_0$ dose of 100 $\mu$g/ml (30 min). In this resistant part of the survival curve, a BLM dose as high as 100 $\mu$g/ml reduces survival to only 15 per cent. Therefore, the toxicity of BLM to HeLa S3 cells seems to be moderate.[13]

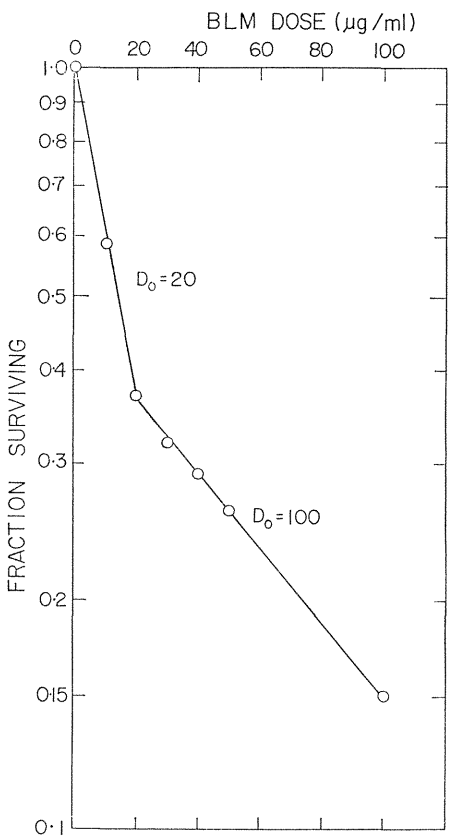


FIG. 1. Effect of BLM (30-min exposure) on survival of asynchronously growing HeLa S3 cells.

*BLM-induced single-strand breaks of extracted HeLa S3 DNA.* Our primary intention here of studying BLM-induced fragmentation of DNA by means of alkaline sucrose sedimentation was to investigate how EDTA, as an ingredient of both cell lysing and sucrose gradient solutions, affects the sedimentation rate of BLM-treated DNA. In addition, the effect of ME, being considered to be essential for DNA breaks

by BLM,[6–9] was also re-examined. For this purpose, highly purified DNA is suitable. The purified HeLa $^{14}$C-DNA was incubated in various reaction mixtures for 30 min at 37°. After reaction or further dialysis, the pH of the material was adjusted to 12·5, followed by alkaline sucrose sedimentation. The results are shown in Fig. 2. The sedimentation profile of the control $^{14}$C-DNA had the same 20S peak with a broad distribution as curve C (so that the former is not depicted in Fig. 2).

In Fig. 2 (left hand panel) the reacted material was not dialyzed before addition of alkali. The sedimentation profiles show that the presence of ME in the reaction mixture fragments DNA extensively and in a similar fashion in either the absence (curve A) or the presence of 50 μg/ml of BLM (curve B). This suggests that the breakdown of DNA may be due to the oxidized product of ME by alkali, as Bode[26] has demonstrated. This contention is obviously valid, because DNA breaks are not recognized when ME is removed by dialysis before the addition of alkali (curve E). In contrast, curve C shows that 0·025 M EDTA present in the reaction mixture completely abolishes single-strand breaks.

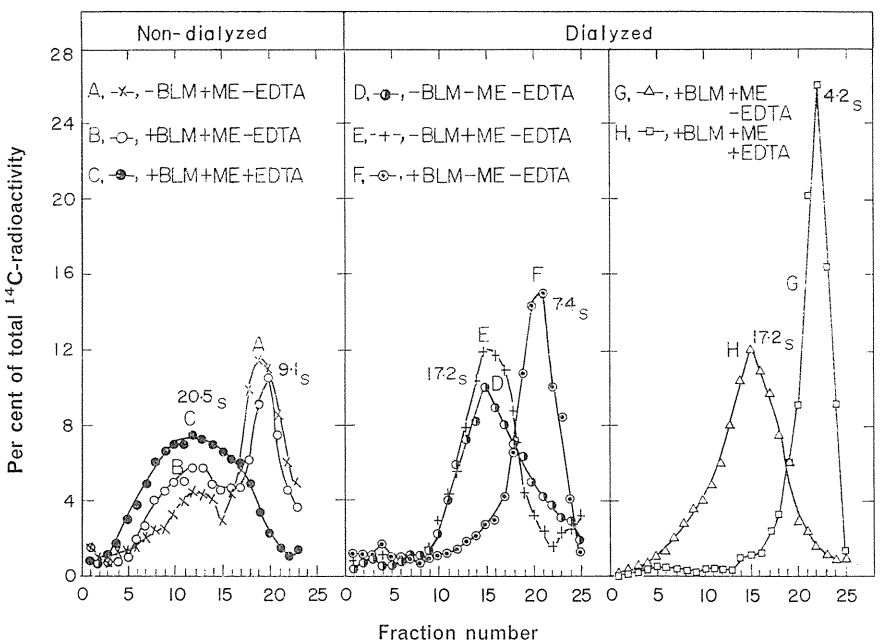


FIG. 2. Effects of BLM, ME and EDTA on the sedimentation profile of extracted HeLa S3 DNA *in vitro*. Purified HeLa $^{14}$C-DNA (5 μg; 2300 counts/min) was incubated for 30 min at 37° in 0·0125 M Tris-buffered reaction mixture (0·2 ml, pH 7·6). Variable constituents (50 μg/ml BLM, 0·003 M ME and 0·025 M EDTA) are indicated by A to H in the upper parts of figures. After reaction, the mixture was alkalinized to pH 12·5 before or after dialysis against 1/10 × SSC for 24 hr at 4°, and layered on top of 5–20% (w/v) alkaline sucrose gradient (pH 12·5), followed by centrifugation at 35,000 rev/min for 5 hr at 20° in a SW39L rotor.

In Fig. 2 (center and right panels), the material reacted in the absence of EDTA was dialyzed against 1/10 × SSC for 24 hr. ME alone, as shown by curve E, has no effect on the sedimentation rate, as compared with curve D obtained without ME. Both peaks of curves D and E position at 17·2S, which is smaller, because of mechanical shearing by dialysis, than that of non-dialyzed DNA (curve C: 20·5S). A charac-

teristic finding is that BLM alone is able to fragment DNA after dialysis (curve F), indicating that ME is not obligatory. However, ME enhances the extent of BLM-induced DNA chain scission, when DNA is incubated in the reaction mixture lacking EDTA (curve G). A prominent peak of curve G sediments at 4·2S, significantly slower than that of curve F (7·4S).

The right hand panel of Fig. 2 concerns the effect of EDTA. Curve H demonstrates clearly that EDTA present during the reaction completely prevents the induced fragmentation of DNA. Moreover, this prevention is not affected by ME (curve H). This result again confirms a profile (curve C) of non-dialyzed DNA, in which EDTA protects against degradation.

*Reaction of intracellular DNA with BLM.* Based on the facts obtained *in vitro*, the following experiments were carried out under conditions in which no ME was used throughout.

Figure 3 illustrates alkaline sucrose sedimentation profiles of DNA from the $^3$H-TdR-prelabeled and BLM-treated cell lysates in an EDTA-containing lysing medium. The control DNA sediments at 76·5S, whose molecular weight is estimated to be approximately 80 $\times$ $10^6$ daltons (Fig. 3, open circle). This agrees with our previous result.[22] The profile of DNA from BLM-treated cells (Fig. 3, closed circle) is similar to that from the untreated control cells. However, a little elevated $^3$H radioactivity is found between the fifteen and twenty-fourth fractions. The amount of fragmented DNA in these fractions corresponds to about 20 per cent of the total radioactivity recovered. Therefore, 25 $\mu$g/ml of BLM breaks a little of the intracellular DNA during a 30-min treatment before the cell lysis.

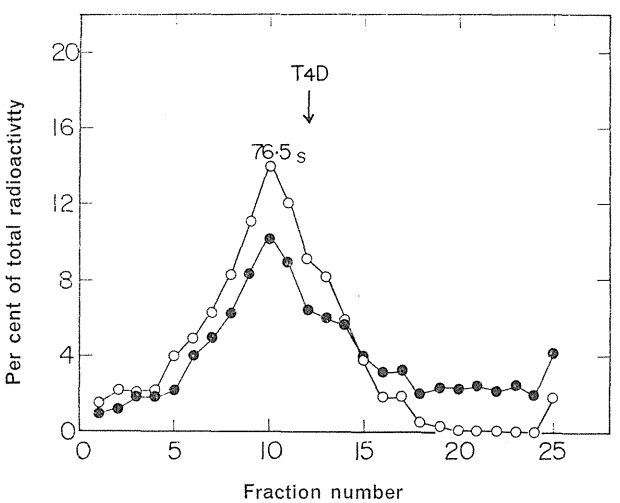


FIG. 3. Effect of EDTA in the lysing solution on the fragmentation of intracellular DNA by BLM. HeLa S3 cells prelabeled with 0·5 $\mu$Ci/ml of $^3$H-TdR for 24 hr and chased for 1 hr were treated with 0 (○——○) or 25 $\mu$g/ml of BLM (●——●) for 30 min at 37°. The cells were lysed in 0·25% (w/v) SDS–0·015 M EDTA–10% (w/v) sucrose–0·15 M $NaHCO_3$, pH 8·0, and digested by addition of an equal volume of pronase (2 mg/ml). After addition of the one-tenth vol. of 3 N NaOH to the lysate, a 0·2-ml aliquot was layered on top of an alkaline sucrose gradient and centrifuged for 2 hr under the identical conditions described in the legend to Fig. 2.

In contrast, Fig. 4 shows typical profiles of DNA from the cell lysates in the EDTA-deprived lysing medium, and indicates that BLM-treated DNA sediments at 24S

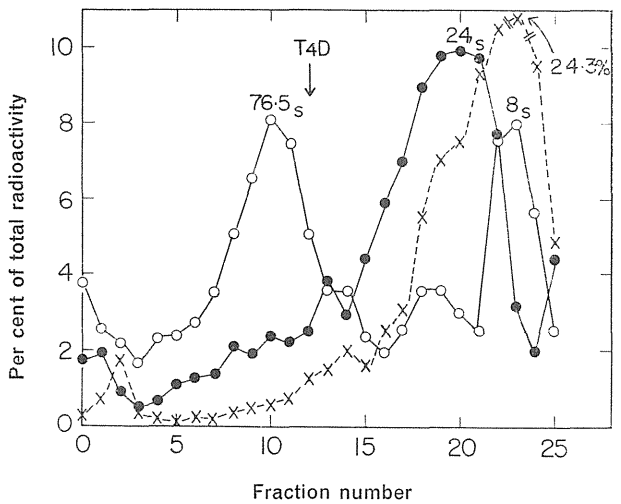


FIG. 4. Effect of cell lysis in the absence of EDTA on the fragmentation of intracellular DNA by BLM. Except that the lysing solution lacks EDTA, all other procedures are the same as described in the legend to Fig. 3. O——O, ×---×, control (no BLM); ●——●, 25 μg/ml BLM-treated.

(closed circle) and has been degraded more than the untreated control (open circle). The control DNA was of course degraded nonspecifically during a 4-hr lysis (Fig. 4, open circle and cross), and the extent of the degradation differs from experiment to experiment, where the extreme proceeds to 8S (Fig. 4, cross). However, the degradation of BLM-treated cell DNA is rather fixed to 24S (Fig. 4, closed circle). As a whole, BLM enhances the degradation of the intracellular DNA during the lysis of cells without EDTA. These results imply that DNase(s) in the lysate preparation in the absence of EDTA may be responsible for the degradation and may recognize BLM damage in DNA. We cannot distinguish whether BLM binds effectively to DNA within the nucleus or if BLM distributed throughout cells exerts its action upon lysis.

*Effect of BLM on newly replicating DNA.* One way of distinguishing whether or not BLM imposes damage, i.e. binding and/or breakage, to intranuclear DNA is to study what kind of new daughter DNA is synthesized on the template DNA of BLM-treated cells. This experiment using $^3$H-TdR pulses, in addition, favors an investigation of the effect of BLM *in vivo* on the joining of short replicating segments, since BLM is considered to inhibit ligase *in vitro*.[12]

Figure 5 shows alkaline sucrose sedimentation profiles of $^3$H-labeled DNA newly replicated for 30 min and 2 hr in HeLa S3 cells. At this time, 25 μg/ml of BLM was added 30 min before the start of $^3$H-TdR pulses and it was present continuously during the pulse-labeling. After a 30-min pulse (Fig. 5A), newly replicating DNA in the control cells consists of three major peaks of 4, 30 and 62S (closed circle), while a single 40S peak from BLM-treated cells predominates (open circle). However, a further 2-hr labeling in the continued presence of BLM (Fig. 5B) makes no obvious difference between the sedimentation rates of DNA newly replicated in both control (closed triangle) and treated cells (open triangle). Both profiles are similar with only one 76–76·5S peak of matured DNA. These results indicate that the enzymic joining of short replicating segments is delayed slightly by BLM in the early period of labeling, but soon resumed. A totally similar figure was obtained in BLM-treated mouse L

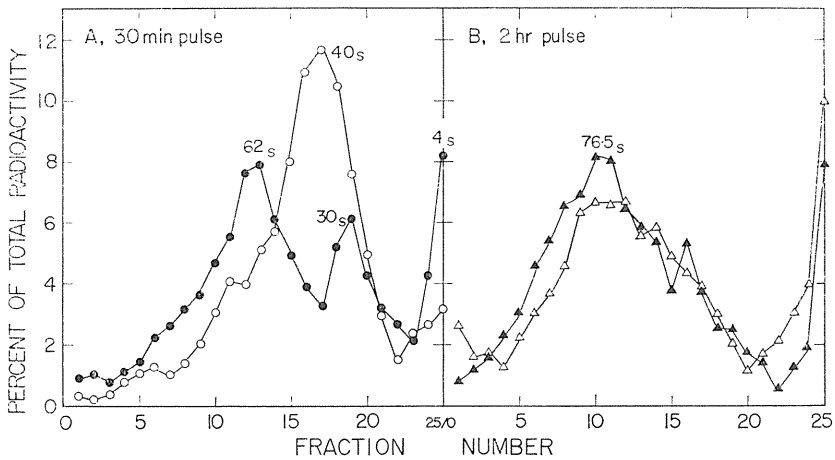


FIG. 5. Effect of BLM on the joining of newly replicating DNA *in vivo*. BLM (25 μg/ml) was added to exponentially growing HeLa S3 cells 30 min before the start of pulse-labeling with 23·4 μCi/ml of $^3$H-TdR and present continuously during the labeling. The procedures for lysing cells and centrifugation in alkaline sucrose gradients are the same as described in the legend to Fig. 3. (A) 30-min pulse; (B) 2-hr pulse. ●——●, ▲——▲, control (no BLM); ○——○, △——△, BLM-treated.

cells (Fujiwara, unpublished data), which cannot excise pyrimidine dimers after ultraviolet light irradiation and hence leaves a gap in newly synthesized DNA during the postreplication repair.[22] Consequently, it seems possible that BLM penetrates so poorly into living cells that it exerts little effect on DNA within the nucleus. Otherwise, BLM incorporated would be readily inactivated within cells.

*Unscheduled DNA synthesis after treatment with BLM.* An autoradiographic study was performed on unscheduled incorporation of $^3$H-TdR into non-S cells of HeLa S3 cultures after a 30-min treatment with 25 and 100 μg/ml of BLM. Table 1 shows recognizable incorporations of $^3$H-TdR in BLM-treated cells over that of the control cells. This indicates that a small amount of single-strand breaks of DNA occurs either by the direct binding of BLM or by indirect action of nuclease, and that, therefore, $^3$H-TdR containing repair patches replace a small amount of BLM damage in the intracellular DNA.

## DISCUSSION

The results reported here indicate that BLM reacts with highly purified HeLa S3 DNA and induces single-strand breaks *in vitro*. This finding agrees with many previous results.[6–10] ME has been reported to be essential for BLM to cause fragmentation of DNA and a decrease in the melting temperature of DNA.[9] However, ME is not obligatory for, but rather enhances the BLM-induced scission of DNA chain under the present conditions (Fig. 2). This view is reconciled with the observation of Haidle.[10]

It should be noticed that EDTA prevents single-strand breaks by BLM both in the presence of ME and after dialysis (Fig. 2). Additionally, EDTA also inhibits the BLM-effected decrease in the melting temperature of DNA in the presence of ME.[9] The

chemical rationale for the inhibitory action of EDTA is not clear, but is assumed to be EDTA-reversible binding of BLM to polynucleotide chains, as found in, for example, luteoskirin and kanchanomycin.[27]

TABLE 1. UNSCHEDULED DNA SYNTHESIS IN HeLa S3 CELLS AFTER A 30-MIN TREATMENT WITH BLEOMYCIN

| Bleomycin ($\mu$g/ml) | No. of non-S cells scored* | Total No. of grains | No. of grains per non-S cell† (unscheduled synthesis) |
|---|---|---|---|
| 0 | 94 | 114 | 1·08 |
| 25 | 101 | 338 | 3·44 |
| 100 | 131 | 455 | 3·48 |

* HeLa S3 cells in log-phase were first prelabeled with 5 $\mu$Ci/ml of $^3$H-TdR for 2 hr before BLM treatment in order to eliminate S cells easily at the time of scoring non-S cells. After a 30-min treatment, the cells were washed and reincubated for an additional 2 hr in media containing the same amount of $^3$H-TdR without BLM. Finally, the cells were subjected to autoradiography[18] with an exposure for 10 days.

† The number of grains per non-S cell includes 0·28 average background grains per cell (= 26 grains/90 cells scored).

Similarly, BLM-induced fragmentation of intracellular DNA is restricted to a low yield (Fig. 3), after EDTA has eliminated nonspecific degradation during cell lysis (Fig. 4). This result is correlated with small amounts of unscheduled DNA synthesis after BLM treatment (Table 1). This conclusion about DNA within cells may contradict previous results[6,8,11] indicating that much lower doses (0·1–10 $\mu$g/ml) of BLM are able to fragment both microbial and mammalian DNA to greater extents by means of alkaline sucrose gradient centrifugation. It is likely that the discrepancy may arise from the procedures for lysing cells. The cell lysates in previous experiments[6,8,11] were obtained by the use of alkaline SDS solution on top of the sucrose gradient according to the method of McGrath and Williams,[28] where EDTA is omitted. Our results obtained after cell lysis in the absence of EDTA (Fig. 4) account presumably for enzymic degradation of DNA which occurs extensively and nonspecifically. We do not know whether or not commercial BLM contains nucleases, but Haidle neglected this possibility.[10] Therefore, it is likely that activation of endogenous nucleases during cell lysis may be responsible for the major part of DNA degradation. Incidentally, the DNA of HeLa S3 cells irradiated intranuclearly with incorporated $^3$H is rendered more susceptible to DNases.[29]

The fact that DNA inside cells is not affected so severely by a 30-min short exposure to BLM (Fig. 3, Fig. 5 and Table 1) suggests that penetration of BLM through the cell membrane may be too difficult to act primarily on the intranuclear DNA as well as the ligase. Another possibility that cannot be ruled out is that BLM might be somehow inactivated promptly even if incorporated into cells. At present, this distinction is difficult because labeled BLM is not available. Moreover, it is apparent that repair-defective mutants (*uvr*$^-$ or *rec*$^-$) of bacteria are no more sensitive to BLM than wild strains.[14,15] Although a linear relationship between BLM dose and strand breaks *in vitro* has been demonstrated,[6–8,10,11] the dose–survival curve of HeLa S3 cells does not show a linear, but rather a biphasic relationship (Fig. 1), being not

very different from other mammalian cell lines tested so far.[13,25] Different and interesting results have been obtained with the closely related antibiotic, phleomycin. Phleomycin-resistant bacteria are unable to repair u.v. damage in their DNA,[30] indicating a correlation between excision ability and phleomycin sensitivity. Perhaps such a correlation is demonstrable in mammalian cells: excision-deficient rodent cell lines (mouse L[22] and Chinese hamster CHO[31]) have a greater resistance to phleomycin than HeLa cells possessing excision ability.[31] These facts do not favor a possibility that BLM causes DNA damage *in vivo* to the same extent as *in vitro*. Recently, Endo *et al.*[32] have presented evidence to indicate that BLM-sensitive mutation in *Escherichia coli*, located closely linked to the *lac* gene, may be involved in the permeability of the cell membrane through which BLM can enter the cells. Therefore, unlike the reaction *in vitro*, the cell membrane is assumed to play a basic role in controlling the toxicity of BLM to cells.

In another aspect, the action of BLM on the cell kinetics is correlated with that of phleomycin. The primary action of phleomycin may be due to a potent anti-mitotic effect, rather than to its inhibition of nucleic acid synthesis, since this antibiotic prevents HeLa cells from entering mitosis after DNA synthesis has been completed and when the concentration is so low as to have little effect on DNA synthesis.[33] Similarly, Barranco and Humphrey[25] reported that BLM interfered with cell progression only during the G2 phase. Therefore, the primary biological action of BLM is assumed to be interference with transcriptional or translational events involved with the synthesis of division-specific protein(s) which is presumably produced at the G2 phase. In this connection, our preliminary experiments with human lymphocyte cultures have shown that both short (4 hr) and prolonged (12 hr) exposures to 20 μg/ml of BLM before chromosome preparation induce chromosome aberrations (chromatid breaks and gaps) non-randomly and to a similar extent at a 10 per cent level of the cells which can enter mitosis successfully (Fujiwara *et al.*, manuscript in preparation).

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# 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 $G_1$ phase, the prolongation of S phase was slight, whereas the $G_2$ phase showed a considerable delay. Bleomycin also affected the entry of asynchronous cells into mitosis by arresting them at the early half of the $G_2$ phase. Interference did not occur with transition of mitosis to the $G_1$ 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.

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## 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 $G_1$ period, 9-hr S period, and 3-hr $G_2$ 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 $1 \times 10^6$ 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 $CO_2$ chamber at 37°. The synchrony procedure was carried out at 37°.

**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 $CO_2$ chamber at 37°. At 3 hr, when cells were attached on coverslips and the whole population of synchronous cells was in $G_1$ 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-$^3$H, 0.5 μCi/ml (5 Ci/mmole, 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 Photoindustry 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



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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 $G_1$ 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°. 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 $G_1$ 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-$^3$H 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 $G_2$ 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 $G_1$ 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 $G_1$-treated cells through the following cell cycle phases was examined by pulse-labeling cells with

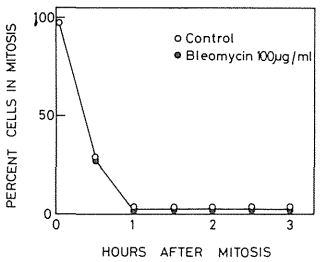


Chart 1. Effect of bleomycin on progression of mitotic cells into $G_1$ phase. The harvested mitotic cells were exposed continuously to bleomycin, 100 μg/ml. At 30-min intervals, samples were taken and prepared for determination of mitotic frequency. For each sample, mitotic frequency was determined by scoring 1000 cells.

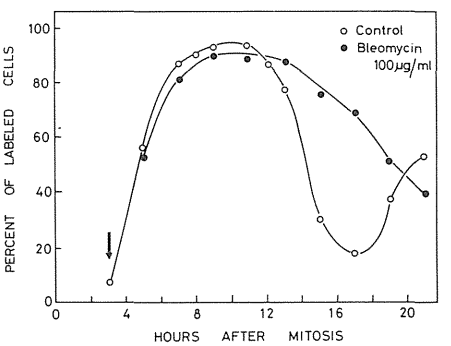


Chart 2. Effect of bleomycin on the DNA-synthetic phase of synchronous L-cells. At 3 hr after mitosis, bleomycin was added into culture medium to a final concentration of 100 μg/ml. Then, samples were taken at intervals and pulse labeled with thymidine-$^3$H (0.5 μCi/ml; 5 Ci/mmole) for 20 min. For each autoradiographic sample, the fraction of labeled cells was determined by scoring 1000 cells.

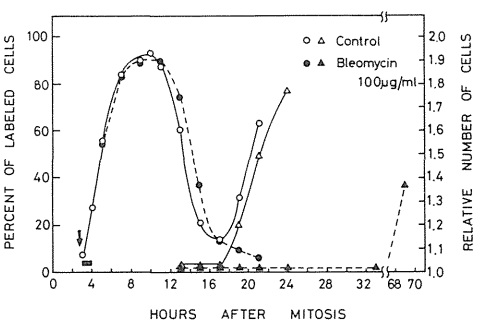


Chart 3. Effect of bleomycin on progression of synchronous cells pulse treated at $G_1$ phase. At 3 hr, bleomycin was added to cultures to a final concentration of 100 μg/ml. After 1 hr of incubation with the antibiotic, the cultures were rinsed twice with F10 medium and fed with fresh F10 medium for further incubation. After the treatment, samples were pulse labeled with thymidine-$^3$H 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).

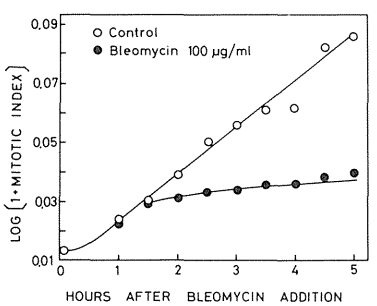


Chart 4. Effect of bleomycin on progression of asynchronous cells into mitosis. Bleomycin (100 μg/ml) and Colcemid (0.1 μg/ml) were added to asynchronous cultures of L-cells at zero time. Then, samples were taken at intervals and prepared for determination of mitotic frequency. *Ordinate*, accumulation of mitotic cells after addition of Colcemid in terms of the collection function log (1 + mitotic index) (10).

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 pulsewise 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

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<特 集>

# 細胞周期依存的感受性変動にもとづいた腫瘍治療の考え方

## "Proposals of Tumor Therapy Based on Cell Cycle Dependent Sensitivity Change."

— 放射線と抗腫瘍剤の併用 —

放射線医学総合研究所生理病理研究部
寺島東洋三

**要旨** 諸種の抗腫瘍剤に対する哺乳類細胞の感受性は細胞周期に依存して変化する。そのパタンから抗腫瘍剤をX線型とハイドロキシウレア型に分け，それらの有効な併用の可能性を求めた。

### はじめに

上手な放射線治療家はあたかも外科医のメスのように腫瘍をとり除くが，そういう治療家にとっても転移は，やりきれない。化学療法の併用はsystemic spread を抑えるかもしれないというところが放射線家にとっての魅力だ。逆に化学療法家の立場からすれば，少しでも systemic damage を減らす，つまり造血系，免疫系を救うという意味で放射線との併用は価値があるということになろう。

放射線家にとってのもう一つの希望は薬剤の腫瘍実質への浸透性である。御承知のように固形腫瘍の内部構造を大まかにみると，血管に近くて酸素の供給もよく細胞増殖のさかんな proliferative (or growing) fraction と，血管から遠くて酸素張力も従って低く，おそらく細胞増殖の乏しい hypoxic fraction と，そして necrotic fraction とがある。放射線家の泣きどころはこの hypoxic fraction で，腫瘍細胞の放射線感受性は2－3倍も低く，ために治療に抵抗して再発のもととなるからである。ある種の化学物質は浸透してhypoxic cells に達するということも知られてきたので，化学療法剤にも大いに望みを托したいのである。

そもそも放射線治療の領域では，腫瘍細胞に対する放射線効果を増強するためにずいぶん昔から増感剤 potentiator というものについて興味がもたれている。上にふれた酸素もその一つであるが，近年は electron-affinic sensitizer と称して放射線によって DNA 分子に生じたラジカルを固定し，細胞死へと導く一群の化学物質 NEM, TAN, NDPP, PNAP などが放射線生物学者達によって研究されている。やや古くは，DNA 分子に組み込まれることによって DNA を放射線に対し感作するハロゲン化ピリミジン (BUdR など) がある。こういう感作剤の他に，放射線により細胞内につくられた損傷の修復過程を阻止することによって放射線の細胞致死効果を高めようとする potentiator も考えられる。ことによると actinomycin D はその1例であるかも知れない。

Toyozo Terasima
Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, Chiba-si.

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potentiatior は相乗効果も考えられて大いに期待されているむきがあるけれども，そして抗腫瘍剤で potentiator であるものが見出されれば大いに意義があるのであるが，いわゆる相加作用 addition も意義が少ないわけではない。むしろ現存の抗腫瘍剤と放射線の併用という面では，どうスケジュールするかということが大事なのではなかろうか。本稿ではその細胞生物学的根拠の，それも一つの局面について述べてみよう。

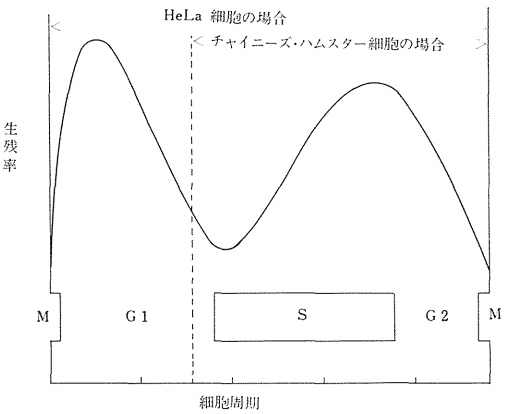
## Ⅰ　細胞周期と放射線感受性

哺乳類の培養細胞の多くは無限増殖能をもっていて，生体内の増殖系のモデルとして諸種の研究に役立っている[13]。それはつまり腫瘍の——正確にいえば proliferative fraction のモデルでもあり，増殖する腫瘍細胞の放射線感受性はその無限増殖能の不活化を集落形成能の存否を知ることによって測定される[14]。一体放射線による細胞の不活化，死はどんな機構で起こるのであろうか。それは未だに明らかではない。そもそもオーガニズムの死というものはきわめて複雑な現象で，直接の死因は心臓麻痺かもしれないが，よって来たるゆえんは多岐である。それでもわれわれはさまざまの間接的な証拠から，細胞の放射線死にはその構造体の青写真である DNA 分子の損傷が主要な役割を占めていると考えている[16]。

細胞は細胞周期と称する生活サイクルを繰り返すことによって増殖してゆくが，そのサイクル，一世代の間に DNA を複製して（DNA 合成期）分裂に備えなければならない。したがって，もし DNA が放射線による細胞死の標的であるとすれば，細胞の感受性は周期依存的変化を示すであろう。この仮説はわれわれの開発した哺乳類細胞の同調培養法を用いてはじめてテストされた[12][18]。

第1図は同調培養された HeLa 細胞の細胞周期依存的な感受性の変動を示す。横軸はM期（分裂期），$G_1$ 期，S 期（DNA 合成期），$G_2$ 期と称する四つのコンパートメントに分けられた細胞の一世代（通例約20時間）をあらわす。この細胞周期の間のそれぞれの時刻（細胞齢，age）において一

第1図　哺乳類細胞における細胞周期依存的なX線感受性の変動

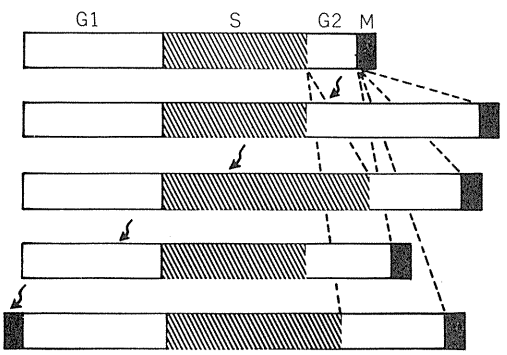



縦軸は単一線量照射後の生残率で感受性を表わす

定の線量を照射し，えられた生残率を模型的に表わすと，$G_1$-S 移行期と $G_2$-M 期に生残率は低く，つまり感受性が高く，$G_1$ 初期と S 後期に感受性が低い。この変動が仮定された標的分子である DNA の感受性なり，量の変化を示すものかどうか，という当初の問題はここでは立ち入って論じないことにする。要するに HeLa 細胞の感受性は細胞周期の間で二つのピークを示して変動するのである。放射線による腫瘍治療が腫瘍細胞の不活化，sterilization を原理とするかぎり，この変動は重要な意味をもつであろうことは多くの研究者によってすぐにさとられた。そしてさまざまの哺乳類培養細胞でしらべられ，感受性変動のパタンは本質的に全く同一であることが明らかとなった。たとえば，チャイニーズ・ハムスターのある種の細胞では $G_1$ 期がきわめて短かく（$G_1'$），ために細胞周期も応じて短かく，分裂期は第1図の破線で示す位置を占める。こういう細胞ではしばしば $G_1$ 初期の生残率のピークを証明しえないが，ある種の処置で $G_1$ 期を延長させると HeLa 細胞と同じ $G_1$ 期のパタンが明瞭になることも知られている。

上に述べられた細胞周期依存性感受性は放射線の致死効果に関するものであったが，同調培養の助けをかりて知られるもう一つの重要な放射線効

**第2図**　細胞周期の各コンパーメントに特異的な，X線による Progression の障害。



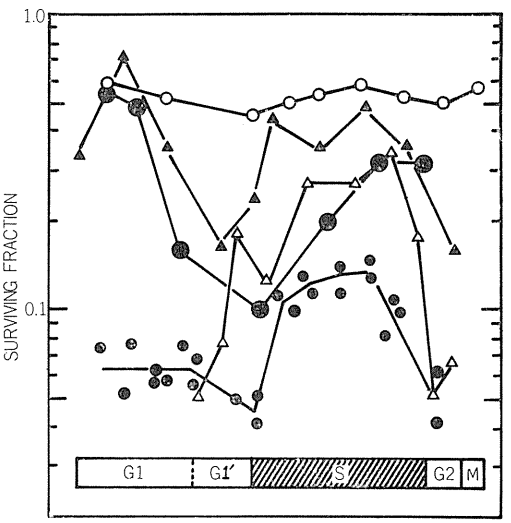

矢印はX線照射の時期を示す。

果は細胞の周期内進行，cell progression に対するものである。これがまた細胞周期内の各ステージに特異的であることが分った。第2図はその模型的表現である。もし細胞が $G_2$ 期で照射されると $G_2$ 期はきわめて鋭敏に反応して延長し，いわゆる分裂遅延が生じる（$G_2$ block）。S期で照射されるとS期自体が延長する他に，その損傷から回復した細胞は $G_1$ 期に入って再びその延長を示す。つまり $G_2$ block のための標的は $G_2$ からSにかけて長い間存在することがわかる。これに対し，$G_1$ 期の照射は $G_1$，S 両期にほとんど影響を与えず，軽度の $G_2$ の延長を示すにすぎない。それは $G_1$，S期の progression に関与する標的が $G_1$ 期にはないからである。このように放射線の cell progression に対する効果は照射をうけるステージに特異的である。その機序はほとんど明らかにされていないけれども，各コンパートメントの細胞のレスポンスは照射をうけた増殖集団の動力学を解くための重要な知見にちがいない。

## II　抗腫瘍剤と細胞周期

放射線効果の細胞周期依存性は抗癌剤をはじめとする細胞毒性物質の効果の研究にも少なからぬ影響を与え，とくに1960年代の後半からそれらの周期依存的作用が明らかにされはじめた。ここではわれわれの研究室でえられている知見と，外国の比較的信頼しうる，かつ細胞周期全体をカバーするデータをあつめて，二，三の図にまとめてみた。細胞系によって細胞周期の各コンパートメントの長さが異なるので，一つの細胞周期（横軸）の上に生残率をプロットする場合，ある程度正確さを犠牲にした時間の変換が行われざるを得なかった。実験によって薬剤の濃度が異なり，作用時間も30分あるいは1時間というようにちがうので，生残率のレベルにも相互のひらきがあるが，それらはそのまま転写された。

**第3図**　各種抗腫瘍剤に対する感受性の細胞周期依存性（I）.

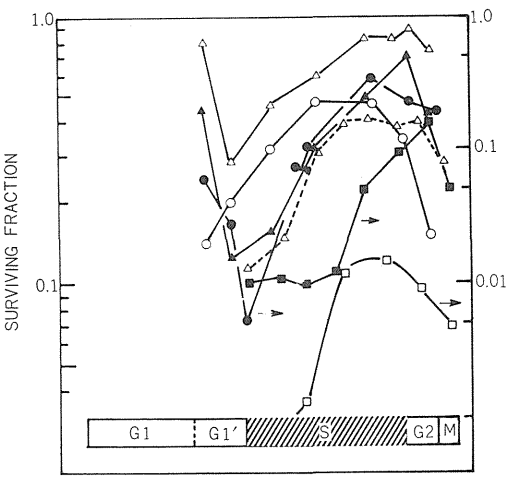



BLM〔マウスL細胞（高部等，未発表）○，HeLa細胞[15]●，バーキット・リンパ腫 P3HR-1 細胞(宮本ら未発表）▲，ヒト・リンパ腫T 1 細胞[4] △〕，MC〔HeLa 細胞[9]●〕

第3図は HeLa 細胞，マウスL細胞，ヒト・リンパ腫T 1 細胞，バーキット・リンパ腫細胞の bleomycin（BLM）致死作用に対する感受性を細胞齢の関数として示したものである。T 1 細胞の $G_1$ 期は他の細胞のそれらに比べて短かいので（$G_1'$），$G_1$ 初期の生残率のピークはみられないが，S期における生残率の高まりは他の細胞系と一致してみとめられる。これらの成績を大づかみにいえば，細胞は $G_1$ 後期と $G_2$-M期に感受性のピーク

をもつ二峰性のレスポンス・パタンをとり，X線のそれと同一であるといえる。なお，mitomycin C (MC) では，S期の生残率の山は明瞭であるが，$G_1$ 期のレスポンスはむしろフラットであるという点で特異的であった。

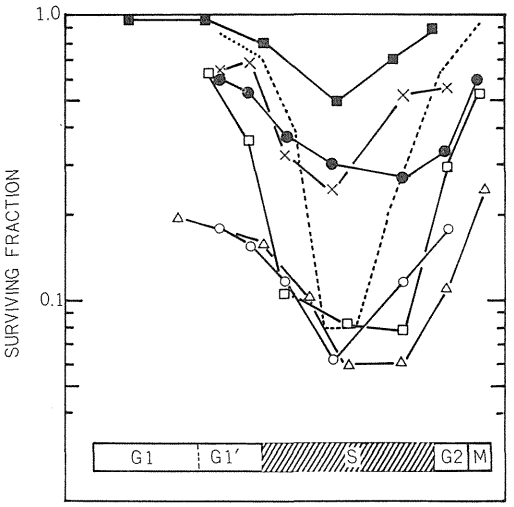
**第4図**　各種抗腫瘍剤に対する感受性の細胞周期依存性（Ⅱ）.




ACM〔チャイニーズ，ハムスター DON 細胞[2]△，同V79細胞[5]△…△〕，NGM〔DON 細胞[2]▲〕，NCS〔DON 細胞[2] ○〕，BCNU〔DON 細胞[2] ●〕，SMD〔V79細胞[8]■〕，NMD〔V79細胞[10]□〕，矢印は生残率のスケールを示す。

第4図はチャイニーズ・ハムスター由来の DON 細胞，V79細胞の諸種薬剤に対する感受性を示している。これらの細胞は $G_1'$ であらわされる短かい $G_1$ 期をもっているので，$G_1$ 期にあるはずの生残率のピークは明らかには検出されないということも考えられる。neocarzinostatin (NCS), nitrogen mustard (NMD), sulphur mustard (SMD) は単峰性のパターンを示すと思われるが，$G_1$ ピークの存在を示唆する actinomycin (ACM), nogalamycin (NGM), BCNU などと共通してS期，とくに後期のピークが明瞭である点，第3図にみた薬剤によるレスポンス・パタンと本質的に同一と考えられよう(ただし sulphur mustard によるピークがSよりむしろ $G_2$ にある点は解釈されない)。

**第5図**　各種抗腫瘍剤に対する感受性の細胞周期依存性（Ⅲ）



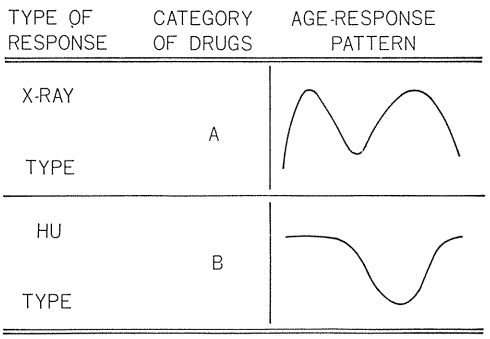

HU〔HeLa 細胞[6]■，V79細胞[11]……〕，ARA-C〔DON 細胞[2]●〕，5AZCR〔DON 細胞[2]○〕，CPTH〔DON 細胞[2]□〕，FUdR〔DON 細胞[2] ×〕，AM〔HeLa 細胞[7]△〕

第5図には前二図とは全く異なったパタンを示す一群の薬剤のそれが示される。hydroxyurea (HU), fluorodeoxyuridine (FUdR), camptothecin (CPTH), adriamycin (AM) などが示されているが，いずれも例外なしにS期細胞に対してより強い致死効果（他の時期の細胞もより低い効率で殺されるけれども――念のため）をあらわすことがわかる。生残率変化のパタンは単峰性であるが，そのピークはS期でなくて $G_2$-M-$G_1$ に位置を占める。

この他にも細胞周期の感受性パタンのしらべられたいくつかの抗腫瘍剤，代謝阻害剤があり，そのあるものはここにまとめられたものと同類であり，他のいくつかは異質である。そういうわけで細胞周期の感受性パタンに関しては薬剤によって若干のずれ，ないし異質性はあるけれども，概括的には第6図に示すようなこのタイプに分類しうるだろう。

一つはいうまでもなく X-ray type と称する二峰性の感受性パタンをもつもので，$G_1$-S 移行期

第6図 各種細胞毒性物質に対する細胞周期依存性感受性変化のパタン




と $G_2$-M 期にもっとも高い感受性を示す。radiomimetic といわれるようなもの，またはアルキル化剤とか，DNA 結合性薬剤とかがこれに属している。これに対し，HU type と類別される一群の薬剤は，機序の上で異なるかもしれないが，すべて DNA 合成阻害の能力をもつものである。そして重要なことはS期に感受性が高い単峰性のパタンで，X-ray type のS期に対し相反的，補足的であるという点である。

細胞周期依存的な感受性の変化は上述の致死効果の面ばかりでなく，cell progression に対する薬剤の効果の面でもみられる。X線の場合には前章で述べたように，細胞周期の各コンパーメントで与えられた損傷が引きつづくコンパートメントの進行にどう影響するかは，十分に定量的でないとはいえ，ほぼ明らかであった。それに比べると薬剤の場合の知見はまだ断片的であることを免れない。

たとえば，mitomycin C で HeLa 細胞の $G_1$ 期を短時間処理すると，$G_1$ は正常に経過するが，S期と $G_2$ 期に顕著な延長が起こされる[9]。bleomycin の $G_1$ 期処理は $G_2$ 期にのみ強い progression の障害，つまり延長を起こす。S期の延長はS期の継続的な薬剤処理によってのみ起こされ，$G_2$ 期処理は $G_2$ 期の延長に結果する[19]。このパタンはX線のそれとかなり類似していると考えられる。BCNU は $G_1$, S 期の他，著るしい $G_2$ 期の延長をおこすといわれる[1]。今までに知られて確からしいことは，progression に対する効果は X-ray type の薬剤の間でも必ずしもX線のそれと同一ではないということである。

HU type の薬剤のうち camptothecin は $G_1$ 期を延長させないが，他の HU type agents，たとえば 5-hydroxyproline, HU, cytosine arabinoside (ARA-C) などは $G_1$ 期の継続的な処理によってS期への進入を阻止し，$G_1$ 期の延長を導くという特長がある[3]。X-ray type の薬剤が概して pulse treatment によって各コンパートメントの progression に影響を与えるのに対し，HU type の作用は可逆的で，continuous treatment によってのみ効果を示すようである。HU type の薬剤の中で daunomycin (DM), adriamycin (AM) だけは $G_2$ 期を延長させるといわれている。これを要するに，両 type の薬剤の progression への効果はそれぞれの作用機序を反映してかなり特異的で，特定のパタンの中に統一しえない。

### III 細胞周期依存的な感受性変動（cell cycle dependent response）を基礎とした腫瘍治療のスケジュール

腫瘍の中の proliferative fraction はいうまでもなく同調増殖をしているわけではなく，ほぼランダムに増殖している集団である。つまり分裂期から $G_2$ 期までのさまざまな細胞齢の細胞がランダムに分布しているので，どの外来性作用体もそれに対し感受性の高いコンパートメントあるいは細胞齢の細胞を選択的に冒すであろう。ここに二種あるいは多種類の薬剤の併用の意義がある。

同時併用と時間差併用とが考えられる。後者の場合には同一の作用体のスケジュールされた投与法に還元されるであろう。いま X-ray type の薬剤を A, HU type の薬剤を B として可能なレジームを挙げてみると第1表のように整理される。

（i）はX線と X-ray type の薬剤の併用の可能性である。A・XはAとX線の同時併用であるが，第6図に示されるような同じパタンをもつ作用体の同時使用は一方の作用体を余計使ったことと同じで意味をなさない。しかししながら，もしAがX線の作用をpotentiateするなら他のpotentiator

**第1表** Possible Regimens of Multiple 2-Shots

| | | |
|---|---|---|
| (i) | a. | A●X……A●X…… |
| | b. | X—X……X—X…… |
| | c. | X—A……X—A…… |
| | d. | A—X……A—X…… |
| (ii) | a. | B●X……B●X…… |
| | b. | X—B……X—B…… |
| | c. | A●B……A●B…… |
| | d. | A—B……A—B…… |
| (iii) | a. | B—B……B—B…… |

●は同時併用，—は時間差併用をあらわす．

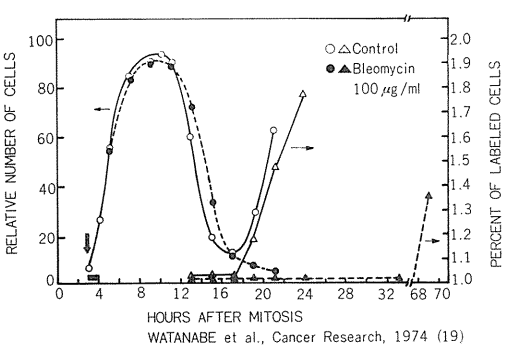
と同様に有用であるかもしれない。actinomycin Dは一つの候補であろう。

X-Xは最初のX線照射，すなわち conditioniug dose で生き残る $G_1$ 初期，S後期の細胞がそれぞれ $G_1$-S 移行期，$G_2$-M 期へ移行した数時間後に第2照射を試みるという方法で，放射線治療の一つの可能性あるスキームである。

同じアイデアで X-A という抗腫瘍剤との併用が考えられる。このような二分割投与とか時間差併用という場合には，mitomycin, neocarzinostatin のような exponential killing agent の方が sigmoidal killing agent より望ましいと思われる．

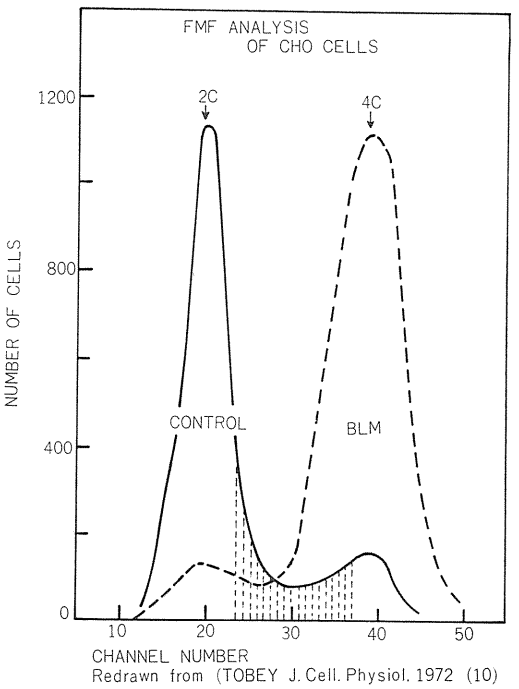
A-X は X-ray type agent の併用のうちでもっとも有望である。第7図の白印はマウスL細胞の

**第7図** ブレオマイシン (BLM) の $G_2$ 期延長効果。



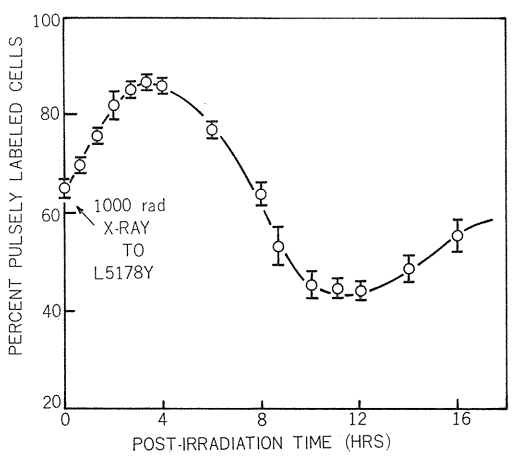

太矢印は BLM の作用時刻，細矢印はスケールを示す。実線は未処理対照，破線は BLM 処理：丸印は DNA 合成細胞の百分率，三角印は細胞数

**第8図** サイトフルオロメトリーによって明らかにされた BLM 処理細胞の細胞当り DNA 量の分布。




横軸は DNA 量の比較値，2C は $G_1$ 期細胞の DNA 量，4C は $G_2$-M 期細胞のそれを示し，影付けされた部分はほぼS期に当る。

**第9図** X線照射によって誘発されるS期細胞の蓄積。

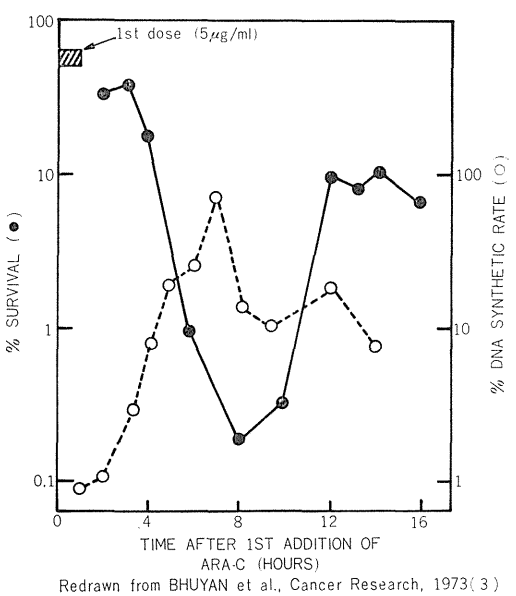


同調培養でみられる DNA 合成期とそれに引き続く細胞の同調分裂を示すが，$G_1$ 期の終り（分裂後3～4時間）で bleomycin の pulse treatment をすると，黒印で表わされるように正常の時間で DNA 合成を終えるが次の分裂は60時間頃まで遅延する[19]．すなわち多くの細胞は $G_2$ 期に蓄積する。第8図はチャイニーズ・ハムスター細胞を同じ薬剤で処理した後に起こる細胞の $G_2$ 蓄積を cytofluorometer で明らかにしたもので，ほとんどの細胞が4cの DNA 量をもつ $G_2$ 期に入っていることを示している[17]。アイデアは薬剤Aで $G_2$ 蓄積を誘発したところでX線照射を試み有効な sterilization を得ようということである。

(ii) は X-ray type と HU type の併用の場合である。B・X という同時併用は第6図の模型から考えて全く合理的である。

時間差併用としては X-B が大いに有望に思われる。前にも述べたようにX線は $G_1$ 期を冒さずにS期の延長を容易に誘導する。例を第9区に示そう。マウスのリンパ性白血病細胞L5178YはG$_1$期のきわめて短かい細胞系であって，通常の増殖時にはS期細胞は約30%を占めている。X線を照射するとS期は遅延し，その上 $G_1$ 細胞のS期への流入も加わってS分画の増加が起こり，4時間後には全集団の90%に達する（渡部，未発表）。この時刻における S-sterilizer としての薬剤Bの投与はもっとも合理的なスケジュールの一つといえる。

第10図 Cytosine arabinoside (ARA-C) の二分割投与による非同調チャイニーズ・ハムスター細胞 (DON) の生残率。




Redrawn from BHUYAN et al., Cancer Research, 1973(3)

上述の二つの併用法と同様に，X線の代りにAをおくこともできる。すなわち A・B および A-B で，その論理はX線の場合と全く同じく合理的である。しかしながら，本稿のはじめにもわずかふれたように，抗腫瘍剤の誘発する systemic damage，つまり正常組織の損傷も並大抵ではないので，いくらよいスケジュールであっても損得は相殺するかもしれない。

(iii) HU type のみで考えられるスケジュールはB-Bである。この種の薬剤は pulse treatment では比較的細胞の progression を冒さないので timing は容易である。第10図はチャイニーズ・ハムスター細胞のランダム集団に ARA-C の2回時間差投与した場合の細胞生残率の変化である[3]。最初1時間の pulse treatment の後，種々の間隔で第2回の pulse treatment (1時間) を行うと，8時間目にもっとも有効な不活化を与えうるが，その後は効果は低下する。第1回投与後の細胞集団におこる DNA 合成率の変化をみると（破線），1時間で不活化した DNA 合成は時間と共に一部回復すると思われるが，主として $G_1$ 期細胞のS期への流入によって増加し，7時間頃ピークに達して後減少する。これは一種の同調増殖の誘発を意味しているのである。DNA 合成率のピークは新らしいS期細胞集団の成立のおおむねの時刻を示しているのであって，つまりもっとも有効な薬剤Bの第2回投与時刻を間接的に教えている。

周期依存的な細胞のレスポンスを土台として腫瘍治療のレジームを考えてみたが，有望と思われるスケジュールは A-X, B・X, X-B であると判

断された。これに関連して2，3のコメントがなされるべきであろう。

(1) 治療の対象となる腫瘍が限局しているときはとくに放射線との併用が望ましい。およそ腫瘍の治療に免疫反応の積極的な関与を仮定するならば，抗腫瘍剤による systemic な損傷，すなわち造血・免疫系の不活化は最少におさえられなければならない。それにつけても，抗腫瘍剤による造血・免疫系の不活化の定量的知見がもっと入手されなければならない。

(2) 時間差併用にはまだ考慮すべき因子が多々ある。X線による時間差投与，すなわち二分割照射を行うとき，治療効果に影響する因子はa．亜致死損傷の回復，b．potentially lethal damage の回復，c．腫瘍細胞の repopulation である。これらの問題は抗腫瘍剤においてはまだほとんど解明されていない。薬剤による細胞の不活化曲線一つにしても，exponential か sigmoidal か，後者のときは亜致死損傷の回復があるか，などは基本的に重要な知見であろう。

(3) 細胞周期依存的なレスポンス変動の知識はいうまでもなく腫瘍の proliferative fraction にのみ妥当なもので，ここに検討されたレジームは non-growing fraction には全く適用されえない。したがって，治療によって誘発されるかもしれない non-growing fraction より proliferative fraction への細胞の動員，移動のカイネティクスも併せ考察されないことには，腫瘍治療のレジームとしては完成されない。

最近われわれの研究室では pleatau phase あるいは non-growing fraction の抗腫瘍剤に対する感受性を測定し，proliferative fraction のそれとの著るしい相異を観察しているが，その機序の解明は治療の完成への重要なステップの一つになるのではないかと考える。

## おわりに

細胞周期依存的な感受性変動の知識にもとづいて考えられるレヂームのうち，放射線と抗腫瘍剤の併用は現在のところもっとも実効性をもっていると考えられた。

しかしながら，最初申し上げたように，ここに検討された事柄は腫瘍細胞のふるまいの知識のほんの一端に基礎をおいているにすぎない，つまりおこがましい議論であったかも知れない。その洗練のためには上述のコメントに指摘したような諸点が抗腫瘍剤のそれぞれについて知られる必要があろうし，そして何よりも腫瘍細胞のsterilizationを中心原理とする放射線治療の幾多の論理が抗腫瘍剤療法に持ち込まれることが必要であろうと思われる。

この稿をおこすに当って，下記の方々の多大の御協力を仰いだ．記して謝意を表わす．

渡辺道典，高部吉庸，勝俣剛志，宮本忠昭（以上千葉大学内科化学療法研究グループ），渡部郁雄，西井正子（放医研，生理病理研究部）．

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## ——COMMUNICATION——

[GANN, 65, 559~560; December, 1974]

# DEMONSTRATION OF REPAIR OF POTENTIALLY LETHAL DAMAGE IN PLATEAU PHASE CELLS OF EHRLICH ASCITES TUMOR AFTER EXPOSURE TO BLEOMYCIN

During the course of experimental Bleomycin chemotherapy of mice bearing Ehrlich ascites tumor, we have found a remarkable increase in colony-forming efficiency of the tumor cells as a function of time after single exposure to Bleomycin. This finding indicates that Bleomycin induced a potentially lethal damage which could be repaired under *in vivo* condition.

Ehrlich ascites tumor cells, kindly given by Dr. M. Urano (National Institute of Radiological Sciences, Chiba), were grown by inoculation of $10^6$ cells in the abdominal cavity of ICR/JCL mice (CLEA Japan, Inc., Tokyo). Most experiments were carried out on the 7th day when tumor cells were in the early plateau phase.

The ascites tumor cells were able to grow in suspension in F10 medium[2] supplemented with 10% calf serum. For assaying the colony survival, the tumor cells appropriately diluted with the growth medium, after counting by the electronic counter, were mixed into 0.3% soft agar (Special Noble Agar, Difco) growth medium in plastic petri dishes (60×15 mm) (Falcon Plastics, Calif.) and grown in a $CO_2$-chamber for 10 days. The plating efficiency varied from 40 to 80% depending on batches of serum.

Bleomycin-complex (Lot #F100AS4) (Nippon Kayaku Co. Ltd., Tokyo) dissolved in distilled water was administered to tumor-bearing mice through a subcutaneous route.

Mice bearing 7-day tumor cells received a single shot of 30 mg/kg dose at 0 hr in Fig. 1. At the indicated time, the ascites was removed and a colony-forming ability of tumor cells was assayed by inoculation of a known number of cells. Repeated experiments consistently showed the increase of colony-forming efficiency which occurred rapidly during the first 3 hr, then slowly, and finally appeared to level off. Roughly 4 times more colony-forming efficiency than the 1-hr value was attained at 9 hr.

These results indicate that the damage given by Bleomycin was not immediately lethal and was repaired with time during which the cells remained *in vivo*. Therefore, it should be assumed that the potentially lethal damage was fixed when cells were brought into the *in vitro* assay system.

Little[3] has shown that potentially lethal damage was induced in the plateau phase culture of Chinese hamster cells by ionizing radiation and a fraction of the damage was repaired with time if the culture was allowed to remain in the plateau phase of growth. Recently, similar finding has been obtained with fibrosarcoma in mice after X-irradiation.[4] Our results present three features: (1) The repair of potentially lethal damage was demonstrated after exposure to the antibiotic,[1] Bleomycin, (2) plateau phase cells in the transplantable tumor were found susceptible to the production and repair of potentially lethal damage, and (3) contrary to other studies where the trypsin dispersion of cultured or tumor cells was always used for *in vitro* assay, the present use of ascites tumor cells did not require any trypsinization and, therefore, a possible change in trypsin sens:-

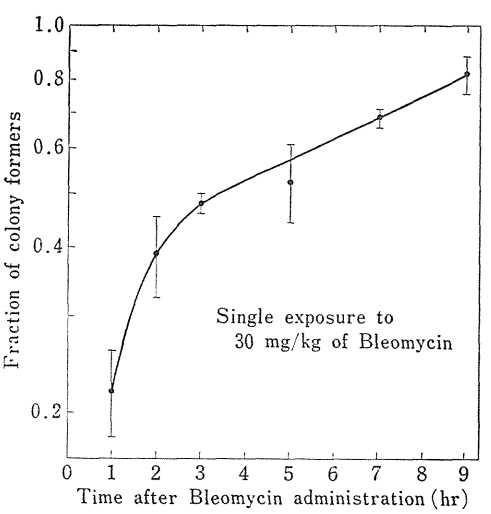


Fig. 1. Change in colony-forming fraction of Ehrlich ascties tumor cells after Bleomycin treatment



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tivity of drug-treated cells can entirely be excluded. The observed repair of potentially lethal damage should concern greatly the rational design of tumor therapy with Bleomycin. Further detail will be reported elsewhere.

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*First Department of Medicine,*
*Chiba University School of Medicine,*
*Inohana 1-8-1, Chiba 280*

Yosinobu TAKABE (高部吉庸)
Michinori WATANABE (渡辺道典)

*Division of Physiology and Pathology,*
*National Institute of Radiological Sciences,*
*Anagawa 4-9-1, Chiba 280*

Tadaaki MIYAMOTO (宮本忠昭)
Toyozo TERASIMA (寺島東洋三)

[Gann, 66, 141～147; April, 1975]

# OPTIMUM FRACTIONATION REGIMEN FOR BLEOMYCIN TREATMENT

Nobuo FUKUDA, Muneyasu URANO, Masahiro ENDO, and Koichi ANDO
*Division of Clinical Research, National Institute of Radiological Sciences**

A theoretical analysis was made on the optimum Bleomycin treatment regimen on the basis of the "binding–saturation model" which was proposed for the Bleomycin dose–cell survival relation. The surviving fraction of tumor cells decreased as a function of the number of fractionated treatments up to the optimum fractionation number if the tumor was treated with the same total dose. The effect of cellular sensitivity to the antibiotic, tumor doubling time, treatment interval, and total doses on the optimum regimen was analyzed. The importance of treatment interval and of tumor doubling time was emphasized and the short treatment interval was recommended for the clinical use of this antibiotic. The optimum number of fractions increased linearly with the increase of the total dose while the optimum single dose was independent of the total dose. A concept of the tumor control probability of tumors treated with the optimum fractionation regimen was introduced and implications of these analyses in the clinical cancer chemotherapy were discussed.

Extensive studies have been made on various effects of Bleomycin on mammalian cells.[14~16,18] It is now well argued that the response of mammalian cells to the antibiotic, either *in vitro* or *in vivo* is characterized by an upward concave survival curve.[1,5,14,16] The drug-resistance is interpreted by the hypothesis that anti-Bleomycin activity is introduced into a cell by the addition of the antibiotic.[14,16] In our previous paper, a hypothesis called "binding–saturation model" was proposed on the basis of these experimental findings.[16] A notable feature of this model is that the dose–response curve is possibly interpreted without assuming multi-components in cellular sensitivity to Bleomycin. The optimum regimen of fractionated Bleomycin treatments, which is a conflicting problem in a clinical tumor chemotherapy, could be obtained by the use of a model and will be analyzed theoretically in the present communication. These analyses might also be useful for chemotherapeutic agents which possess the saturation property[2,4] in their dose–cell survival curve.

**Mathematical Analysis of Dose Fractionation Regimen** The binding–saturation model is based on the assumptions that the Bleomycin molecule combines with the critical target in a cell in a rapid reversible manner and that surviving fraction of the Bleomycin-treated cell decreases with the increase of inactivated fraction of the target. The resulting expression for the surviving fraction, $S(D_1)$, of tumor cells after a single dose of $D_1$ mg/kg is as follows:

$$S(D_1) = \exp\left(-\frac{\sigma D_1 \ln S_{\min}}{\ln S_{\min} - \sigma D_1}\right) \tag{1}$$

where the initial sensitivity, $\sigma$, and minimum survival, $S_{\min}$, are defined by

$$\sigma = \lim_{D_1 \to 0}\left(-\frac{1}{S(D_1)}\frac{dS(D_1)}{dD_1}\right) \tag{2}$$

$$S_{\min} = \lim_{D_1 \to \infty} S(D_1) \tag{3}$$

The surviving fraction of tumor cells after equally fractionated $\nu$ doses of total $D$ mg/kg given at constant time interval, $T_i$ days, is

$$\ln S(D, \nu) = (\nu - 1)\frac{T_i}{T_d} + \frac{\sigma \nu D \ln S_{\min}}{\sigma D - \nu \ln S_{\min}} \tag{4}$$

where assumptions are made that tumor cells

* Anagawa 4-9-1, Chiba 280 (福田信男, 浦野宗保, 遠藤真広, 安藤興一).



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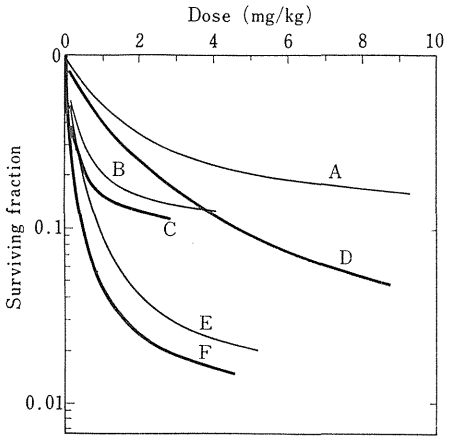


Fig. 1. Single Bleomycin dose-cell survival curves obtained theoretically on the basis of the "binding-saturation model"

Curves A~F reveal those of mammalian cells with various Bleomycin sensitivities. Sensitivity parameters employed are: $\sigma=1.0$ for curves A and D, 5.0 for B and E, 10.0 for C and F, $S_{min}=0.1$ for A, B, and C, and 0.01 for E, D, and F.

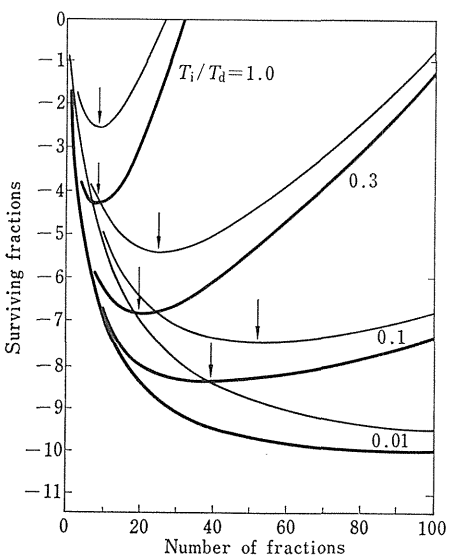


Fig. 2. Effect of $T_i/T_d$ on the dose fractionation regimen, expected when tumor cells with $\sigma=5.0$ and $S_{min}=0.1$ (thin lines) or 0.01 (thick lines) were treated with $D=5.0$ mg/kg

Arrows indicate the $\nu_{opt}$. With the decrease of $T_i/T_d$, $S(D, \nu_{opt})$ decreases while $\nu_{opt}$ increases

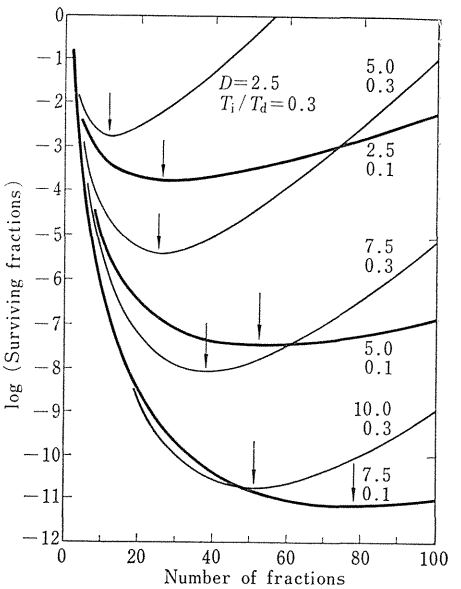


Fig. 3. Effect of $\sigma$ on the dose fractionation regimen expected for tumor cells treated with $T_i/T_d=0.3$ and $D=5.0$ mg/kg

$S_{min}$ is fixed at 0.1. $S(D, \nu_{opt})$ decreases exponentially with $\sigma$ and $\nu_{opt}$ increases linearly with $\sigma$.

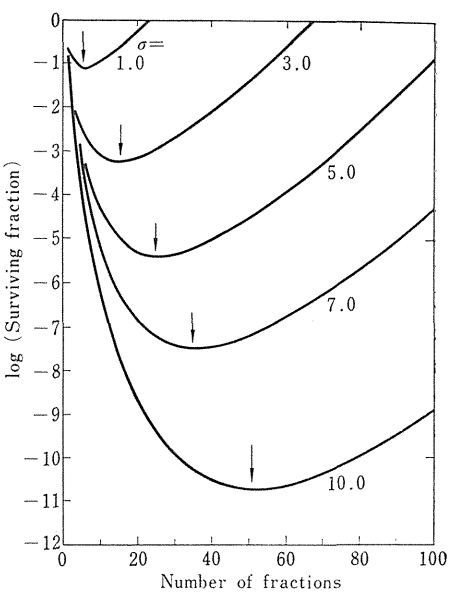


Fig. 4. Effect of $D$ on the dose fractionation regimen expected for tumor cells treated with $\sigma=5.0$ and $S_{min}=0.1$

With the incresae of $D$, $S(D, \nu_{opt})$ decreases exponentially while $\nu_{opt}$ increases linearly.

repopulate exponentially and asynchronously with tumor doubling time, $T_d$ days, throughout the treatment period and that equally fractionated doses are the most effective (see Appendix). It should be noted that $T_d$ is assumed independent of Bleomycin dose, and that the $S(D, \nu)$ vs. $\nu$ relation depends on four parameters, i.e., $\sigma$, $S_{min}$, $T_i/T_d$, and $D$. In Figs. 2~4, many examples of the effect of each one of these parameters on dose–fractionation regimen are presented while other three parameters are fixed. These curves are based on several model tumor systems in which tumor cells exhibit a single Bleomycin dose–cell survival relation presented in Fig. 1 and clearly demonstrated that $S(D, \nu)$ decreases as a function of $\nu$ up to the optimum number of fractions, $\nu_{opt}$, which reduces $S(D, \nu)$ to the minimum and is indicated by an arrow in Figs. 2~4.

**Effect of Some Factors on Dose–Fractionation Regimen**

Effect of $S_{min}$ and $T_i/T_d$: The effect of $S_{min}$ and $T_i/T_d$ on the $S(D, \nu)$ vs. $\nu$ curves is illustrated in Fig. 2, where $D$ and $\sigma$ are fixed at 5 mg/kg and 5.0, respectively, and thin and thick lines indicate $S_{min}$ of 0.1 and 0.01, respectively. It is notable from Eq. (1) that $S(D, \nu)$ is reduced proportionally with the decrease of $S_{min}$. This relation is also reflected in the $S(D, \nu)$ vs. $\nu$ curves, while the effect of $S_{min}$ is not so large as that of $T_i/T_d$.

As far as $T_i/T_d$ is concerned, the $S(D, \nu)$ vs. $\nu$ relation shown in Fig. 2 indicates that the smaller the $T_i/T_d$, the larger the $\nu_{opt}$ is, and that a fractionation schedule which reduces the $T_i/T_d$ as small as possible would sterilize tumor cells most effectively. For instance, if Bleomycin therapy is applied for a tumor which possesses $T_d$ of 10 days, 1-day interval schedule ($T_i/T_d = 0.1$) should be selected rather than 3-day interval (0.3) or 10-day interval (1.0) schedule. If a tumor whose $T_d$ is 1 day is concerned, even 1-day interval treatment might not reduce $S(D, \nu)$ to the surviving fraction required for the tumor control. The 3-day interval schedule which is used routinely in clinic would be applicable for a tumor which possesses $T_d$ of more than 30 days.

Effect of $\sigma$ and $D$: Several examples of the effect of $\sigma$ and $D$ on the $S(D, \nu)$ vs. $\nu$ relations are presented in Figs. 3 and 4, respectively. These curves demonstrate that $S(D, \nu)$ decreases with the increase of $\sigma$ or with that of $D$, if the same number of fractions is applied, and suggest an approximate exponential relation between $S(D, \nu)$ and $\sigma$ or $D$, which will be shown in Fig. 7. It is also notable that $\nu_{opt}$ increases linearly with the increase of $\sigma$ or $D$. These linear relations are shown in Figs. 5 and 6, and could be interpreted by minimizing Eq. (4) with respect to $\nu$, i.e.,

$$\nu_{opt} = C\sigma D \tag{5}$$

where,

$$C = \frac{(T_i/T_d)\ln 2 \ln S_{min} + \{(T_i/T_d)\ln 2\}^{1/2}(-\ln S_{min})^{3/2}}{(T_i/T_d)\ln 2(\ln S_{min})^2} \tag{6}$$

and $$(T_i/T_d)\ln 2 + \ln S_{min} < 0, \quad C > 0 \tag{7}$$

Eq. (5) also indicates that an optimum single dose is independent of $D$, e.g., if a tumor with a fixed sensitivity of $\sigma = 5.0$, $S_{min} = 0.1$, is treated with a fixed $T_i/T_d$ of 0.3, an optimum single dose is 0.2 mg/kg without regard to $D$ administered.

$TCD_{50}$ (BLM, $\nu_{opt}$): The 50% tumor control dose ($TCD_{50}$) has been introduced as a radiation dose which yields a local tumor control in one-half of irradiated tumors on the basis of multi-target response law.[11,12] A relevant analysis could be applied to the Bleomycin dose which yields a tumor control in one-half of Bleomycin-treated tumors. To derive the expression for $TCD_{50}$ (BLM, $\nu_{opt}$) given in the optimum fractionation regimen, let us redefine $S(D, \nu)$ in Eq. (4) to be that of tumor cells at $T_i$ days after the termination of treatments and insert Eq. (5) into Eq. (4). Then an approximate exponential relation as shown in Fig. 7 is obtained between $S(D, \nu_{opt})$ and $D$ as follows:

$$\ln S(D, \nu_{opt}) = -C\sigma\left(\frac{T_i}{T_d}\ln 2 + \frac{\ln S_{min}}{1-C\ln S_{min}}\right)D \quad (8)$$

$$= -D/D_0 \quad (9)$$

$$S(D, \nu_{opt}) = e^{-D/D_0} \quad (10)$$

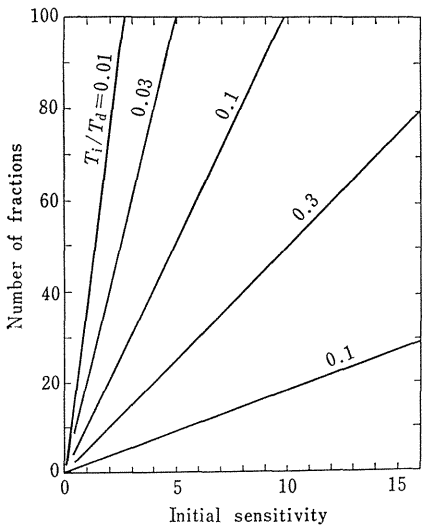


Fig. 5. Relation between $\nu_{opt}$ and $\sigma$
Linear relation is observed if tumor cells are treated with a fixed $T_i/T_d$.

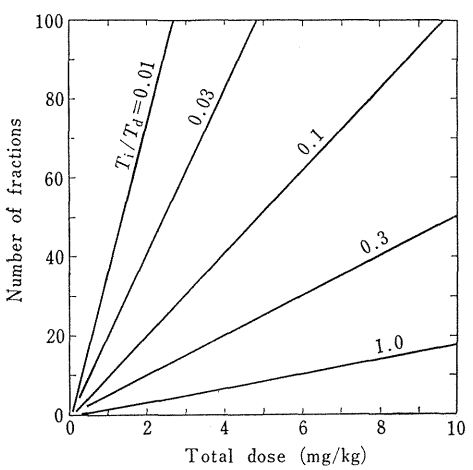


Fig. 6. Relation between $\nu_{opt}$ and $D$
Linear relation is observed if tumor cells are treated with a fixed $T_i/T_d$.

$$D_0 = \frac{1}{C\sigma\left(\frac{T_i}{T_d}\ln 2 + \frac{\ln S_{min}}{1-C\ln S_{min}}\right)} \quad (11)$$

where $D_0$ implies a Bleomycin dose which decreases the surviving fraction from 1 to 1/e in the exponential portion of the optimum fractionation dose–cell survival relation, and is proportional to $1/\sigma$ in the single dose–cell survival curve.

Provided that a single tumor cell surviving the treatments could produce a tumor recurrence and that the probability of tumor control, $p$, obeys the Poisson's law, the probability of tumors initially containing a total of $M$ cells and treated with total $D$ mg/kg of Bleomycin in an optimum fractionation regimen is defined by

$$p = e^{-S(D, \nu_{opt})M} \quad (12)$$

and $TCD_{50}$ (BLM, $\nu_{opt}$), i.e., Bleomycin dose which produces the local tumor control with $p$=0.5 is expressed as

$$TCD_{50}(BLM, \nu_{opt}) = D_0(\ln M - \ln\ln 2) \quad (13)$$

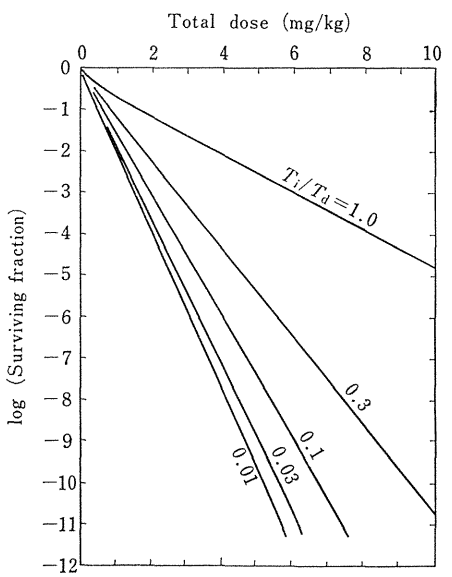


Fig. 7. Relation between $S(D, \nu_{opt})$ and $D$
Exponential relation is observed, indicating that the optimum single dose is independent of $D$, if tumor cells are treated with a fixed $T_i/T_d$.

## Discussion

### Implications of the Model in Human Cancer Treatment

Many informations on tumor control mechanism have been provided by radiation biology.[7,11] It is argued that the tumor could be controlled by a radiation dose after which several hundred tumor cells might remain without receiving the lethal damage.[10,11,17] In one of our previous papers,[17] it was proposed that cell population kinetics of non-treated and sublethally irradiated tumor cells should be taken into account in the analysis of tumor control probability, and the division probability model[9] was applied for this purpose. Assuming the same procedure for the tumor control after fractionated Bleomycin treatments, Eq. (13) could be rewritten as follows:

$$\mathrm{TCD}_{50}\,(\mathrm{BLM}, \nu_{\mathrm{opt}}) = D_0\,[\ln\{M(2Pd-1)(2Pd_x-1)\}-\ln\ln 2] \qquad (14)$$

where $Pd$ is the division probability of tumor cells before treatment and $Pd_x$ is that of tumor cells surviving fractionated treatments. The fraction of tumor cells which are able to undergo at least one more division before or after treatment is represented by $(2Pd-1)$ or $(2Pd_x-1)$, respectively, and the latter was introduced on the basis of the extinction probability instead of assuming that one surviving tumor cell could produce a tumor recurrence.[17]

In Fig. 8, $\mathrm{TCD}_{50}$ (BLM, $\nu_{\mathrm{opt}}$) required when tumors containing $10^9$ tumor cells were treated with the optimum fractionation regimen are presented as a function of $\sigma$, provided that $Pd$ and $Pd_x$ are 0.55 and 0.505, respectively (cf. Ref. 17). There are also shown other values (scale B-D) useful for tumors which contain different number of

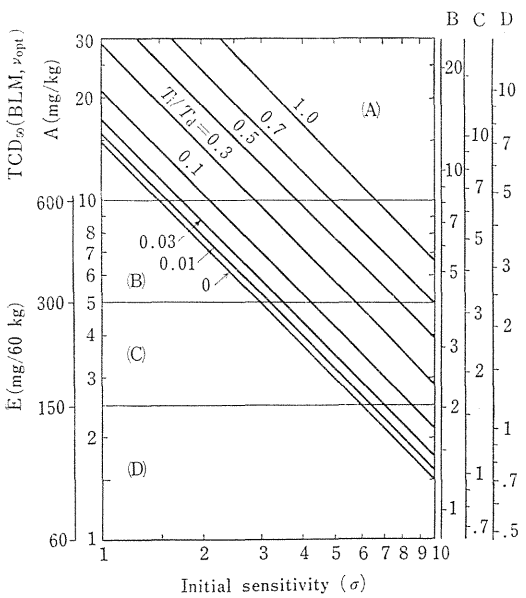


Fig. 8. $\mathrm{TCD}_{50}$ (BLM, $\nu_{\mathrm{opt}}$) and $\sigma$

Scales A~F are for tumors containing different number of tumor cells or with different $Pd$. $M$ and $Pd$ for scales A~D are: A, (1) $10^9$, 0.55, (2) $10^8$, 1.0; B, (1) $10^9$, 0.505, (2) $10^8$, 0.550, (3) $10^7$, 1.0; C, (1) $10^8$, 0.505, (2) $10^7$, 0.550, (3) $10^6$, 1.0. Or scales B~D might be applicable for paliative therapy of tumors of, e.g., $M=10^9$ and $Pd=0.550$. Scale E is for patients of 60 kg body weight and values correspond to scale A. Area (A) means non-effective, (B) resistant, (C) relatively sensitive, and (D) sensitive.

Table I. Optimum Number of Fractions and Optimum Single Dose

(A) Optimum number of fractions and $T_i/T_d$

| Expected SF after treatments | $T_i/T_d$ 1.0 | 0.3 | 0.1 | 0.03 | 0.01 | 0 |
|---|---|---|---|---|---|---|
| $5\times10^{-7}$ | 24 | 30 | 44 | 73 | 122 | ∞ |
| $5\times10^{-6}$ | 20 | 25 | 37 | 62 | 102 | ∞ |
| $5\times10^{-5}$ | 16 | 20 | 30 | 50 | 83 | ∞ |
| $5\times10^{-4}$ | 12 | 15 | 23 | 38 | 63 | ∞ |

SF = Surviving fraction of tumor cells

(B) Optimum single dose and $T_i/T_d$

| $\sigma$ | $T_i/T_d$ 1.0 | 0.3 | 0.1 | 0.03 | 0.01 | 0 |
|---|---|---|---|---|---|---|
| 1.0 | 2.5 | 1.0 | 0.50 | 0.24 | 0.13 | 1/∞ |
| 2.0 | 1.43 | 0.50 | 0.25 | 0.12 | 0.067 | 1/∞ |
| 3.0 | 1.0 | 0.33 | 0.16 | 0.081 | 0.044 | 1/∞ |
| 5.0 | 0.56 | 0.20 | 0.096 | 0.048 | 0.027 | 1/∞ |
| 7.0 | 0.40 | 0.14 | 0.069 | 0.035 | 0.019 | 1/∞ |
| 10.0 | 0.28 | 0.10 | 0.048 | 0.024 | 0.013 | 1/∞ |

Values in mg/kg.

Note that the optimum number of fractions is independent of $\sigma$, and the optimum single dose is independent of $D$. $S_{\min}$ is fixed at 0.1.

tumor cells or possess different *Pd* (cf. legend for Fig. 8). In this graph, linear relation is observed between ln $TCD_{50}$ (BLM, $\nu_{opt}$) and ln $\sigma$, while $\nu_{opt}$ for $TCD_{50}$ (BLM, $\nu_{opt}$) depends only on $T_i/T_d$ and is independent of $\sigma$, as already shown in Fig. 6 and Eq. (5). Numerical examples of $\nu_{opt}$ for corresponding $TCD_{50}$ (BLM, $\nu_{opt}$) are listed in Table I, together with the optimum single doses which are independent of $D$ as described above.

The importance of $T_i/T_d$ for tumor control probability is again demonstrated in Fig. 8. For instance, if a tumor with $\sigma$ of 5.0 is treated with $T_i/T_d$ of 1.0, $TCD_{50}$ (BLM, $\nu_{opt}$) would be as high as 14 mg/kg, while it could be 6.0 or 3.5 mg/kg if treated with $T_i/T_d$ of 0.3 or 0.1, respectively. However, it is difficult to select an adequate $T_i$ since $T_d$ of tumors, especially that of tumors under treatment, is usually unpredictable in human patients. Human tumors are usually reported[8] to have a long $T_d$ and the repopulation capability of tumor cells during the treatment period is often neglected in the analysis of fractionated treatment.[9] The cell progression throughout the cell cycle was found to be delayed in $G_2$ phase after Bleomycin.[1,19] On the other hand, some experimental results demonstrated rather rapid repopulation of treated cells. Mouse mammary carcinoma cells exhibited $T_d$ as fast as the cell generation time after a large single X-ray dose.[13] An evidence found in our previous study was that 4 fractionated administrations of 25 mg/kg each of Bleomycin delayed the growth of mouse mammary carcinoma for 6 days and this fact also suggested such rapid repopulation of treated tumor cells.[16] Therefore, a short $T_i$ should be recommended for clinical Bleomycin therapy, while the shortest $T_i$ should allow the complete excretion of the antibiotic from the treated cells, i.e., approximately 4 hr in a cultured mammalian cell line,[14] for the reduction of Bleomycin resistance.

It is certainly important to treat a cancer patient without producing a severe side effect. Therefore, the optimum treatment regimen should mean a regimen which allows the maximum therapeutic ratio, $TR(D)$. The $TR(D)$ after total $D$ mg/kg Bleomycin could be represented by

$$TR(D) = f\{H(D), e^{-MS(D,\nu_{opt})}\} \quad (15)$$

where $H(D)$ is an approximate measure of the side effect.

Let us tentatively assume that the side effect is lethal and is measured by the following surviving fraction of cells in critical normal tissue, $S_n$(D),

$$S_n(D) = e^{-D/D_n} \quad (16)$$

Hence,
$$TR(D) = e^{-D/D_n} e^{-MS(D,\nu_{opt})} \quad (17)$$

Eq. (17) is interpreted as the probability that the number of surviving tumor cells is reduced to zero and the critical normal tissue does not receive any destructive effect after drug treatments. Then the true optimum dose, $D_{opt}$, of the antibiotic which maximizes $TR(D)$ is given by

$$D_{opt} = D_0 \ln \{M(D_n/D_0)\} \quad (18)$$

The corresponding surviving fraction of tumor cells, $S(D_{opt})$, and of normal tissue cells, $S_n(D_{opt})$, are represented respectively as follows:

$$e^{-MS(D_{opt})} = e^{-D_0/D_n} \quad (19)$$

$$S_n(D_{opt}) = (MD_n/D_0)^{-D_0/D_n} \quad (20)$$

A difficulty to obtain $TR(D)$ in the clinical Bleomycin therapy is that the cellular sensitivity to Bleomycin varies from tumor to tumor and from tissue to tissue.[16] It is now known that a total of 150 mg/kg could be administered without inducing pulmonary fibrosis, i.e., the most severe side effect of this antibiotic, while a total of 300 mg/kg produces it in a few percent of the patients.[3] In Fig. 8, $TCD_{50}$ (BLM, $\nu_{opt}$) is divided into 4 dose ranges, i.e., sensitive ($<150$ mg), relatively sensitive (150～300 mg), resistant (300～600 mg), and non-effective ($>600$ mg), and for clinical convenience these values are indicated in scale E which corresponds to patients of 60 kg body weight in scale A. It is noted that a total of 300 mg of Bleomycin might control 50% of tumors which contains $10^9$ tumor cells with $\sigma$ larger than 3.0 and a total of 150 mg could control tumors con-

taining $10^9$ tumor cells with $\sigma$ larger than 6.0 if an adequate $T_i/T_d$ is selected.

Finally, we would like to point out that the present analyses were based on some assumptions such as that tumor cells repopulate exponentially with constant $T_d$ throughout the fractionated treatments. Also the age-dependent Bleomycin sensitivity is not included. These assumptions might constitute a vulnerable point in this study. However, an exponential repopulation is likely if the short treatment intervals are applied. Age-dependent sensitivity of tumor cells has never been successfully demonstrated in solid tumors treated with X-rays which are typical age-dependent agents, while $T_d$ is shown to be dose dependent.[17] Therefore, more detailed analysis in which these inevitable factors are taken into account could follow more precise experimental or clinical studies, especially those of experimental animal tumors.

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## Appendix

### Equi-partition

Eq. (4) in the text was based on an assumption that the equi-partition (equally fractionated doses) is the most effective fractionation regimen. This assumption could be justified if it is proved that the solution of the following extreme problem

$$\frac{\partial}{\partial D_{1j}}\{\sum_{i=1}^{n} \ln S(D_{1i})\}=0 \quad (j=1, 2, \ldots, n) \tag{A1}$$

with a constraint

$$\sum_{j=1}^{n} D_{1j}=D \tag{A2}$$

is given by

$$D_{11}=D_{12}=\ldots=D_{1n}=\frac{D}{n} \tag{A3}$$

By using the Lagrangian undetermined multiplyer, $\lambda$, Eq. (A1) and (A2) are rewritten as

$$\frac{\partial}{\partial D_{1j}}\{\sum_{i=1}^{n} \ln S(D_{1i})+\lambda(\sum_{i=1}^{n} D_{1i}-D)\}=0 \quad (j=1, 2, \ldots, n) \tag{A4}$$

Hence, $$\frac{\mathrm{d} \ln S(D_{1j})}{\mathrm{d}D_{1j}}=-\lambda \quad (j=1, 2, \ldots, n) \tag{A5}$$

Since the right-hand side of Eq. (A5) is independent of $D_{1j}$ $(j=1, 2, \ldots, n)$, one can equate the arguments of the left-hand side as Eq. (A3), providing the single valuedness of the function which is equivalent to the following inequality.

$$\frac{\mathrm{d}^2 \ln S(D_{1j})}{\mathrm{d}{D_{1j}}^2}=\frac{-2(\sigma \cdot \ln S_{\min})^2}{(\sigma \cdot D_{1j}-\ln S_{\min})^3} \neq 0 \quad \text{for } \ln S_{\min}<0 \tag{A6}$$

特　集

## 第6回放医研シンポジウム*

# 腫瘍治療の生物学的基礎

## 腫瘍治療の生物学的基礎　序

寺　島　東洋三**

**Introduction to "Biological Basis of Tumor Therapy"**: T. Terasima (Division of Physiology and Pathology, National Institute of Radiological Sciences)
Preferential sterilization of tumor cells, a major principle of tumor therapy, constitutes a formidable problem. This can not readily be effected by present radio- and chemo-therapy. However, a great improvement would be expected through studies on proliferation kinetics in tumor as well as normal tissues.
One should also look for other possibilities of tumor cure in investigations for control of metastasis and for immunological, endocrine as well as differentiational control of tumor cells.
Key words : Tumor therapy, Proliferation kinetics
*Jap. J. Cancer Clin.*, **21**(9): 651～652, 1975.

この小文は表題のシンポジアムについての企画者の見解・意図を述べたものとご了解願いたい．

現在，腫瘍治療は3つの異なるアプローチに基礎をおいている．すなわち外科的治療，放射線療法，化学療法である．外科的なアプローチはいうまでもなく原発巣と所属リンパ腺を根こそぎとり去ることによつて多くの primary cancer に対し大変有効であるが，ひとたび播種された病巣に対しては手を施すことができないという限界ももつている．したがつて放射線やとくに抗腫瘍剤に少なからぬ期待が寄せられるのであるが，そこには逆に相当程度の systemic な損傷を覚悟せねばならぬというジレンマがある．そういう状況でわれわれは腫瘍治療について考えを深め拡め有効な方途を探ろうというわけである．

元来，腫瘍の治療というものは外科的な腫瘍切除によつて象徴されるように，腫瘍細胞の Vernichtung を基本原理とするものであつたし，今でもそうである．そのとき治療にとつての肝要な条件は高い治療効果比をうることができるかどうかという点である．つまり正常組織とその機能を温存しながら，腫瘍細胞を選択的に破壊することが望まれる．化学療法におけるような systemic な agents においてとくにしかりである．

20世紀における医学・生物学を支配する思想の1つは Ehrlich の "Seitenkettentheorie" に基づく特異性の概念である．この考えは免疫学の発展にかんしては言うまでもなく，感染性寄生体の preferential sterilization に圧倒的成功をおさめたが，これが抗腫瘍剤への適用は必ずしもみるべき成果をもたらさなかつたように思われる．そもそも細胞に対する薬剤作用の選択性は，腫瘍細胞と正常細胞との間で薬剤の1）蓄積性が異なる，2）活性化する能力に差がある，3）不活化する力に差がある，4）作用点が質的，量的に異なる，ときにあらわれる．現在までにえられた薬剤はこうした選択性の面でまだ十分に成功しているとは思われない．近年発見されたブレオマイシンは腫瘍特異性を示さないとはいえ，組織選択性をもつた顕著な1例であつて，治療効果比の改善にかなり意義のあるものであると考える．

他方，放射線作用においては，1906年 Bergonié et Tribondeau によつて "分裂している，あるいは分化度の低い細胞がとくに高い感受性を示す" ということが記載され，以来放射線癌治療の基本原理の1つとみなされてきた面もある．この観察の重要性は，一般に何となく考えられているように，正常細胞と腫瘍細胞の放射線感受性の差を指摘したことではなくて，むしろ感受性は組

* 於放射線医学総合研究所
1974年12月13，14日
** 放射線医学総合研究所生理病理研究部

癌の臨床　21，651-652，1975

織の増殖力 proliferative potential（その kinetics を含めて）に関連していることを示唆している点にある．私見では細胞の増殖能を指標とする限り，正常細胞と腫瘍細胞の放射線に対する感受性は変らない．二，三の実験結果ではむしろ正常細胞がより高い感受性を示しさえする．しかしながら，治療という practice を経てわれわれが観察し，そこから抽出しうるものは腫瘍組織と正常組織の間の“tolerance”の差であつた．これは細胞レベルの感受性と組織レベルのメカニズムとの複雑なからみあいの結果と解釈されよう．それでは組織レベルのメカニズムとは具体的に何であろうか．腫瘍組織にせよ正常組織にせよ，それらの外来性因子に対するレスポンスはいくつかの因子に依存するであろう．すなわち，1）構成細胞の量，2）増殖相や細胞周期に依存する細胞の感受性，修復力，3）幹細胞や non-dividing clonogenic fraction の存在，そのプールサイズ，4）repopulation や cell loss の速度，5）細胞分化の能力，6）間質の量などがこれである．これらを包括的に cell proliferation kinetics とよぶならば，これが治療比を生み出す根底となつているのではないだろうか．

このシンポジアムの前半は多かれ少かれ今述べたようなバックグランドにおいて理解されるであろう．とくに治療効果比に深く立ち入るための基盤として，1）cell proliferation の知見の拡大，2）それに基づいていかに有効な腫瘍細胞の sterilization をうるか，3）担癌個体における正常組織の機能，正常細胞の感受性などの諸点について努力が傾けられるであろう．

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腫瘍はちようど外来性の infectious agents のように，方法はともあれ，sterilize されるべきであるという考えが正統的な考え，あるいは態度であつたといえよう．しかしながら悪性腫瘍が宿主の構造と統御機構の枠組の中に生活する寄生生物である以上，当然宿主から独立していることはありえないし，たとえある種の自律性をもつているとしても，そういう抽象はことがらの正しい把握には至らないだろう．つまり癌という新しい組織は構造的にも機能的にも宿主によつて支持されたり規制されたり，しながら生きている．この宿主との関係，あるいは“やりとり”の中から治療へのいとぐちを探ろうという努力がこのシンポジアムの後半に課せられる．

すなわち，1）悪性腫瘍の増殖様式のきわだつた局面である転移と宿主側の要因，レスポンスを通じて，それから2）体液性統御機構，分化機構あるいは特異的，非特異的免疫機構の腫瘍への影響，腫瘍との相互作用を通じて，また治療によるこれらの相互関係の修飾，活性化などを通じて，腫瘍の認識，制御，共存，変質，排除の可能性を求めるつもりである．

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シンポジアムの終りにおいて癌治療および研究の将来像について考えたいと思う．そのためには従来の治療の成果，癌という疾病の生態学などについて検討されるかもしれない．それらの知見をふまえて治療の実効性とか意義，つまり治療の個人，社会，種族への関連という点にもできれば考え及びたい．

このシンポジアムは多くの学会の分科会にみられないような multidiciplinary な構成をとつているので，それぞれの専門領域の間の相互認識に役立つであろうと期待される．本シンポジアムのもう1つの意義として付け加えておこう．

# 1. 腫瘍治療と細胞の感受性

寺島東洋三* 渡辺道典
高部吉庸*** 宮本忠昭**

**Cell Sensitivity to Exogenous Agents as a Basis of Tumor Therapy:**
T. Terasima*, M. Watanabe, Y. Takabe*** & T. Miyamoto** (*Division of Physiology and Pathology, **Hospital, National Institute of Radiological Sciences & ***First Department of Medicine, Chiba University School of Medicine)
Dose-survival relationship of mammalian cells to exogenous agents, as determined by *in vitro* and *in vivo* assays, provides a basis for tumor therapy. The dose-survival response can be altered by several cellular factors: (1) cell cycle dependence of sensitivity, (2) growth phase dependence of sensitivity, (3) repair of sublethal damage, (4) repair of potentially lethal damage, and (5) cell-drug interaction. All these factors involves deeply in a therapeutic design of tumor.
In the present paper, repair of potentially lethal damage and cell-drug interaction, the most recent development in studies of these factors, were mentioned in relation to bleomycin action on mammalian cells. On the basis of these cellular factors the pros and cons of different therapeutic designs by bleomycin were discussed. Cell sensitivity (cell cycle, growth phase) repair (sublethal damage, Key words: potentially lethal damage) dose-survival response
*Jap. J. Cancer Clin.*, **21**(9): 674～680, 1975.

## 1. 細胞の感受性

腫瘍治療の原理は tumor cell sterilization, つまり細胞の増殖力の不活化である. したがつてその定量的知見, すなわち細胞の "dose-survival relationship" (放射線領域では通例, 線量効果関係などと称するが, ここでは薬剤投与量, 作用量などの意味もあるので, 仮に survival response と略記しよう) は放射線治療でも化学療法においてもその治療デザインの確立のために等しく重要である.

survival response の決定には *in vitro* assay と *in vivo* assay とがある. 前者はガラス器内における細胞の無限増殖能の測定, すなわち集落計数法であり, 培養細胞, *in vivo* の細胞に適用される[1]. 後者は *in vivo* の細胞を *in vivo* あるいは *in vitro* で作用体の処置を受けさせ, 単離細胞にしてから *in vivo* に接種するもので, (1) 脾, 肺に増殖細胞の集落をつくらせて計数する nodule counting[2-4] と, (2) 増殖細胞の50%定着率を示す接種サンプルの稀釈率から増殖細胞濃度を求める $TD_{50}$ assay[5] とがある. この他に正常組織 (腸上皮, 脾, 皮膚) を構成している細胞のレスポンスを知る方法として, endogenous colony assay[6] が用いられることがある.

放射線のレスポンスに関しては, *in vitro*, *in vivo* assay の結果はほぼ一致しているので, この survival response は腫瘍細胞の不活化を推定するに役立つている. しかしこの assay の結果が必ずしも *in vivo* の細胞の不活化動態を予言しないような実験系も知られてきて, 新しく大きい問題を提起しつつある (たとえば, 後述の potentially lethal damage の回復). これに対し, 薬剤では事情はさらに複雑である. *in vivo* での薬剤の吸着, 不活性化, 活性化, あるいは細胞への蓄積, 透過性などさまざまの因子によつて, *in vitro* assay の結果は *in vivo* の状況を正しく反映しないことが多い. *in vitro* assay と *in vivo* assay の等価性, 対応は *in vitro* の研究の拡大によつて将来に解決を期するものとしてひとまず措き, *in vitro* における細胞の感受性を修飾する要因について考えてみよう.

### 1. 細胞周期と感受性

1961年 Terasima & Tolmach[7,8] によつて哺乳類細胞の放射線感受性は, 細胞周期に依存して変動すること

* 放射線医学総合研究所生理病理研究部
** 放射線医学総合研究所病院部
*** 千葉大学医学部第一内科学教室

癌の臨床 21, 674-680, 1975

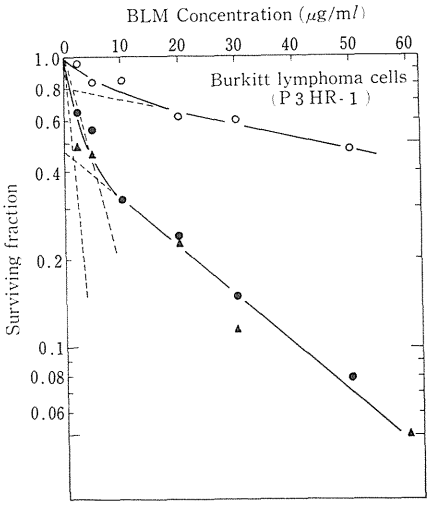


**図 1**　バーキット・リンパ腫細胞の増殖相とブレオマイシン感受性[14]

白丸は plateau phase 細胞の，黒丸は対数増殖期の細胞の dose-survival response を示す（宮本，未発表）．

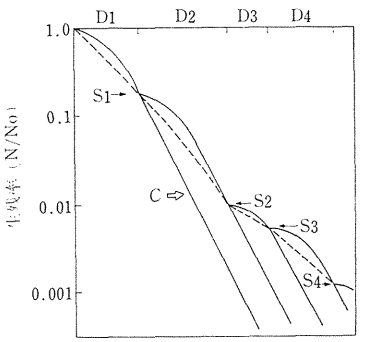


**図 2**　放射線の分割照射による哺乳類細胞の不活化

線量 $D_1$, $D_2$, $D_3$, $D_4$ の生残率をそれぞれ $S_1$, $S_2$, $S_3$, $S_4$ とすると，分割照射の生残率 N/No はそれらの積で与えられる．C は初めの集団の dose-survival response を示す．

が初めて明らかにされ，以来一定の変化のパタン（age-response function）があらゆる細胞系で確認されてきた[9,10]．その結果，哺乳類細胞はその細胞周期の間で2つの感受性のピーク（$G_2$-M 期と $G_1$-S 期移行期）をもち，したがつて2つの抵抗性の山（$G_1$ 初期と S 後期）をあらわすことになつた．この感受性の変化の原因は単一の仮説では説明されず，各時期についてそれぞれ異なつた機序が働いているものと思われる（これに関しては別の機会に譲りたい）．

周期依存性感受性変動は放射線によるばかりでなく，諸種の細胞毒性物質（抗腫瘍剤，代謝阻害剤など）でもみられるという知見が1960年代の後半からあらわれた．このことは治療に対するレスポンスを理解し，腫瘍の治療デザインを組立てる上に役立つ基本的な知識となつた（他誌を借りてやや詳細に述べてあるので参照されたい[11]）．

## 2. 細胞の増殖相と感受性

培養細胞における対数増殖期と定常期とでは細胞の感受性の著明な差異がみられる．この差はX線では明瞭でないが，5-フルオロウラシル（5-FU）[12]，フレオマイシン[13]，ブレオマイシン[14]では顕著で，後者では図1にみるように対数期細胞は定常期細胞に比べて3～4倍も感受性が高い（逆の結果もある）．その原因の主なものとしては，(1) 細胞の薬剤取り込み能，(2) 薬剤の活性化，不活性化能，(3) 定常期集団における特定な細胞周期の蓄積，(4) 薬剤による細胞損傷の回復力の差，が考えられる．図1にみられるバーキット・リンパ腫細胞とブレオマイシンとの間では (3)，すなわち定常期にはブレオマイシン感受性の低い $G_1$ 期細胞が蓄積しているということでおおむね説明される[14]．腫瘍における非増殖細胞（non-dividing fraction），あるいは $G_0$ 期細胞と増殖相（proliferative phase）の細胞との間にもこの程度の感受性差の存在することが予言される．

## 3. sublethal damage の回復

細胞が放射線（低 LET の）で照射された場合，致死損傷の他に回復可能な多くの sublethal な損傷が誘発されることが1960年 Elkind & Sutton[15] によつて見出された．哺乳類細胞の放射線による survival response はほとんど例外なしにシグモイド形をなすが（図2，C），彼らの研究によると，その shoulder 部分は致死損傷の発現に先立つ sublethal な損傷の蓄積の大きさを示すものであつた．したがつて細胞が sublethal な損傷の回復に十分な時間をおいて繰り返し照射されるときは，最初の細胞集団の survival response (C) と同じ response をとるため，破線で示されるような不活化をうることになるだろう．つまり分割照射による腫瘍の不活化速度の変更は，1回の照射量と shoulder 部分の形によつておおむね決まつてくるといえる．同じような現象は抗腫瘍剤のあるものにおいても期待されるだろう．

### 4. potentially lethal damage (PLD) の回復

1969年以来 Little, Hahn らによつて，放射線をうけた plateau phase の培養細胞に見出され，研究された[16,17]．plateau phase の細胞を照射してからインキュベートし，経時的にトリプシン分散して生存細胞数を測定すると，直後より生残細胞数は増加し，約6時間で最高値に達する．この生残率の上昇は放射線で誘発された PLD の回復によつて説明される．その発現の条件としては，(1) 細胞密度の最大に近い培養状態 (density-inhibited) が保たれること，かつ (2) その時の培養メジウム (conditioned medium) が必要で，(3) $G_1$ 期の細胞で起こりやすい．

近年は移植腫瘍でもこの種の回復がみられている．また，5-FU，サイクロフォスファマイド，ブレオマイシンなど化学療法剤投与の後にも発生することが指摘されている[18,19]．

### 5. cell-drug interaction

抗腫瘍剤により細胞に感受性の変更を誘発する現象で，1972年 Terasima らによりブレオマイシン処理細胞で見出された[20]．

上述のいずれも細胞の感受性を大きく変化させる要因であつて，腫瘍治療の細胞生物学的基礎として重要であるが，ここではとくに近年の発展である後二者についてブレオマイシン (BLM) 処理細胞を例として述べ，*in vitro* の survival response から治療に対してどんな原理が提供できるかを探つてみたい．

## 2. cell-drug interaction はブレオマイシンの特徴である

抗腫瘍剤ブレオマイシン (BLM) の哺乳類細胞に対する致死効果は，1970年以来，寺島，梅沢らによつて詳細に記載された[20,21]．その survival response の特徴は図3に見るように濃度に対して upward-concave であることであつた．この形のレスポンスはハイドロキシウレア，チトシン・アラビノシドなどでもみられるが，それらの upward concavity は主として薬剤の DNA 合成期細胞に対する選択的毒性によるものであつた．しかしながら，BLM の場合は同調された $G_1$ 期集団を用いても同じ upward-concave なレスポンスが得られるので，細胞周期の特定のステージの選択的致死によつては説明されないのである．以下に述べるマウス L 細胞の BLM による時間—不活化曲線の解析は，この特異な survival response に説明を与えるとともに，治療への有用な知識をもたらすと考えられる．

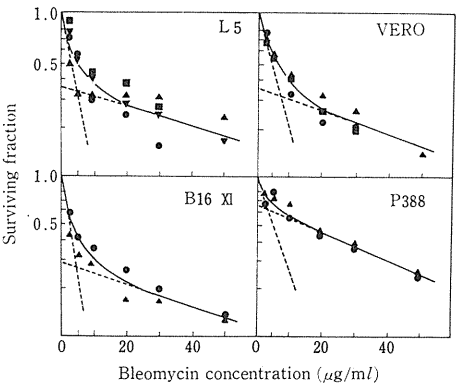


**図3**　培養哺乳類細胞のブレオマイシンに対する survival response[21]

L5：マウス線維芽細胞 L 株，B16-XI：マウス B16 メラノーマ由来のクローン，VERO：ミドリザル腎由来細胞，P388：マウスリンパ性白血病細胞

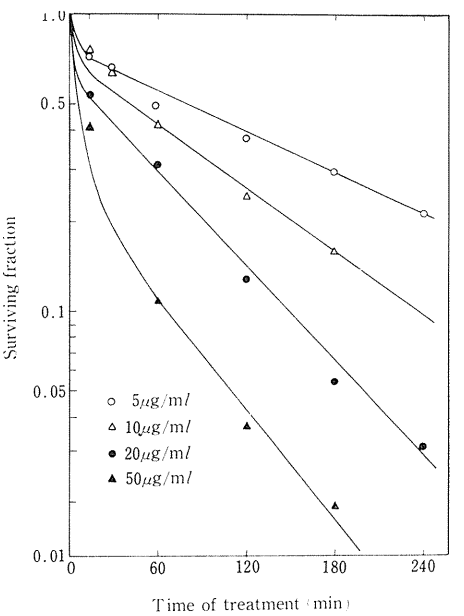


**図4**　マウスL細胞のブレオマイシンによる不活化曲線 (time-kinactivation curve)[21]

ブレオマイシンの濃度にかかわらず，二相性不活化を示す．

図4は種々の BLM 濃度でインキュベートされたときの細胞の不活化曲線で，その特徴は明らかな二相性であつた．この抵抗性部分は ① BLM 抵抗性変異細胞の存在，② 細胞周期内の BLM 感受性の変動，③ BLM の

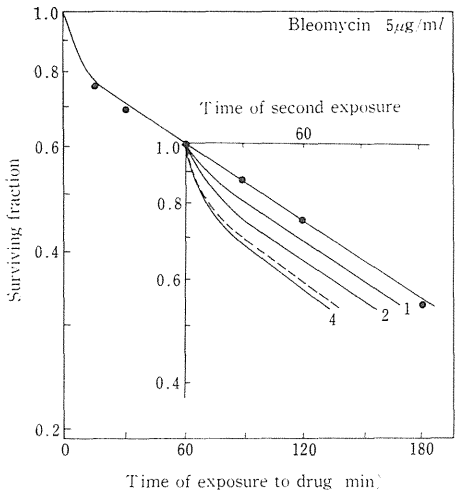


**図 5**　二分割投与実験による感受性復帰の証明[21]

黒丸は 5 μg/ml ブレオマイシンによるマウス細胞の不活化曲線．最初の60分の第1投与の後，薬剤を含まない培地で 0，1，2，4 時間インキュベート（挿入図に示すごとく），その間に起こる感受性の変化を同濃度の薬剤の第2投与によつてテストした．第2投与の結果，抵抗性のスロープ（挿入図内の黒丸）を示す生残細胞集団はインキュベーションの時間とともに感受性を回復し，4時間でほぼ元来の感受性（破線）に復帰する（ここでは模型的に表現した）．

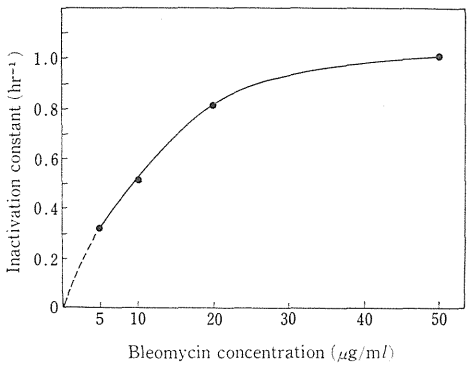


**図 6**　不活化曲線（図4）の terminal slope とブレオマイシン濃度との関係[21]

細胞の不活化効率は低い薬剤濃度においてより高いことがわかる．

熱失活または代謝的失活，④ BLM による抵抗性の誘導の4つの仮説のいずれかによつて説明されなければならないだろう．仮説①，②は二，三の実験によつて容易に否定されたが，残る仮説は図5に示される二分割投与実験を俟つてはじめて検討された．

細胞は BLM とインキュベートすることによつて急速に不活化するが，15～30分後に薬剤に対する比較的な抵抗性を獲得し，ゆるやかなスロープを示すようになる．二分割投与実験とは，この第1投与で現われた抵抗性が，BLM を除いたのちどう変化してゆくかを第2投与に対するレスポンスでテストしようとするものである．図5に見るように60分後にただちに新しい BLM メジウムに変えてもその抵抗性は変わらない（挿入図黒丸印）．それゆえ BLM の失活は二相性を説明しないことは明らかである．さらに，2つの BLM 投与の間 BLM を含まないメジウムでインキュベートすると，その間隔が長くなるにつれて感受性が高まること（挿入図実線），しかし4時間を超えても元来のレスポンス（破線で示される）以下になることはないこと，がわかつた．つまり薬剤で細胞に誘導された抵抗性は薬剤の取り除きによつて崩壊し，4時間でまつたくもとの感受性に復帰する．このことは，挿入図実線に模型的に示されるように，復帰細胞が再び抵抗性誘導をうける能力をも回復することを意味している．そしてもし BLM 投与の間隔を適当にとるならば，抵抗性の誘導と消滅は何回も繰り返えされうることがその後の実験でも明らかになつた[21,22]．機序について本稿では触れないが，この現象は cell-drug interaction と仮称される．そして survival response の upward concavity（図3）は抵抗性の発生に基因することはいうまでもない．

以上の知見はすでに腫瘍治療のデザインに有用な示唆を与えている．

（a）まず BLM の持続投与を考えるとき，細胞の不活化は図4の terminal slope に従うだろう．図6に見るように，この terminal slope の速度定数は濃度の増加に比例せず，upward-convex になる．つまりもつとも効率のよい腫瘍の不活化はより低い濃度の薬剤で成就され，高い濃度は無駄が多いということである．図7は L 細胞と同じ感受性をもつ仮説的な腫瘍において，薬剤の1回の投与が一定濃度を1時間だけ維持しうると仮定した時，5 μg/ml と 50 μg/ml の薬剤を持続させて得られる細胞の不活化を図示している．もし腫瘍細胞を $10^{-8}$ まで不活化するに要する総薬剤量を比較するなら，5 μg/ml 濃度では 300 μg となり，50 μg/ml の 1/3 で，上に指摘したとおりである．

（b）腫瘍細胞の sterilization に terminal slope を利用する持続投与の不利なことは当然で，誘導される抵抗性を避けるため間歇的投与が望ましい．図7の破線は 5 μg/ml の分割投与による不活化のあり方を模型的に示

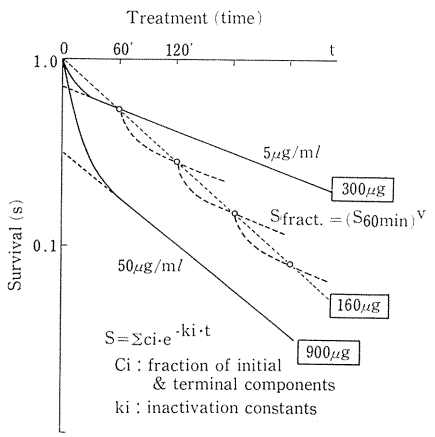


**図 7**　腫瘍細胞の仮説的不活化曲線

細胞の二相性不活化曲線は図下部の式によつてあらわされる．本文中に示した仮定によると，5 μg/ml，50 μg/ml の薬剤の持続投与によつて $10^{-8}$ の不活化をうるにはそれぞれ 300，900 μg の総薬剤量を要する．各投与間に感受性復帰のための十分な時間をおくと，不活化は破線に従い，同じ 5 μg/ml の薬剤で $10^{-8}$ に達するに総量 160 μg を要するにすぎない．

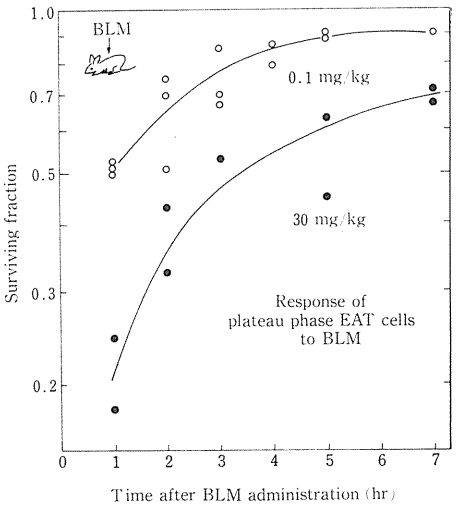


**図 8**　エールリッヒ腹水腫瘍の plateau phase 細胞における potentially lethal damage の回復[22]

plateau phase 細胞をもつマウス皮下に 0.1 mg および 30 mg/kg を投与し，1時間後より経時的に腫瘍細胞をとり出し，軟寒天培地で集落形成能をしらべる．細胞が腹水中にある時間とともに，損傷は回復し，生残率はそれぞれ1時間値の2および4倍に上る．

しているが，抵抗性の消失を許すに十分な間隔で行われる時には点線の示す速さで，不活化され，$10^{-8}$に達するに要する総量は持続投与のそれの50%になろう．図3の upward-concave な survival response から知られるように，単一投与は低い濃度でとくに効率が高いので，低濃度の薬剤の間歇投与が増殖細胞の sterilization にもつとも望ましいスキームと考えられる[23]．

浦野ら[24]は BLM を用い C3H マウス乳癌の実験治療を行い，単一投与に比べて分割投与の腫瘍増殖阻止力のより優れていることを報告している．

## 3. *in vivo* で potentially lethal damage (PLD) の回復はあるか

はじめにふれたように，plateau phasel に達した培養細胞では放射線による PLD の回復がみられる．このようなレスポンスが ① 腫瘍の，それも non-dividing fraction の，細胞で起こるか，② 放射線ばかりでなく，抗腫瘍剤でも誘発されるか，を知ることは腫瘍治療の細胞生物学的基礎の面ではきわめて深刻な問題である．ここでは再び BLM を用いて，エールリッヒの腹水腫瘍の plateau phase 細胞に対する効果を紹介しよう．

plateau phase の腫瘍細胞をもつマウスの皮下に種々の濃度の BLM を投与し，1時間後に腹水中の腫瘍細胞を取り出して軟寒天培地内に培養し生残細胞の率をみると，ちようど図3に示したように upward-concave な survival response が得られる．これは通例，当腫瘍細胞の BLM に対する感受性を示すものと解釈されるが，実際に体の中ではそうではないことが知られてきた[19]．図8にみるように，0.1 mg/kg，30 mg/kg の単一投与をうけたマウスから経時的に腫瘍細胞をとり，その生残率を測定すると，値は1時間後より急速に増加し，やがて緩かに増え，4～7時間でほぼ最大値に達する．1時間値に対するその増加率はそれぞれ2倍，4倍である．これは ① BLM によつて与えられた PLD が時間とともに回復して増殖力を再獲得すること，② この回復は *in vivo* 条件下（高い細胞密度，腹水など）においてのみ起こり，ひとたび *in vitro* の検定環境にもたらされると，PLD は致死損傷として固定されると考えることによつて説明されるだろう．その機序はどうあろうと，この回復は細胞の感受性を変更する大きな *in vivo* の因子であることはまちがいない．

それではこの現象の治療への関与はどうであろうか．腫瘍細胞の不活化という面から考えると，前章で指摘された分割投与をもし plateau phase の細胞に適用した

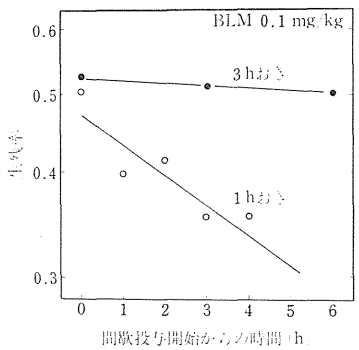


**図 9**　エールリッヒ plateau phase 細胞の間歇投与による不活化[25]

黒丸は3時間おきに 0.1 mg/kg の薬剤を投与した場合の各1時間後の生残率，白丸は同じく1時間おき投与のさいの細胞の不活化．3時間おきでは，PLD の回復が大きく有効な腫瘍細胞の不活化に至らない．

ら，それは PLD の回復のための時間を貸すにすぎないだろう．といつて持続的投与は前にも述べたように，誘導された抵抗性によつて不活化効率の低くなる不利益を免れない．これら2つの相反する因子の大きさは用いられる薬剤の濃度にも依存するので，単純な解決は直ちに期待できない．図9の実験成績は，同じ plateau phase の細胞に対する1時間と3時間の2つの分割間隔で与えられた BLM の細胞不活化曲線を示している．これによると，0.1 mg/kg という低い濃度（実際の治療濃度に近い）では短い時間間隔の分割スケジュールの方が長い時間間隔のそれより有効に細胞を不活化するようにみえる．つまり，この濃度では3時間に起こる PLD の回復量はその間に消滅する抵抗性より大きいからであろう．また投与後1時間においては，誘発された抵抗性はまだ成立しているにもかかわらず，1時間おきの間歇投与は細胞の不活化を進めている．この所見を拡張するならば，PLD の回復に時間を貸さない持続投与はより効果的ではなかろうかと推論される．

以上の結果を要約すると，① BLM で治療する場合には実際的な最少濃度を用いること，② 増殖細胞群に対しては間歇投与により，③ plateau phase の細胞には持続投与によりもつとも効率的な sterilizationを行いうると推論される[23]．

## 結　語

腫瘍はいうまでもなくさまざまの点で不均質なものである．proliferative fraction や nondividing, clonogenic fraction などから成り立つ．酸素分圧も不均等である．当然，外来性作用体に対するレスポンスも一律ではありえない．つまり，単一の原理で腫瘍治療を行うことは少なくとも合理的ではない．それは上述の BLM の諸実験でも明らかにされたとおりである．はじめに細胞の感受性の変更要因としていくつかの細胞生物学的機序をあげたが，それらの関与する survival response の知見は，直ちに *in vivo* の腫瘍細胞の不活化動態を定量的に示すものではないけれども，治療に有用な原理の提供と問題点の指摘を行うことができるだろう．

また，変更要因の物質的基礎の研究は治療効果の増幅のいとぐちを与えるものであることはいうまでもない．

本研究は奥田邦雄教授（千葉大学医学部第1内科）および栗栖明部長（放医研病院部）の多大の御理解と御協力によつて遂行された．また，実験とその整理に関しては安川美恵子，西井正子氏の協力に依つた．記して感謝の意を表わす．

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# —NOTE—

[Gann, 66, 701～703; December, 1975]

## COMBINED EFFECT OF X-RAY AND BLEOMYCIN ON CULTURED MAMMALIAN CELLS*1

Toyozo TERASIMA,*2 Yosinobu TAKABE,*3 and Mieko YASUKAWA*2

*Division of Physiology and Pathology, National Institute of Radiological Sciences,*2 and First Department of Medicine, Chiba University School of Medicine*3*

Investigations were carried out with cultured mammalian cell lines to assess the effect of combination of radiation and Bleomycin. When cells were treated with the antibiotic before and during exposure to X-rays, a slight potentiating effect was consistently found. The magnitude of potentiation of the radiation effect appeared to depend on the concentration of Bleomycin. When the cells were treated pulsewise at various periods after irradiation, the potentiating effect was found only during the first 2 hr. Simultaneous application of X-rays and Bleomycin provided the greatest effect.

Using a murine epidermoid carcinoma, Jørgenson first demonstrated that the combination of X-ray and Bleomycin treatment produced a synergistic action.[3] Such a combination is currently being used in clinical trial.[2] However, Bleehen *et al.*[1] could not find such a synergism with several bacterial and mammalian cell lines, except for a particular strain of *E. coli*. Present communication describes results of similar experiments which showed a definite difference from those of Bleehen *et al.*

### Materials and Methods

**Cell Strains and Survival Assay** In most of the present experiments, L5 cells, a clonal derivative of mouse fibroblast L strain (B929-L2J), were used. Several particular experiments were carried out with HeLa S3-9IV cells. All these cell strains were grown in F10HI medium supplemented with 10% calf serum and antibiotics.

Cultures seeded in plastic petri dishes with an appropriate number of trypsin-dispersed cells were incubated for 4 hr before initiation of experiments. The antibiotic treatment was carried out by adding Bleomycin into the culture before and during, or after, irradiation. The cultures were incubated for a desired period and then rinsed repeatedly with F10 medium. Colony survival was assayed as described previously.[4]

Sensitivity of cells to X-ray was presented in terms of *Do* (mean lethal dose) and *n* (extrapolation number); the former is the amount of radiation needed for reducing survival down to 37% in the exponential portion of the survival curve, and the latter is a value on the ordinate to which the exponential portion of the suvival curve is extrapolated.

**Bleomycin** $A_2$ and $A_5$ compounds were supplied by Nippon Kayaku Co., Tokyo. The antibiotics were dissolved in distilled water and kept in a freezer as the stock solution. The solution was diluted with F10 medium just before use.

**X-ray** Radiations were delivered from an X-ray therapy unit (Sin-ai, Shimadzu, Kyoto) operated at 200 kVp, 20 mA, with added filtration (0.3 mm Cu+1.0 mm Al). Cultures grown in plastic dishes were irradiated at room temperature at an exposure-rate of 119 R/min.

### Results and Discussion

Fig. 1 represents the results of experiments in which mouse L5 and HeLa S3 cells were treated with 10 μg/ml of Bleomycin 30 min

*1 This study was supported by the Project Grant for Development of Bleomycin from the Science and Technology Agency.

*2 Anagawa 4-9-1, Chiba 280 (寺島東洋三, 安川美恵子).

*3 Inohana 1-8-1, Chiba 280 (高部吉庸).



GANN 66, 701-703, 1975

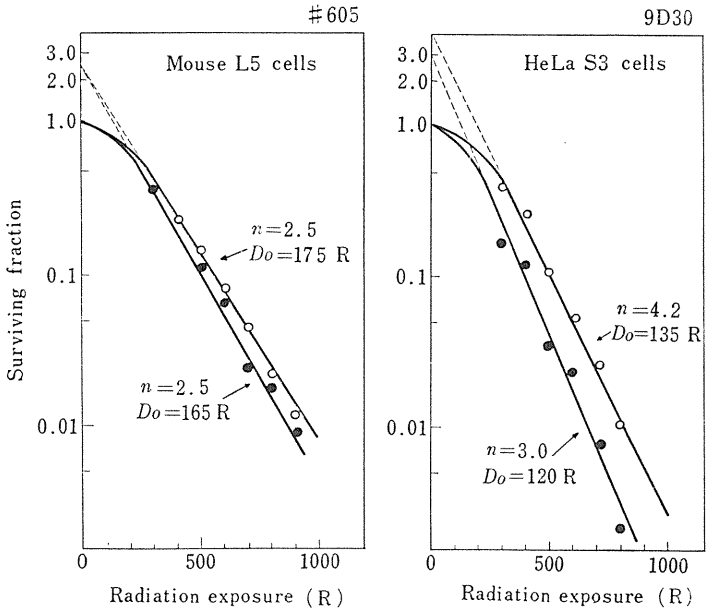


Fig. 1. Effect of Bleomycin-pretreatment on X-ray survival of cultured mammalian cells

Cells were treated with 10 $\mu$g/ml Bleomycin $A_2$ for 30 min, then exposed to X-ray. For HeLa S3 cells Bleomycin $A_5$ compound was used. All survival curves were normalized to the survival of unirradiated dishes irrespective of treatment with Bleomycin. Curves were fitted by naked eyes. ○ Untreated cells, ● Bleomycin-treated cells.

before and during exposure to graded doses of X-ray. As compared to the survival curves for both untreated controls, those for treated two cell lines manifested about 6 and 11% reduction in *Do* value, respectively. Change in *n* value was not consistent among several experiments.

Similar experiments were carried out by Bleehen *et al.*[1] They found no sensitization when several bacterial and mammalian cell lines were treated with 0.75 to 2 $\mu$g/ml of Bleomycin for 1 hr before and/or during irradiation. The difference from our results might be attributed to the difference in the concentration of the antibiotic used.

The extent of potentiation was examined for various concentrations of Bleomycin. Clutures treated with the antibiotic for 1 hr were exposed to a single 500 R irradiation immediately thereafter. The ratio of surviving fraction after antibiotic treatment and X-

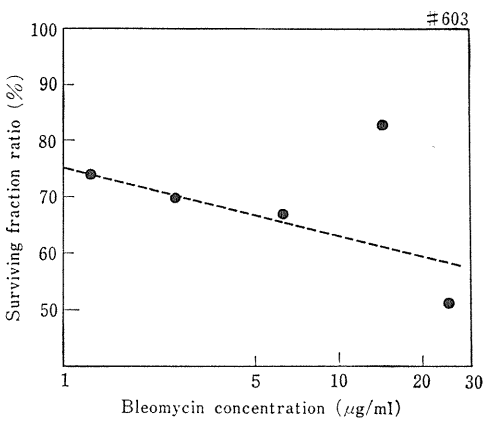


Fig. 2. Relationship between potentiating effect and concentration of Bleomycin

L5 cells treated with varying concentrations of Bleomycin for 1 hr were exposed to 500 R X-ray. Surviving fraction ratio was obtained from the surviving fraction after Bleomycin + X-ray divided by surviving fraction after X-ray alone.

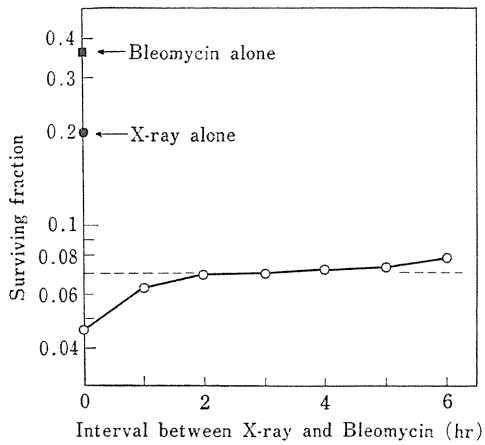


Fig. 3. Effect of post-treatment with Bleomycin on X-ray survival

After exposure of L5 cells to 400 R X-ray, pulse-treatment with Bleomycin (20 $\mu$g/ml, 1 hr) was given at the indicated time. ○ Survivals, ● survival levels after a single dose of X-ray, ■ survival after a single dose of Bleomycin. Additive effect by these two agents is expected to be a product of surviving fractions to each agent and is shown by a broken line.

irradiation to surviving fraction of X-irradiation alone was determined. The results illustrated in Fig. 2 showed that the ratio decreased as a function of concentration ranging from 1.25 to 25 $\mu$g/ml of Bleomycin. Therefore, the potentiating effect of Bleomycin on radiation effect appears to depend on its concentration.

Fig. 3 shows results of post-treatment of X-irradiated L5 cells with Bleomycin. In the experiments illustrated, cells exposed to 400 R were pulsely treated with Bleomycin (20 $\mu$g/ml, 1 hr) at the indicated time. The survival (open circles) increased rapidly for the first 2 hr and then stayed almost constant until 6 hr. The final survival level (0.07) was exactly the one which would be expected when the effect of two agents was simply additive, since the surviving fraction after single doses of X-ray and Bleomycin was 0.2 and 0.36, respectively. Therefore, it would be justified that the lower survivals found during the first 2 hr were a result of potentiation. Further, it can be noted that the shorter the interval between the two administrations, the greater was the potentiation. This may be due to either enhancement of X-ray damage or inhibition of repair process after X-ray by Bleomycin.

More pronounced effect of combined treatment is being found with transplantable tumor cells,[5] and this finding, together with the present results, will be discussed at a later date.

The authors acknowledge the kind supply of Bleomycin from Nippon Kayaku Co. throughout the present studies. Technical assistance of Miss M. Nishii is greatly appreciated.

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＜総　説＞

# 細胞に対するブレオマイシン作用と腫瘍治療

*千葉大学医学部第一内科
**放射線医学総合研究所病院部

渡辺　道典*　　高部　吉庸*　　宮本　忠昭**

**要旨**　ブレオマイシンの培養細胞および動物腫瘍に対する効果を解析し，こうした基礎的研究で得られた結果が合理的な薬剤投与方法を考えるうえでどのように役立つかを述てみた。

## はじめに

最近の癌化学療法の進歩は新しい抗癌剤の開発によるところが大であるが，加えるに *in vitro* 培養細胞系で確立されている方法を利用して生体内癌細胞の増殖動態（各種癌細胞の世代時間，cell cycle parameter，growth fraction 等）と抗癌剤へのレスポンスがしだいに解明され，それ等に基づいたより合理的な抗癌剤の投与方法が工夫されていることにもよるであろう。従って，現在では癌の化学療法を行う場合，①治療の対象となる癌細胞の増殖様式，②治療に用いる抗癌剤の作用機序（特に細胞致死作用，cell cycle への効果等）に関する十分な知識が cancer chemotherapist に要求されるようになってきた。今なお，癌化学療法は多剤併用療法が主流であるが，それとは別に抗癌剤の細胞，特に cell cycle への効果を重視した投与方法も芽生えつつある。例えば Lampkin[1] 等は Ara-C を用いて生体内白血病細胞の partial synchronization を誘導させ，より効率的に細胞を殺そうと試みているが，これは薬剤の cell cycle への効果を臨床的にうまく利用しようとしている点で一つの新しい方向を示しているといえるであろう。

このように，従来ずいぶんとかけ離れた感のあった抗癌剤の細胞レベルでの研究と臨床的投与方法との距離は現在ではしごく近接したものであり，今後臨床家にとっても合理的癌治療を行ううえで cell biology に関する知識はますます重要になると思われる。本文では我々の行ってきた bleomycin の研究，特に細胞致死作用，cell cycle へ及ぼす効果，また比較的新しい概念である potentially lethal damage の回復等を中心として，それ等が臨床的投与方法とどのように関係してくるかを考えながら述べてみたい。

### 1. Bleomycin の構造

Bleomycin (BLM) は1966年梅沢等により Streptomyces verticillus より分離された抗癌抗生物質であり[2]，現在 $A_1$, demethyl-$A_2$, $A_2$, $A_2'$-a, $A_2'$-b, $B_2$, $A_5$, $B_4$ および $A_6$ の各成分が単離されている[3]。その構造は図1に示すような糖ペプチッドの複合体で[4]，末端アミンの部分を除いて各成分間にあまり相違はない。BLM $A_2$ の超遠心で得られた分子量は約1550である[5]。

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Action of bleomycin on mammalian cells as a basis of tumor chemotherapy.
Michinori Watanabe, Yosinobu Takabe, Tadaaki Miyamoto**
*First Dept of Medicine Chiba University School.
**National Institate. of Radiological Sciences. Hospital.

癌と化学療法　2，923-933，1975

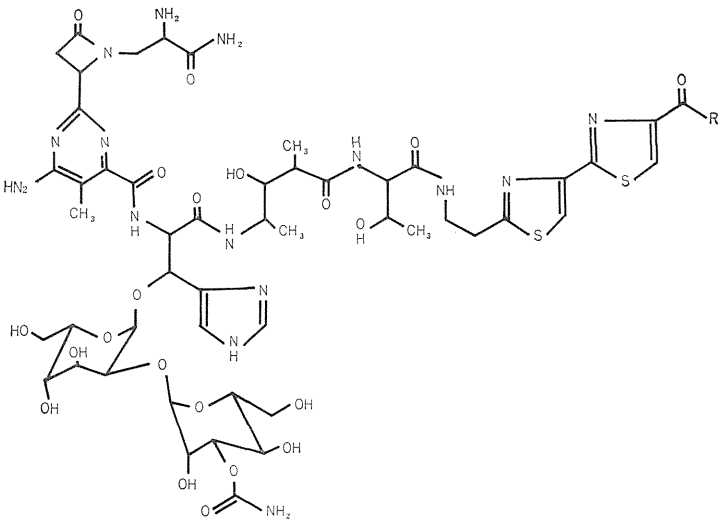

図 1　BLM の構造．R：末端アミン

## 2. BLM の高分子合成及び DNA に対する効果

BLM の作用機序についてはその発見以来多数の報告がなされているが，現在ではその主たる作用点は DNA であろうと考えられている。ここでは BLM の高分子（DNA, RNA, 蛋白）合成及び DNA に対する作用について簡単に触れておこう。

細胞に BLM を作用させると細胞の DNA 合成が抑制される事が最初に報告されたが[6,7]，この事実は我々の実験[8]によっても確認された。すなわち，マウスL細胞に種々の濃度の BLM を投与すると DNA 合成が最も強く，次いで蛋白合成，RNA 合成の順に抑制される（図2）。では，BLM 処理を受けた細胞の DNA はどのような損傷を受けているであろうか。寺島[9]は BLM 投与によりマウスL細胞内 DNA の一重鎖及び二重鎖切断が引起こされる事，及び一重鎖切断は細胞を BLM を含まない培地に移すとよく修復される事を認めた（図3）。このような生細胞内 DNA の一重鎖切断は HeLa 細胞でも認められるが[10,11]，この場合反応液中より EDTA を取り除くと DNA の切断が起こりやすいという報告もある[11]。BLM

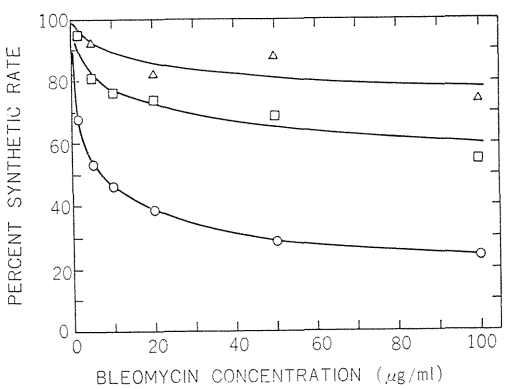


図 2　BLM の高分子合成に対する効果（マウスL細胞）
○：DNA 合成，□：蛋白合成，△：RNA 合成

によるこのような細胞内 DNA の損傷（一重鎖及び二重鎖切断）はかなりよく修復されるようであり[12]，最近寺島は BLM 投与後ごく短時間のうちに細胞内 DNA の一重鎖切断が起こる事及びこの切断は BLM 存在下でさえも修復されてくる事を認めている。ただし，BLM 自体は DNA 合成酵素である DNA polymerase の作用は阻害しないが DNA ligase の作用をむしろ抑制する事が報告されている[13,14]。

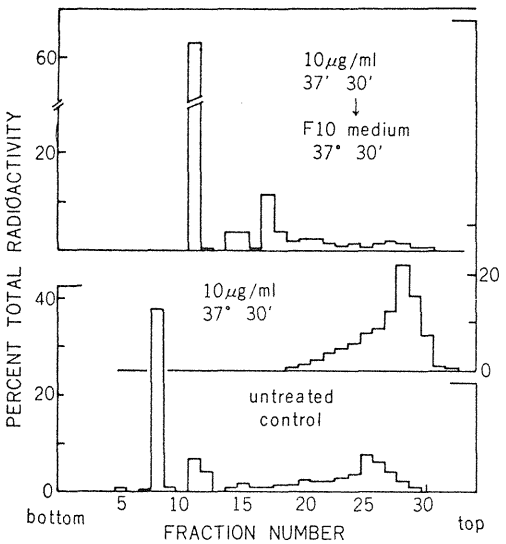


図 3　BLM 処理マウスL細胞の DNA 一重鎖切断とその修復

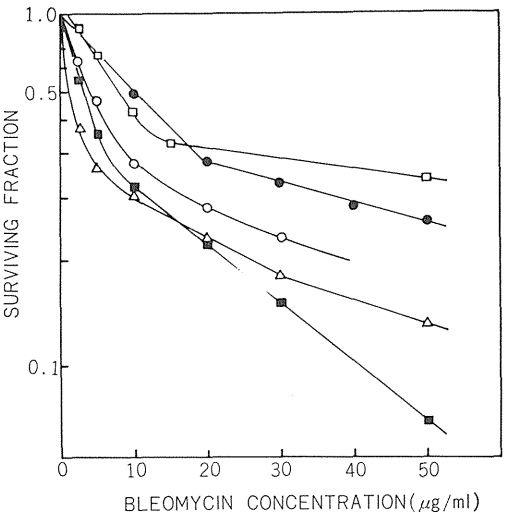


図 4　BLM による各種細胞の Dose Response Curve.
○: マウスL細胞[22], ●: HeLa 細胞[11], □: CHO 細胞[23], ▩: バーキットリンパ腫 (P3HR-1) 細胞 (宮本, 未発表データ), △: マウスメラノーマ(B16-X1) 細胞[22]

*in vitro* で BLM を直接 DNA に作用させた場合，DNA 分子に引起こされる変化については多数の研究がありそれ等を要約すれば次のようになろう。① BLM は *in vitro* で DNA の低分子化（一重鎖及び二重鎖切断）を起こし，SH 化合物の存在はその作用を増強する[10,11,15,16,17,18,19]。②BLM と反応した DNA からは thymine[19] あるいは thymine も含めたすべての種類の塩基[20]が遊離される。③ BLM は SH 化合物あるいは過酸化水素の存在下で DNA の熱変性曲線に影響を与え，その融解温度を下降せる[16,17,21]。

### 3. BLM の細胞致死作用

a) BLM による細胞致死曲線の特徴

抗癌剤の細胞致死作用を知る事は化学療法を行うにあたり我々に最も基礎的な情報を与えてくれる。現在では，薬剤の細胞致死効果は薬剤処理後のコロニー形成率をコントロールのそれと比較する事により *in vitro* で定量的に調べる事ができる。図4は種々の濃度の BLM を細胞に作用させた時に得られる生残曲線 (dose response curve) である。細胞株により感受性の差はあるが，ここで特徴的な事実は曲線が2相性を示す事，すなわち低濃度範囲では細胞の BLM に対する感受性が高く，高濃度範囲では低くなるという事である。次に一定濃度の BLM を種々の時間細胞に作用させた場合 (time inactivation curve) にも全く同様の，初期（30分以内）に感受性が高くそれ以後感受性の低い2つの components よりなる2相性の曲線が得られる（図5）。このような細胞致死曲線は，例えば Daunomycin が細胞を指数関数的に殺すのと比較すると BLM に特異的である。その原因として次のような可能性が考えられる。①BLM の培養液中における生理的代謝乃至温度による不活化，②細胞周期依存的な感受性変動（後述）による，③遺伝的に BLM 抵抗性細胞が存在する，④ BLM 投与により細胞に可逆的な抵抗性が誘導される。このうち①～③の可能性は実験的に否定され，結局 BLM 投与により細胞が損傷を受けると同時に BLM に対する抵抗性をも獲得しこのため細胞致死曲線が2相性となる事が次に述べる BLM 分割投与実験により明らかとなった。

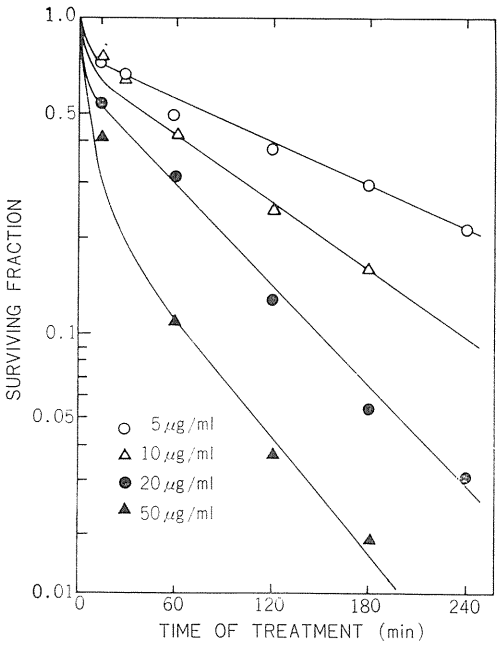


図 5　マウスL細胞の BLM による Time Inactivation Curve.[24]

b)　BLM 分割投与実験

BLM 投与により細胞に抵抗性が誘導されるならば，この抵抗性は細胞を BLM を含まない培地で培養すれば消失し，もとの高い感受性がもどるはずである。この事は図6に示す BLM 分割投与実験で証明される。細胞を120分間持続的に BLM 処理した場合（図6の実線）に較べ，最初60分間 BLM 投与後細胞を BLM を含まない培地で培養しその後さらに第2回目の BLM 60 分間投与を行った場合（図6の破線）では明らかな感受性の増加（すなわち抵抗性の消失）が認められる。同様の実験を繰返した結果，BLM 投与によって誘導される抵抗性は細胞を BLM を含まない培地に移せば2～4時間のうちに消失する事が明らかとなった。従って *in vitro* でより良い細胞致死効果を得るためには，BLM 持続投与よりも2～4時間以上の interval をおいて分割投与を繰返した方がはるかに効率がよい。図7は5時間の interval を置いて BLM 5 μg/ml 60 分間投与を繰返した

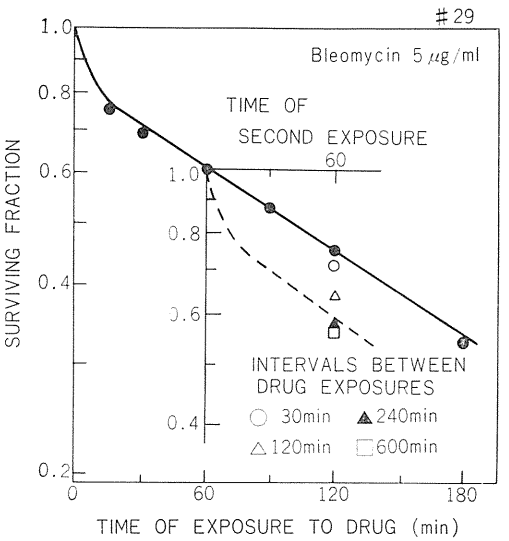


図 6　BLM 分割投与実験[22]

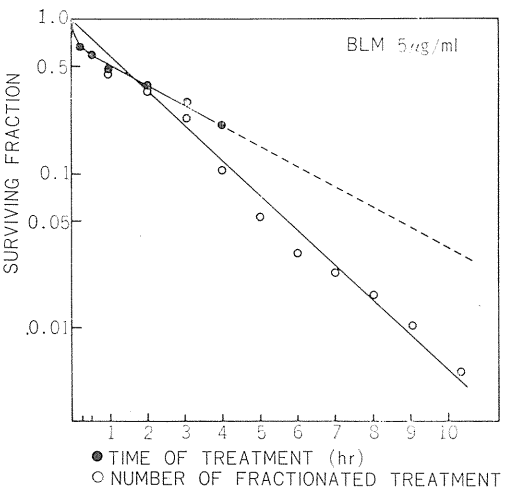


図 7　BLM 持続投与（●）と頻回分割投与（○）との比較[25]

場合と持続投与を比較したものである。前者が原点より直線的に細胞を殺してゆき，結果として同じ BLM 投与量でありながら持続投与とは大きな差がでてくる事がお判りになると思う。

このような *in vitro* の結果より BLM 臨床投与法についてどのような示唆が得られるであろうか。重要な事は①高濃度あるいは長時間 BLM 投

与では細胞の感受性が低くなり細胞致死効率が悪い。② BLM 投与中止後2～4時間以内に細胞は最初の高い感受性を回復するという事である。従って，低濃度の BLM を分割投与するのが最も効率のよい臨床的投与法という事になろう。例えばここに我々の得たデータを基にして行った計算例を示そう（詳しくは文献22）を参照されたい)。$10^8$ 個の細胞よりなる腫瘍の 90% 治癒に要する BLM 総量は 50μg/ml. 持続投与では 910μg であるのに対し，5 μg/ml 持続投与では 317μg で明らかに低濃度の方が有利である。さらに同じ5 μg /ml 投与でも60分間の分割投与を繰返した場合では BLM 総量はわずか 168μg（持続投与の半分量）でことたりる。こうした BLM 低濃度分割投与の有利性は後述する potentially lethal damage の回復という現象で修飾されるものの *in vitro* の実験でも証明されておりまた事実臨床例ではホジキン病の場合 1 mg/day の投与で十分なレスポンスが得られるようである[26]。

c) *In vivo* における BLM 分割投与

*In vivo* の実験動物腫瘍においても，BLM 投与後の腫瘍細胞致死曲線は *in vitro* のそれと本質的に全く同様の低濃度域で高感受性（表1の Initial portion），高濃度域では低感受性（表1の Terminal portion）の2つの components よりなる。表1に各種動物腫瘍で得られた細胞致死曲線の $D_{37}$ 及び変曲点（表1の Infrexion point で，高感受性域と低感受性域の境界量）を示す。前項で述べたように1回投与量としてはこの変曲点以下の BLM を用いた方が腫瘍治療効率がよい。さらに浦野等[28]は表1に示すマウス乳癌を用い BLM 1回投与と分割投与の腫瘍発育に及ぼす効果を比較している。図8はその結果を示すが，BLM 総量 100mg/kg を1回に投与した場合よりも変曲点（この腫瘍では 30mg/kg）以下の 25mg/kg を 12～24時間の間隔で4回分割投与した方が明らかに腫瘍発育を抑制している。

こうした *in vitro* 及び *in vivo* で得られた結果を臨床面で適用する際の課題は各種の人癌において細胞致死曲線の変曲点を決める事，すなわち1回投与量の検討であろう。ちなみに，1例のホジキン病でしらべられた腫瘍のレスポンスから変曲点は 2.5mg に相当し，1回投与量はこれ以下の量が合理的なようである[26]。

表 1.　各種動物腫瘍の BLM に対する感受性

| Tumors | Sensitivity to BLM(D37*:mg/Kg) | | Inflextion point(mg/Kg) |
|---|---|---|---|
| | Initial portion | Terminal portion | |
| mouse(ICR/JCL) Ehrlich ascites tumor(Takabe, unpublished data) | 0.15 | 23 | 0.25 |
| mouse(WHT/Ht) squamouse carcinoma27) | 0.1 | 2.7 | 0.2 |
| mouse(C3H/He) mammary carcinoma28) | 12.8 | 85 | 30 |
| mouse Ehrlich carcinoma29) (subcutaneous solid form) | 0.5 | 8.5 | 0.78～1.56 |

*D37：37%生残率を示す時の薬剤量

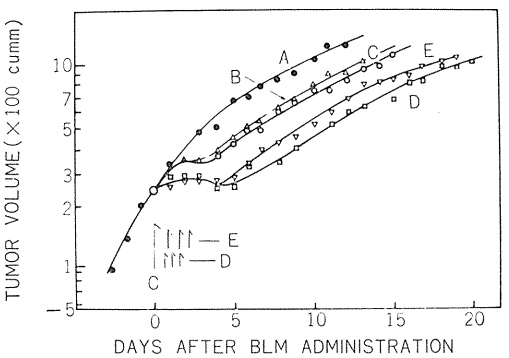


図 8.　マウス(C3H)乳癌の腫瘍発育に及ぼす BLM の効果

A：コントロール，B, C は各 BLM 50, 100mg/kg 1回投与，D, E は BLM 25mg/kg を 12, 24時間間隔で4回分割投与。

## 4.　エールリッヒ腹水腫瘍の BLM による治療：potentially lethal damage とその回復

エールリッヒ腹水腫瘍細胞接種後7日目のマウス（腹水細胞は stationary 期にある）に BLM を 0.1mg/kg 及び 30mg/kg 皮下注射し，以後1時間目から経時的に腹腔外に細胞を取り出しその生残率を求めると図9の如くとなる[30]。細胞生残率は 0.1mg/kg の投与でも 30mg/kg の投与でも1時間後に最低となり，以後経時的に上昇していく。

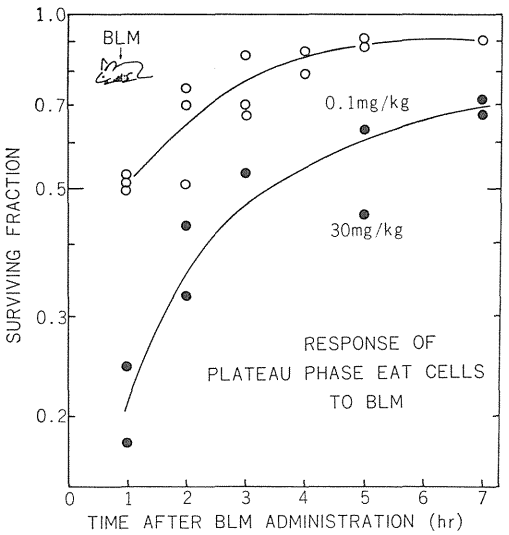


**図 9**　BLM　1回投与によるエールリッヒ腹水腫瘍細胞の生残率の経時的な推移。

すなわち，BLM 投与後1時間で細胞に与えられた damage は lethal ではなくいわば potentially lethal の状態で，細胞を腹腔外に取り出してしまえばその細胞は死に至るが腹腔内にそのままおいておくと potentially lethal の状態から回復して生き残るということを示している。この現象は，1969年 Little 等[31]が報告した radiation による potentially letha. damage (PLD) の回復と同一の現象であると解釈される。前に述べたように，抵抗性の誘導とその崩壊という観点からの BLM 分割投与法は，*in vivo* においてはさらに PLD の回復という現象が加わってきたため問題が複雑になってきたわけである。

さて，エールリッヒ腹水腫瘍の BLM による治療を考えた場合，1回投与量は表1に示した Inflexion point 以下の量（0.25mg/kg 以下）を使用することにより効果的な細胞不活化率を得る事ができる。次に投与間隔を考えるにあたって次の2つの要因が問題となってくる。① BLM 投与による PLD の発現とその回復。② BLM 投与後の抵抗性の誘導とその崩壊。ここで BLM 0.1mg/kg を1時間及び3時間間隔でマウスに注射し，

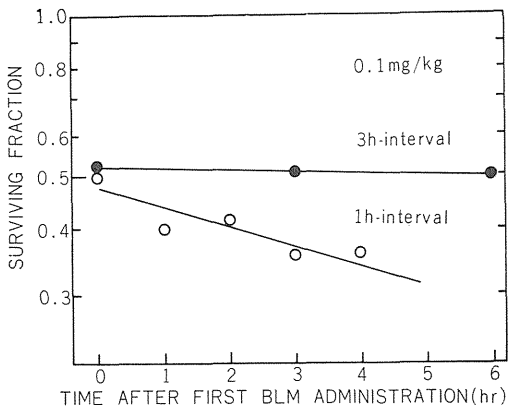


**図10**　BLM 間歇投与によるエールリッヒ腹水腫瘍細胞の生残率の経時的推移。

細胞不活化率がどのような推移を見せるかを検討して投与間隔の問題を考えてみた。図10はその結果であるが，3時間間隔よりも1時間間隔の BLM 投与でより強い細胞致死効果が期待される。これは一見したところ，*in vitro* の実験から得られた分割投与法と矛盾した結果のように思われる。この矛盾は，しかし次のように説明する事ができる。すなわち，0.1mg/kgという低濃度では BLM 投与後に誘導される抵抗性が僅少であることが明らかになっており，投与間隔を考えるには PLD の回復のみを考慮すればよいと思われる。PLDの回復は図9に示す如く BLM 投与後3時間値が1時間値よりも大であり，そのため1時間間隔の BLM 投与の方が3時間間隔よりも PLD の回復による生残率の増加の影響を少なくすることができるわけで，それだけ細胞不活化率も大きくなると考えられる。

従って，BLM で腫瘍治療を行う場合，1回投与量としては Inflexion point 以下の低濃度を用いるべきであるが，BLM 投与間隔については前項で述べた抵抗性の誘導とその消失という事を基礎として決めるよりも，むしろ PLD の回復の影響を最少とするような投与間隔――例えば，エールリッヒ腹水腫瘍では持続注入法がより効果的であると考えられる。

### 5. BLM の細胞周期依存的（cell cycle dependent）な作用

近年，腫瘍細胞の生体内における増殖様式が解明されてくるにつれて，抗癌剤の cell cycle dependent な作用は注目されてきつつある。ただ，抗癌剤の cell cycle への効果という場合，往々にして細胞周期依存的な感受性変動（cell cycle dependent survival response）と細胞周期内進行（cell progression）に及ぼす効果とが混同されている事があるのでここではおのおのにつき別に述べてみたい。

a) BLM に対する感受性の細胞周期依存性（cell cycle dependent survival response）

細胞の一世代は M 期（分裂期），$G_1$ 期，S 期（DNA 合成期），$G_2$ 期という4つの phase に分けられ，抗癌剤に対する感受性も各 phase で異なる事が多くこれを細胞周期依存的な感受性変動と呼んでいる。図11に各種細胞の BLM に対する cell cycle dependent survival response を示す。各種細胞の BLM に対する response は大体において共通しており，$G_1$ 後期及び $G_2$-M 期に感受性を示し $G_1$ 前期及び S 期は抵抗性である二峰性のパターン（X-ray type）をとる。このような response を各種抗癌剤について検討する事は，併用療法を行う際の効果的な薬剤の組合せを考えるうえで重要であり，例えば BLM と他の抗癌剤との同時併用療法を考えた場合，組合せとして S 期細胞致死効果を示す薬剤（HU-type, DNA 合成阻害剤が多い）との併用が合理的といえよう[32]。

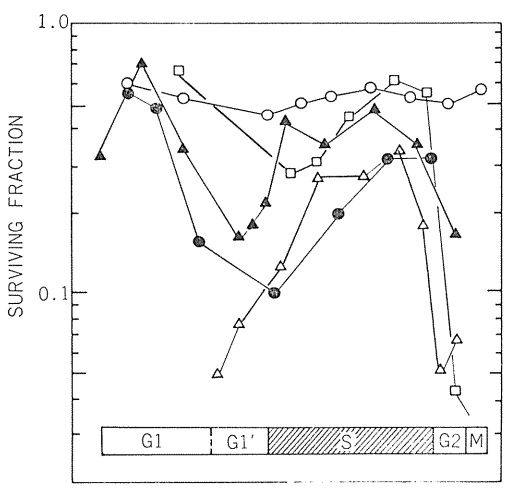


**図11** BLM に対する感受性の細胞周期依存性
○: マウスL細胞[32]，●: HeLa S3 細胞[33]，△: ヒトリンパ腫 T1 細胞[34]，▲: バーキットリンパ腫（P3HR-1）細胞[32]，□: CHO 細胞[23]，（T1 細胞の G1 期は他の細胞の G1 期に比し短かいので，便宜上 G1′ とした）

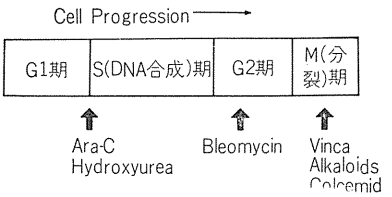


**図12** 各種薬剤の Cell Progression 阻害点

b) BLM の細胞周期内進行（cell progression）に及ぼす効果

細胞がM期→$G_1$ 期→S 期→$G_2$ 期→M期と cell cycle を進む過程を細胞周期内進行（cell progression）と称し，抗癌剤がこの過程のどこを阻害するかは抗癌剤による生体内癌細胞の部分的同調（partial synchronization）の誘導とも関連して近年注目されている。図12に BLM 及び参考として他の2, 3の抗癌剤の cell progression 阻害点を示した。*in vitro* では，BLM は $G_2$ 期において cell progression を阻害し（$G_2$-block），細胞の $G_2$ 期から分裂期への移行を妨げるので結果として細胞分裂は抑制される[23,35,36,37,38]。このような $G_2$-block は BLM による細胞分裂に必要な蛋白あるいは RNA 合成の抑制[37]，$G_2$ 期細胞染色体への損傷[38]によるものかも知れない。さらに，BLM の cell progression に及ぼす効果としては他に次のような事が知られている。① $G_1$ 期の細胞に pulse treatment を行った場合，その細胞の $G_2$ 期がやはり特異的に延長される[37,39]。② BLM はその存在下で細胞の S 期を延長させる[37,38]（S→$G_2$ 期への移行阻害）。

*in vivo* においても，Okagaki 等[40]は兎 VX2

癌で BLM 注射後直ちに分裂細胞数が減少してくる事を認めており，また Cohen 等[41]はマウス腸管上皮細胞の分裂が BLM 注射により抑制されるがこれは S→$G_2$ 期への移行障害に基づくものである事を報告している。

さて，このような抗癌剤のもつ cell progression への効果は生体内癌細胞の partial synchronization を誘導する。この効果を治療に応用しようという企図についてここで少し触れておきたい。これは，first treatment として cell progression 阻害作用をもつ薬剤を投与して癌細胞を cell cycle 内の特定の phase に集めることができるならば（partial synchronization），second treatment としてその期に強い致死作用を有する抗癌剤を投与して効率的に癌細胞を不活化することができるという考えに基づいている。ただし，生体内で癌細胞の synchronization を有効に起こさせるためには次のような条件が必要となる。①抗癌剤の cell progression 阻害点が cell cycle 内の1つの phase に限られる事。②阻害作用は reversible である事。③対象となる腫瘍の growth fraction（分裂細胞と非分裂細胞の比）が大きい事。さて，BLM は前述の如く主として $G_2$ 期に阻害作用をもつ。Barranco[42] 等は悪性黒色腫（癌細胞の世代時間は約3日間）の患者にBLM 15mg～25mg/日，4日間投与し synchronization を誘導しようと試みている。これ等の患者では，BLM 投与終了後2日目で癌細胞の Labeling Index（S期細胞の比率）が投与前に比べて 1.5～4 倍に増加しており，これは BLM により$G_2$ 期に蓄積された癌細胞がその後同調的にS期に入った為であるとしている。BLM が Synchronizing agent として適当かどうかについては疑問があるが，少くとも他剤あるいは放射線との併用療法を考える時 BLM のこうした cell progression への効果を一応考慮に入れる必要がある[32]。

### 6. 細胞増殖期による BLM 感受性の相異

非分裂細胞の放射線及び薬剤抵抗性は腫瘍治療の最大の難関の1つである。BLM はホジキンリンパ腫やある種の扁平上皮癌に作用してその体積をさ激減せる。これ等の癌を構成する細胞はその大部分が分裂を止めた状態にあると考えられるので BLM が非分裂細胞に特別な致死作用を及ぼす可能性が推察される。培養細胞の増殖期は通常，対数増殖期と定常期の2つに分けられ，定常期にある細胞は非分裂細胞の *in vitro* モデルとして BLM のこの作用を検討する格好の材料である。

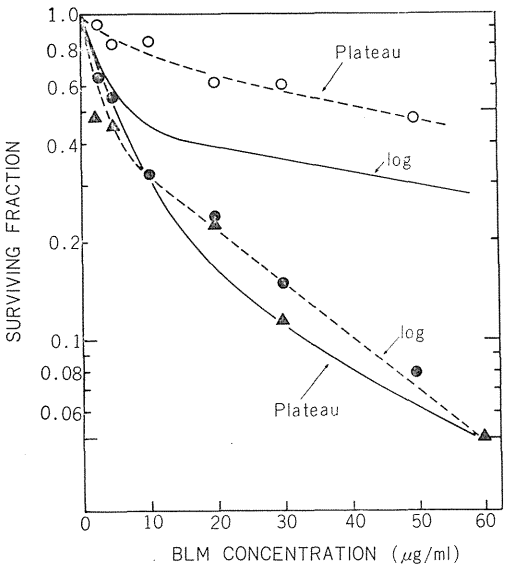


**図13**　細胞増殖期による BLM 感受性の相異。
破線：バーキットリンパ腫（P3HR-1）細胞（宮本，未発表データ），実線：CHO 細胞

さて，BLM は定常期細胞に対し対数増殖期細胞と同様に抵抗性を誘導し2相性の致死曲線を描く[43,44,45,46]。また BLM の除去により誘導された抵抗性は崩壊する[43]。しかしこの両期の細胞の BLM 感受性は著しい相異をあらわす（図13）。Barranco 等[42]は定常期にある CHO（チャイニーズ・ハムスター）細胞が対数増殖期細胞より BLM にはるかに感受性があると述べているが，これに反し Twentyman 等[45]は EMT-6（マウス）細胞で，Mauro 等[43]はV79（チャイニーズ・ハムスター）細胞で逆に対数増殖期細胞が定常期細胞より BLM に感受性があると報告している。我々はこの問題を浮遊培養系であるバーキットリンパ腫細胞と単層培養系であるマウスL細胞を用いて検討した。バーキット細胞では定常期にある細胞は対数増殖期細胞より BLM に抵抗性であり後者の報告と一

致している（図13)。これは，図11に示したように BLM に著しく抵抗性を示す $G_1$ 期細胞が定常期細胞集団の大部分を占めているためと考えられる。L細胞でも同様に定常期細胞には抵抗性があったが，L細胞の場合この抵抗性は細胞増殖期に関係なく細胞密度が増加することにより生じてくる。しかし，単層培養系であるL細胞での重要な所見は，細胞を定常期のまゝ長期培養するとこの抵抗性は失われてくる事，すなわち定常期の初期と後期とでは BLM 感受性が大きく変化してくることである。最近 Twentyman 等[47]は単層培養系である EMT-6 細胞で全く同様の結果を得ている。この定常期後期細胞の BLM に対する感受性は，BLM の固形腫瘍に対する効果を説明する一つの鍵となるかも知れぬ。何故なら，前述の如く固形腫瘍では辺縁部を除きその大部分が非分裂細胞で占められ，これ等の細胞は単層培養系定常期後期細胞と非常に類似した状態にあると推察されるからである。

*in vitro* 実験で得られた諸家の結果の相違は定常期細胞をすべて同一と考えた所に起因していると思われる。定常期細胞の BLM 抵抗性は，定常期に集積された $G_1$ 期細胞の抵抗性による以外に，単層培養系では細胞の増加によっても誘導される。しかし長期培養によっておそらく代謝活性の低下したと思われる定常期後期細胞では未知の因子により感受性はきわめて増加する。この因子に関し4項で述べた PLD の回復を第一の候補として挙げることは当を得た帰結と思われる。

## おわりに

BLM の培養細胞，動物腫瘍に対する効果を中心として，これ等が臨床面においてどのような意味あいをもつかを念頭におきながら述てみた。基礎的実験で得られた結果をうまく臨床面に応用するという事はなかなか容易なことではない。例えば BLM の投与法にしても PLD の回復という化学療法上はなはだ厄介な現象を解明することなしには完成しない。しかし，細胞あるいは動物レベルでの抗癌剤の振舞をよく理解し，治療対象となる癌の増殖様式とそのレスポンスを把握して始めて癌化学療法は論理的基盤をもって前進するのであり，そうした見地から本稿で述べたような cell level での研究の重要性を強調したい。

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特集

# 第6回放射線による制癌シンポジウム*

## ——基礎と臨床の対話——

# 腫瘍—宿主関係からみた腫瘍治療

## 序　論

寺　島　東洋三**

放射線をはじめとする外来性作用因子による腫瘍の治療が，腫瘍細胞の増殖力の不活化を原理とする以上，細胞の生残率の測定は，治療の定量的基礎として欠くことのできない最初の情報である．これは今から20年前にT.T. Puck によつて開発された，哺乳類細胞のクロン培養技法によつて成就された．Puck，その他の人々によつて，この培養法で調べられた哺乳類細胞，癌細胞の放射線に対する線量効果関係は，ほとんど例外なくシグモイド型であつて，不活化曲線の外挿値（n）と傾き（$D_0$）によつて記述される．$D_0$ 値はあらゆる哺乳類細胞で100～200 rad に分布し，舌癌その他の治療総線量を説明するのにほぼふさわしいものと考えられた．

しかしながら，1960年代に入ると，この *in vitro* の感受性の測定が，果たして体の中の細胞のレスポンスをどの位忠実に反映するものであろうか，という反省と疑問がいくつも提出されてきた．*in vivo* では細胞は高い密度で存在し，なんらかの相互依存の機構によつて比較的生残力が高いのかも知れないし，逆に *in vitro* の細胞の享受する高い栄養と酸素張力は，*in vivo* におけるより細胞に対して高い生残力を与えるかも知れない．他方，臨床経験からしても，放射線照射後まつたく局所的改善の得られない例も知られており，*in vitro* で測定された細胞の感受性というもの，つまり放射線治療の細胞生物学的基礎にもう一度問い直しがなされなければならないと思われる．

第1のセッションでpotentially lethal damage の回復現象がとり上げられたのは，これが前述の疑義に対する放射線生物学よりの1つの回答であると考えられるからである．*in vivo* では腫瘍細胞はきわめて密度の高い状態で存在するので，この種の回復は当然期待されるものであり，もし回復反応の完成後に腫瘍細胞の clonogenicity（集落形成能）を測定するならば，従来知られている single cell の感受性より数層倍も低い感受性がえられるであろう．われわれはこの回復が *in vivo* 特有の細胞のレスポンスを説明するものと期待したい．

さらに近年の放射線生物学のトピックスについてふれたい．1つは臨床経験として長い間認知されている高温の腫瘍治療効果である．これが新たに細胞生物学の基盤の上に考え直されることは，まつたく exciting なことであろう．oxic あるいは hypoxic な細胞ではどういうレスポンスをとるであろうか．放射線との併用によつて協調的効果をもつならば，その機序は両者の損傷の間の interaction によるのであろうか，または一方の修復を他方が阻止するためであろうか．

他の1つは hypoxic sensitizer の試みである．放射線によつて発生した電子が，酸素の代わりの電子親和性の高い物質によつて固定されれば，生じたアニオン・ラジカルは生物学的標的を有効にアタックするであろう．酸素はたいへん代謝されやすいので，血管のそとたかだか150 $\mu$ しか到達しないが，もし電子親和性が高く，代謝的に安定で，毒性も少ない物質がえられたならば，いわゆる hypoxic region の細胞を oxic region の細胞と同じように不活化することができるであろう．これまでの感作剤は腫瘍細胞と同様に正常細胞をも感作してしまう傾向がある．しかし，hypoxic sensitizer は oxic な細胞を感作することがないので，その作用はまつたく選択的というべきである．放射線生物学の近年のもつとも輝かしいアイデアの1つといえよう．

以上は細胞の感受性に立脚して腫瘍治療を考えてきたのであるが，本当に細胞の感受性だけで腫瘍の放射線に対するレスポンスが説明しきれるであろうか．異種の癌はもちろん，同一種の癌でも組織型，分化度によつてレスポンスの異なるという知見は多い．予後というものは

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** 放射線医学総合研究所

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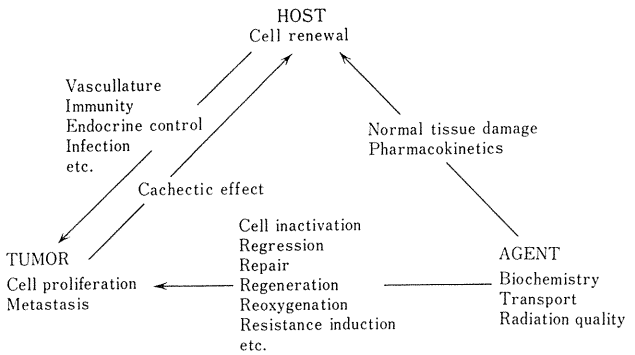


腫瘍の location などの病巣因子，あるいは，生体側の因子，たとえば年齢，内分泌機能などによつても左右されるので，可能であればその局所効果をもつて，腫瘍のレスポンスを判断することが望ましく思われる．照射をうけた腫瘍細胞の核腫大が，腫瘍そのもののレスポンスを強く反映しているという所見は，治療のガイドラインとしてきわめて有用であるばかりでなく，同時に腫瘍の感受性における腫瘍細胞の感受性の役割の大きさ，重要性を示すものといえよう．

それにしても細胞の感受性だけでは理解しきれない腫瘍組織固有の，つまり組織構築に依存する，レスポンスというものがあるのではないだろうか．たとえば，細胞増殖と退行の動態，腫瘍細胞と間質（血管）との関係，周囲組織との関係などである．それには腫瘍という新しい寄生生物の構造や生態が，細胞の増殖動態の面からもう一度検討される必要があると思われる．これはヒト腫瘍も実験腫瘍も含めて，病理家の助けをかりてなされなければならない．移植腫瘍は原発腫瘍と作用体に対するレスポンスを異にすることはすでに明らかであるが，だからといつて原発腫瘍のみ尊いとは思われない．科学は抽象する能力であるとすれば，移植腫瘍は1つの抽象生物系として大いに役割を果たすはずだからである．

第3のセッションにおけるヌードマウスの寄与を論じようとする意図は，まさに上述の腫瘍生物学への志向に沿つたものである．ヌードマウスは実験医学における近年の特筆すべき開発の1つで，ヒトの癌の生物学ばかりでなく，その作用体に対するレスポンスを実験的に扱いうる非常なメリットをもつていると思われる．ヌードへの移植はどういう性格のヒト癌で可能か，マウス間質との関係はヒトのそれと同じか，ヌード環境で腫瘍細胞の選択が起こるであろうか，移植癌をヒトの癌と同一に考えてよいかなど，多数の情報をヌードへの移植経験は与えてくれるであろう．このシンポジアムのタイトルである宿主一腫瘍関係をヒト腫瘍を用いて実験的に扱いうるという迫力に期待したい．

最後のセッションは治療における腫瘍一宿主関係の意義を考えるためのものである．

前回の制癌シンポジアムにおいて，筆者は腫瘍治療が図に示されるような関係――真実はさらに複雑でさえあるが――にあることを指摘した．多くの研究は外来性作用体の腫瘍細胞ないしは腫瘍への効果を取り扱い，そこでは細胞不活化，腫瘍退縮，修復，再生，reoxygenation 抵抗性誘導などが明らかにされる．他方，作用体は宿主体内で特異的動態を示し，骨髄，脾，消化管，リンパ腺などの再生系に対し，また腫瘍間質に対し少なからぬ影響を及ぼすことも知られている．このことは必然的に腫瘍一宿主間のデリケートな関係――それは血管系，免疫，感染，内分泌機能，年齢因子などの腫瘍への効果――に影響し，治療効果を修飾するであろう．外来性作用体ばかりでなく，腫瘍自体も toxic effect を宿主に与え，それが再び腫瘍との関係を変化させるということも考えられる．このような見地から，今回はとくに宿主の免疫機能に焦点を合わせ，これを修飾するであろうとみられる諸因子の効果を実験と臨床の両面から探ろうと試みた．しかしながら，免疫の機構はきわめて複雑な多数のステップ，細胞間の相互作用，それにもとづく制御機構より成り立つているので，どのステップへの効果が免疫治療効果の基盤であつたかを指摘することは直ちに可能ではないであろう．当シンポジアムは“臨床と基礎の対話”をキャッチフレーズとして出発したが，それは一言にしていえば，臨床をいかに科学として捉えるか，基礎へいかなる impact を臨床は与えうるか，という命題の実践であり，これは医学生物学にたずさわるものの間に，絶えることのない法灯のようなものであると考える．本会議の成否は次回への発展を占う大事なステップであるので，諸賢の熱意ある討議への参加を期待する．

I Dose-fractionation (2)

# 5. 分割治療における reassortment の役割

寺　島　東洋三*

## はじめに

腫瘍とその宿主にはそれぞれの有機体にふさわしい cell kinetics があり，外来性作用体 (exogenous agents) には放射線なら線質，薬剤ならその生化学がある．腫瘍の治療とはこの三者の相互作用で成立つきわめて人為的な歪みの集りなのである．作用体の腫瘍へと向けた力は腫瘍に対し，直接的な効果を与えるばかりでなく，宿主への効果を通しても腫瘍と宿主との相互関係を変えるような三角関係がそこに在ることを知らせる．ここで述べられることがらはその三角関係の一辺，作用体と腫瘍のレスポンス，それもごく一次的なレスポンスについてであつて，葭の髄から天井を覗くような趣きもあるが，これが腫瘍治療に何を寄与できるか，その可能性を探ろうという試みなのである．

分割治療は元来正常組織を温存しながら，腫瘍の sterilization を目指したものであつた．その分割間隔には正常組織ばかりでなく腫瘍組織にも種々のレスポンスがあらわれてくる．たとえば，細胞の増殖力の回復 (recovery)，組織の再生，交代 (repopulation)，reoxygenation，あるいは作用体が薬剤であれば cell-drug interaction などがある．それらの1つ reassortment とは，腫瘍細胞集団における細胞齢の新しい組み合わせの成立である．これが次にくる作用体と腫瘍との出合いにどういう効果をもつかは，治療家にとつて一考に値することだろう．

## 1. 細胞齢依存性レスポンス (age-dependent response)

reassortment はなぜ起こるのか．それは放射線に対する細胞の感受性が細胞齢* に依存して異なるからである[6]．まず第1には，放射線の致死作用に対して感受性の高い細胞はそれの低い細胞より余計不活化をうける (selective cell killing)．第2に，放射線の亜致死的 (sublethal) な損傷は細胞齢あるいは細胞期**に特異的な周期内進行 (cell progression) のおくれ，または停滞を引き起こす (age-specific progression delay)．これらは一括して細胞齢依存性レスポンスといわれ，reassortment の基礎となるものである．

細胞齢依存性レスポンスはまつたく放射線生物学の領域で育つたものであるが，最近10年間同じようなことが細胞毒性物質，代謝阻害剤などでも知られてきた．むしろある種の抗腫瘍剤ではその依存性はとくにいちじるしいので，腫瘍治療との関連において reassortment を考える場合に，抗腫瘍薬剤の効果をぬきにして論じることは非現実的であるというべきだろう．本項では放射線のそれを中心として諸種薬剤の細胞齢依存性レスポンス・パタンを survival response と progression delay に分けて述べてみる[7]．

図1はやや模型的に表わされた哺乳類培養細胞の，周期内の survival response のパタンで，単一線量の生残率の変化としてかかれている．それによると G1-S 移行期と G2-M 期がもつとも感受性が高く，G1初期とS後半に抵抗性を増すことがわかる．HeLa 細胞で得られたこの二峰性のパタンは，今ではあらゆる培養細胞でその一般性が確認されている[5]．チャイニーズ・ハムスター細胞など G1 期のきわめて短い細胞系では通例 G1 初期の抵抗性のレスポンスをあらわさない．

抗腫瘍剤でも同じパタンを示すものが数多くみつかつてきた．たとえば Bleomycin, Actinomycin, Nogalamycin, Neocarcinostatin, BCNU などである．その他 G1 細胞を一般に不活化する Mitomycin, あるいは Ni-

* 放射線医学総合研究所生理病理研究部

癌の臨床　22, 120-124, 1976


*age．または cell age．細胞周期内の位置を示すパラメーターで，分裂期 (M) からの時間 ($\tau$：$0 \leq \tau \leq T$，T＝世代時間) によつて規定される．

**cell cycle period または cell cycle stage．DNA 合成を指標として区別された細胞周期の各時期，すなわち $G_1$, S, $G_2$, M 期のこと．

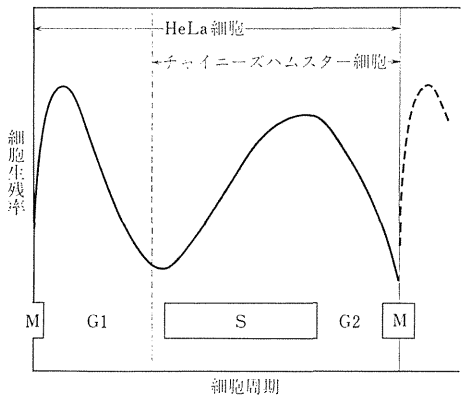


**図 1**　哺乳類細胞の細胞周期に依存したX線感受性の変化
縦軸は単一線量照射後の細胞の生残率で，感受性を表わす．

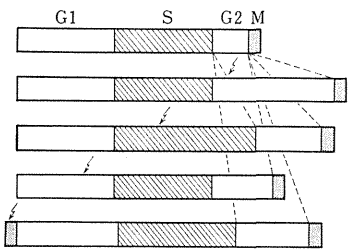


**図 3**　細胞周期内の各期に特異的な，X線による進行（cell progression）の阻害．矢印はX線照射の時期を示す．

trogen mustard なども本質的には同類ではなかろうか．

これに対してS期の細胞に特異的に致死効果をあらわす一群の抗腫瘍剤がある．たとえばHydroxyurea(HU)を代表とする，FUdR, Camptothecin, Cytosine arabinoside, Adriamycin などで G2-M-G1 に高い生残率を示す単峰性のパタンをとる．以上を模型的に示すと図2のように X-ray 型（A）と HU 型（B）とに分類しうる．前者は概してアルキル化剤，DNA 結合性薬剤であり，後者は一般に phase-specific drugs と呼ばれるもので，DNA 合成阻害作用で共通している．重要な点はS期の感受性パタンが両型の作用体の間で相反的，相補的なことである．

細胞齢依存性レスポンスは周期内進行の面でもみられる．図3は HeLa 細胞の各細胞期に X 線照射を行ったとき，それぞれの細胞期に特異的なパタンで進行の遅延が起こることを示している．これらのパタンを要約すると，(1) G2期はおかされやすいステージで，G2期に損傷が与えられたときばかりでなく，それに先立つ細胞

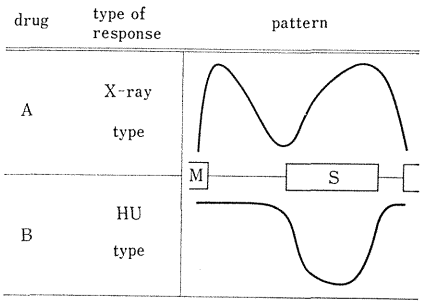


**図 2**　諸種の外来性作用体の細胞致死作用に関する細胞周期依存性感受性変化のパタン

期につくられた損傷にも感受性を示す，(2) S期に照射をうけると DNA 合成率が低下しS期は延長する，(3) G1期は照射によりほとんど遅延しないし，引き続くS期も延長しない，これは G1 期制御に関与する放射線感受性の機構がすでに役割を終えているためであろう，などである．このように放射線の周期内進行に対する効果は細胞期特異的である．

同じように Bleomycin は G2 期に作用していちじるしい G2 延長（G2-block）を起こすばかりでなく，G1期細胞に対しても G1，S期に変化を与えることなく G2 延長を誘発する[8]．これに反し Mitomycin は G1期に作用してS期，G2期のいちじるしい延長を起こす．S期の延長はおそらく DNA に誘発された未修復損傷によるのであろう．X-ray 型薬剤の進行に対する効果はまだ詳細に知られていない．概括的にはX線のそれに似て G2期に対し強く影響を与えながら，G1期の進行をおかすことがない．またS期に対する効果は薬剤によつてさまざまであるといえる．

HU 型薬剤は Camptothecin を除いてすべて G1 細胞のS期への進入を阻止し，G1後期への細胞の蓄積を起こす（late G1-block）のが特徴である[1,2]．S期の進行が事実上停止することはいうまでもないが，いずれの作用も可逆的であつて，X-ray 型薬剤の進行に対する作用が損傷由来であるのと対照的である．

## 2. 細胞齢依存性レスポンスと治療デザイン

腫瘍の増殖細胞群はおおむねランダムな細胞分布をもつているので，外来性作用体の1回投与により腫瘍細胞は，選択的不活化（selective killing）と特異的進行遅延（specific progression delay）をこうむる．つまり reassortment が起こり，これが分割投与または 2 種以上の作用体を併用するための契機となる．併用には同時併用と時間差併用とが考えられるが，分割投与は同種作用体

表 1　Possible Schedules of Multiple Z-Shots

| |
|---|
| Ⅰ. X-ray type agent alone |
| 1. A・X…A・X… |
| 2. X－X…X－X… |
| 3. A－X…A－X… |
| Ⅱ. Combination of X-ray type and HU type agents |
| 1. B・X…B・X… |
| 2. X－B…X－B… |
| 3. A・B…A・B… |
| 4. A－B…A－B… |
| Ⅲ. HU type agent alone |
| 1. B－B…B－B… |

・は同時併用，－は時間差併用
…は単位治療の間隔を示す．

の時間差投与として理解されよう．

reassortment を利用して腫瘍治療を考えるときのもう1つの重要な原理は腫瘍細胞の recruitment である．すなわち細胞を非増殖状態（non-cycling state）から，細胞周期へ誘導する時間をつくることで，そのためにはスケジュールされた単位治療が適当な間隔で繰返されなければならない（これについては後に触れる）．

いま図2にしたがつて両型の作用体をA（放射線をX），Bとし，意味のある組み合わせをとつてみると表1のごとくである．

Ⅰ．2種の X-ray 型の作用体の同時投与は，細胞齢依存性感受性パタンが同一なので元来意味をなさない．しかし，reassortment の意義から離れるけれども，Aが細胞へのX線効果を増強する能力のある場合に限り，A・X は有効である．Ⅰ-2は conditioning dose で生き残つた G1 初期，S後期の細胞がそれぞれ G1-S 移行期，G2-M 期へ移るであろう数時間後に therapeutic dose を与えるという考え方であるが，二分割照射線量の和を1回で与えたときに達せられるであろうより大きな細胞不活化を得るには，やや大きな therapeutic dose を要するかもしれない．これは分割間隔に sublethal な損傷の回復が完成してしまうためで，そういう意味からすると，therapeutic dose にX線と違つて細胞の指数関数的不活化をする薬剤を用いることは有利かもしれない．Ⅰのなかでもつとも有力な考えは A-X と思われる．たとえば Bleomycin はその単一投与によつて G1 細胞にさえいちじるしい G2-block を惹起させることができる．つまりこの種の薬剤で conditioning をするなら，細胞周期内のほとんどの細胞を G2 期に集め，これをX線によつて効率よく不活化することができるであろう．その成功は，いかに多くの G2 細胞を集めうるか，X線照射のタイミングを正しく把みうるか，にかかつている．

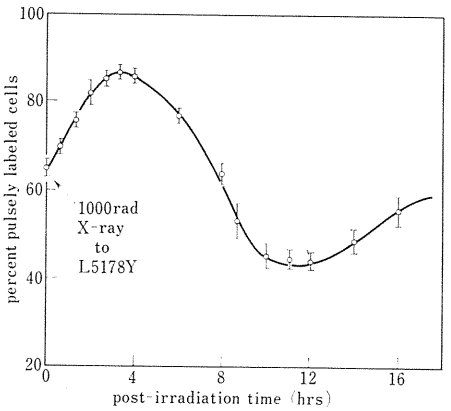


図 4　X線照射後に誘発されるS期細胞の蓄積（S-retention）．
1,000 rad の照射をうけたマウス白血病細胞（L5178Y）の培養から経時的にサンプルし，DNA 合成細胞率をオートラジオグラフによつて測定した（渡部郁雄氏の好意による，未発表データ）．

Ⅱ．デザイン Ⅱ-1 は原理的にもつとも矛盾が少ないが，G1 初期の抵抗性部分をどう処理するかが問題として残されている．この点はつぎの Ⅱ-2 によつてもつとも実際的な解決を得るように思われる．前項で述べたように，X線は G1 細胞のS期への流入をおかすことなしに，S細胞の G2 期への移行を遅らせる．結果としてS期細胞群の一時的な拡大が起こるであろう（S-retension）．図4はマウスリンパ性白血病細胞（L5178Y）の照射の後に起こる DNA 合成細胞の百分率の変化を示している（渡部，未発表）．この成績からもわかるように，4時間後に約90%の細胞がS期にたまり，S-sterilizer であるB薬剤のよい標的を形成する．

Ⅱ-1，2 と同じことが Ⅱ-3，4 でも実行可能であるが，全身的に拡がつた疾病でない限り抗腫瘍剤の及ぼす systemic な損傷を避けるのが望ましい．

Ⅲ．これは phase-specific agents の時間差投与ということで，とくに白血病細胞の sterilization を目指して研究されてきた．詳しくはその方面の専門誌に譲りたい[2]．HU 型作用体に総じていえることは，S期細胞を特異的に不活化する薬剤であるから，その1回投与量はS期集団を不活化する以上である必要がない．またこの種の薬剤は周期内進行に対する効果が可逆的なので，時間差投与に有利な面があることが指摘される．

### 3. Reassortment を土台とする治療の問題点

細胞齢依存的な感受性変動は放射線ばかりでなく，種

々の抗腫瘍剤についても証明され，まだ分類できないものもあるけれども，それらの survival response のパタンは大筋において相補的な2つの型に入ることが知られた．

また，薬剤には腫瘍細胞の周期内進行に対し，X線とは異なる効果を起こすものがみいだされる．それゆえもし腫瘍細胞の reassortment に治療デザインの基礎を求めるとなれば，放射線ばかりでなく抗腫瘍剤の作用を援用することは当然といわなければならない．

(1) この小論では単一投与による周期依存性レスポンスのパタンを基礎として分割あるいは併用について考えてきたが，とくに時間差併用においてはまだ考慮すべき kinetics がある．たとえばX線の二分割照射の効果に影響する因子は，i. sublethal な損傷の回復，ii. potentially lethal な損傷の回復，iii. 細胞の repopulation などであるが，まつたく同じ因子が抗腫瘍剤投与後において検討されなければならない．最近われわれの研究室は，Bleomycin が potentially lethal な損傷を誘発すること，*in vivo* の腫瘍細胞はそれらを回復する能力をもつこと，を明らかにした．この種の回復が他の抗腫瘍剤によつても誘発されるか否かはまもなく明らかになるであろう．また，細胞の不活化曲線で shoulder を示す BCNU その他の薬剤における sublethal な損傷の存否，損傷回復の可能性についても知見を蓄積しなければならない．

(2) これまで論じられた cell cycle kinetics と細胞齢依存性レスポンスとは，すべて培養細胞での成果に依存しているが，ヒトの腫瘍細胞にそのまま適用しうるか否かについてはかなり疑問がある．図5にみるように，腫瘍細胞の世代時間はそのタイプによつてかなり異なるが，とくにヒトの腫瘍では移植腫瘍と違つて世代時間の分布の大きいものがあると考えられる[3]．種々の腫瘍型について知見を得るためには，human tumor biology の開拓に俟たなければならない．

(3) reassortment はいうまでもなく増殖細胞の動力学なので，その限りでは培養細胞の研究成果は原理の提供に役立つであろうけれども，腫瘍の構造にかかわるレスポンス，つまり放射線をうけた腫瘍に起こる repopulation とか，reoxygenation という cell kinetics，を無視しては治療として成り立たない．腫瘍のなかの cycling fraction はここにあげたタイプの作用体によつて特異的な細胞死と同時に，除去 (elimination) をうけるであろう．このことは腫瘍内に homeostasis を維持する demand となり，non-cycling fraction より cycling fraction への動員 (recruitment) とそれに続く repopulation を引き起こす．表1に示された単位治療の反復というデザインの完成にとつて，上述の cell kinetics は避けられない課題として残されている．

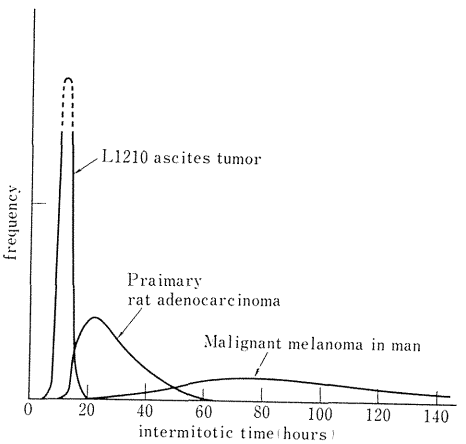


**図 5**　各種の腫瘍で推定された intermitotic time の分布 (Lamerton, 1972).
*Brit. J. Radiol.* の許可により転載

## おわりに

感受性の変動は外来性作用体に対する生体の homeostasis の1つの表われ，平たくいえばショックをいなし生体を守るメカニズムの一つともいえるが，本稿においては逆に腫瘍細胞の感受性変動を利用して，いかにこの細胞を sterilize するかだけが問題とされてきた．ここでわれわれは正常組織の cell kinetics と腫瘍のそれとの相違，作用体に対するレスポンスの相違，つまり感受性変動が治療効果比に寄与するものについて考察しなければならないのであるが，それはこの小論の規模を超えていると同時に，知見の一層の熟成を待つべきであると考えたため触れられなかつた．将来の機会を期したい．

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[Gann, 67, 157～163; April, 1976]

# COMBINED USE OF BLEOMYCIN IN RADIOTHERAPY OF A MOUSE MAMMARY CARCINOMA

Muneyasu URANO, Koichi ANDO, Sachiko KOIKE, Kyouko OHARA, and Naofumi NESUMI
*Division of Clinical Research, National Institute of Radiological Sciences**

Experiments were carried out to determine $TCD_{50}$ (50% tumor control dose) of 3rd generation isotransplants of a C3H mouse mammary carcinoma treated or not treated with Bleomycin. If the antibiotic was injected 30 min before a single X-ray dose, $TCD_{50}$ was reduced. This reduction in $TCD_{50}$ was independent of Bleomycin dose of more than 15 mg/kg, because of the upward-concave nature of Bleomycin dose–cell survival curve. The combined effect, when tested by $TCD_{50}$ assays, appeared less than additive. This effect was further examined by a series of $TD_{50}$ assays which revealed that these tumor cells were capable of repairing the potentially lethal damage induced by X-rays and that induced by Bleomycin. It was also found that the potentially lethal damage after combined X-ray and Bleomycin treatments was repaired. These findings indicated that the combined X-ray and Bleomycin treatment resulted in additive effect if the repair of potentially lethal damage in tumor cells were taken into account.

Many attempts have been made to use chemotherapeutic agents together with ionizing radiation in the treatment of malignant tumor. Except for some chemicals, such as halogenated pyrimidines or electron-affinity compounds which possess radiosensitizing ability,[1,6,16,26] theoretical basis for these attempts includes the following: Employment of different age-dependent sensitivities between chemicals and radiation, the likelihood for specific agents to reduce the capability of tumor cells to repair sub-lethal radiation damage, or a synergistic action of the agent and X-rays or of two chemical agents which might occur because of different modes of action.

Bleomycin,[23] now extensively used in the clinics as one of effective anticancer antibiotics,[10,13] is reported to have a different effectiveness in cultured mammalian cells as a function of age in the cell cycle.[18] It has been observed also that Bleomycin induces strand breaks in DNA.[19] Its potentiating effect on X-ray damage has been reported to be very limited,[4] e.g., response to X-rays was enhanced by only a factor of ～1.1 when HeLa cells were irradiated in the presence of Bleomycin (T. Terasima, personal communication).

On the basis of these experimental findings it seemed desirable to study the efficacy of this antibiotic in radiotherapy of tumors using an experimental model tumor system. Present attempts were made to study the influence on tumor therapy of age-dependent sensitivity to Bleomycin and to X-rays; and the capacity of Bleomycin to inhibit the repair of sublethal radiation damage in tumor cells.

## MATERIAL AND METHOD

Experimental animals were 8- to 10-week-old C3H/He mice supplied by Funabashi Farm Co., Chiba. They were kept in a small animal facility in our Institute where constant temperature was maintained. Animals had free access to standard Purina pellets and chlorinated water. Third generation isotransplants of a mammary carcinoma

* Anagawa 4-9-1, Chiba 280 (浦野宗保, 安藤興一, 小池幸子, 大原恭子, 根住直史).



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which had arisen spontaneously in a C3H/He female mouse were used throughout the present study. The procedure to obtain 3rd generation isotransplants involved storing the spontaneous and 1st generation tumors in a liquid nitrogen refrigerator and proliferating the latter in several female mice.[24]

Tumor cell suspensions were aseptically prepared from 2nd generation tumors in two different ways. The suspension of monodispersed cells was obtained as described in a previous paper[27] and was used for the determination of tumor control dose. The second or enzyme procedure was employed for $TD_{50}$ studies. The intact tumor tissue was removed and roughly minced. The mince was trypsinized by stirring at 37° for 30 min in a flask containing the Dulbecco balanced salt solution, which was free of Mg and Ca, and contained 0.2% trypsin (Difco 1:250). The flask was placed in the crushed ice and the suspension was allowed to settel for 15 min. Then the supernatant was removed by a syringe, passed through a Swinny filter, and centrifuged for 5 min at 1600 rpm. The sediment was resuspended in a sufficient amount of Hanks' medium containing 5% fetal calf serum. In both procedures, viable tumor cells were counted in a hemocytometer with the aid of Trypan Blue staining and 10 $\mu$l of this suspension was subcutaneously transplanted into the right thigh of each recipient mouse.

The method used to irradiate animal tumors was the same as reported elsewhere.[28] Briefly, the procedure was as follows: An X-ray machine was operated at 200 kVp and 25 mA without added filtration. The half-value layer was 0.8 mm Cu and target–tumor distance was 15 cm. Two tumors were irradiated simultaneously through a 2.5× 3.5 cm field and, to give a homogeneous dose distribution in the tumors, the animals were turned over in the middle of the irradiation. The dose-rate at the tumor center was ~660 rads/min. Animals were anesthetized by intraperitoneal injection of 60 mg/kg of Nembutal before irradiation.

Local irradiations were given under two different conditions. One was of air-irradiation, i.e., tumors were irradiated when animals were breathing normal air without disturbing the local blood circulation. The other was made under condition of hypoxia which was obtained by applying a heavy brass clamp above the tumor 2 min before and during irradiation.

Two experimental assay methods were employed. $TCD_{50}$ assay, determination of radiation dose which yields a local control in 50% of irradiated tumors, was applied as described elsewhere.[14, 28] Animals were randomly arranged in experimental groups after transplantation. Tumors were locally irradiated when they reached 8 mm in average diameter. After irradiation they were examined for tumor recurrence by palpating the irradiated area once a week for at least 120 days. If a tumor relapsed and regrew to over 10 mm in average diameter, it was scored as a tumor recurrence. If the animal died without any palpable tumor before 120 days, it was excluded from the assay. If the animal died with a small tumor, the tumor recurrence was examined by autopsy. Based on the tumor control frequency in 120 days, $TCD_{50}$ was calculated by the method of logit analysis. The second method was $TD_{50}$ assay, i.e., an assay to determine the number of tumor cells to transplant a tumor in one-half of the animals.

The enzyme procedure was applied to prepare a single cell suspension from the treated tumors. The suspension was serially diluted in 2- to 3-fold by Hanks' medium into 6 to 8 doses and each dilution was transplanted into the right thigh. The scoring method for tumor take was similar to that of tumor recurrence in $TCD_{50}$ assays, and $TD_{50}$ was calculated by logit analysis from the tumor-take frequency in 80 days. In both methods, 40~60 animals were used for a single assay and approximately equal number of male and female mice were used. Usually 4~5 assays were made simultaneously in one experiment and all the $TCD_{50}$ assays were at least duplicated.

Bleomycin complex (copper free) was kindly supplied by Nippon Kayaku Co., Tokyo. The antibiotic was diluted in 0.9% NaCl solution in order to inject a desired amount in 0.01 ml/g body weight. This solution was administered intraperitoneally.

## Results

In the 1st series of experiments, the effect of a single Bleomycin dose on the $TCD_{50}$ was examined. Administration of the antibiotic was followed 30 min later by local tumor irradiation which was given under hypoxic condition. $TCD_{50}$ of tumors pretreated with Bleomycin was found smaller than that of untreated tumors, but it did not change with Bleomycin dose over the dose range tested. These results are tabulated in the upper half of Table I, where the last column on the right, headed "observed", lists the surviving fraction of tumor cells after Bleomycin only and the next-to-last column, headed "expected", shows the surviving fraction of tumor cells calculated on the basis of the Bleomycin

dose–cell survival curve obtained in an earlier experiment.[27] The "observed" surviving fractions were obtained as follows:

$TCD_{50}$ after single X-ray dose can be expressed as

$$TCD_{50}=D_0(\ln m+\ln M+\ln \ln 2)$$

where $m$ and $D_0$ are extrapolation number and radiation dose to reduce the surviving fraction by a factor of 1/e in the straight portion of the survival curve, and $M$ is the number of tumor cells which should be sterilized for tumor control.

Hence,

$$\ln M=TCD_{50}/D_0-\ln m+\ln \ln 2.$$

If $M$ is reduced to $M'$ by a given amount of Bleomycin before irradiation, $TCD_{50}$ of this tumor, say $TCD_{50}$ (B), will be

$$TCD_{50}\,(B)=D_0(\ln m+\ln M'-\ln \ln 2)$$

Then the "observed" surviving fraction after Bleomycin was obtained as the ratio of $M'$ to $M$.

Present results indicated that "observed" surviving fractions were larger than the "expected" ones, and may imply that the combined treatment did not sterilize tumor cells as effectively as predicted from both the X-ray and Bleomycin dose–cell survival curves; that is, the combined effect was less than additive.

This "less than additive" effect was again observed in a split-dose study. At 2 min before the 1st X-ray dose, a heavy brass clamp was applied above the tumor and then Bleomycin was administered intraperitoneally. The 2nd X-ray doses were given also under hypoxic condition 5 hr after the conditioning dose. As shown in the lower half of Table I, a large recovery dose, if the recovery dose is defined as a difference between $TCD_{50}$ (two doses) and $TCD_{50}$ (single dose), was observed, i.e., ~1300 (=6870—5560) and ~1500 (=6700—5190) rads without or with Bleomycin, respectively. This suggests that the antibiotic did not inhibit the capability of these tumor cells to repair sublethal radiation damage.

The effect of Bleomycin in the fractionated X-ray doses was investigated in the next series of experiments. The 1st to 4th X-ray doses were 800 rads each, while the 5th administration was carried out with graded doses to estimate the $TCD_{50}$. The purpose was to examine the effect of fractionated treatments and the length of the interval between Bleomycin and X-ray administration on the tumor control probability. The $TCD_{50}$ of tumors which had received Bleomycin and X-rays was less than that of tumors treated with X-ray alone, while it was independent of time interval between Bleomycin and X-rays (Table II). Based on the radiation dose–cell survival relations, the effect of combined treatment was again found to be less than that anticipated. It is notable that the com-

Table I. $TCD_{50}$ of C3H Mouse Mammary Carcinoma Treated with Bleomycin and X-rays

| 1st treatment X-ray (rads) | 1st treatment Bleomycin (mg/kg) | Time interval (hr) | $TCD_{50}$ in 120 days (95% confidence limit) (rads) | Surviving fraction after Bleomycin Expected | Surviving fraction after Bleomycin Observed |
|---|---|---|---|---|---|
| — | — | — | 5560 (5430~5690) | 1.0 | 1.0 |
| — | 15 | 0.5 | 5180 (5050~5300) | 0.26 | 0.38 |
| — | 30 | 0.5 | 5040 (4840~5240) | 0.13 | 0.26 |
| — | 45 | 0.5 | 5090 (4830~5360) | 0.067 | 0.30 |
| 2500 | — | 5.0 | 6870 (6660~7080) | 1.0 | 1.0 |
| 2500 | 22.5 | 5.0 | 6700 (6390~7040) | 0.20 | 0.65 |
| — | 22.5 | 5.0 | 5190 (5040~5340) | 0.20 | 0.39 |

All the tumors received 2nd treatment of X-irradiation after a listed time interval. Values employed for calculation of "surviving fraction after Bleomycin" are $m=5.0$ and $D_0=390$ rads.[28]

Table II. $TCD_{50}$ of C3H Mouse Mammary Carcinoma Treated with Fractionated X-rays and Bleomycin

| Daily treatment | | | | $TCD_{50}$ in 120 days (95% confidence limit) (rads) | Surviving fraction after Bleomycin (observed) |
|---|---|---|---|---|---|
| Bleomycin (mg/kg) Day 1~5 | Time interval (hr) Day 1~5 | X-ray Dose (rads) Day 1~4 | X-ray Condition Day 1~5 | | |
| — | — | 800 | Air | 5950 (5760~6150) | 1.0 |
| 7.5 | 0.5 | 800 | Air | 5630 (5490~5770) | 0.44 |
| 7.5 | 4.0 | 800 | Air | 5560 (5290~5840) | 0.37 |
| 7.5 | 8.0 | 800 | Air | 5490 (5240~5750) | 0.31 |
| — | — | 800 | Hypoxia | 7130 (7010~7250) | 1.0 |
| 7.5 | 0.5 | 800 | Hypoxia | 7080 (6850~7320) | 0.88 |
| 7.5 | 8.0 | 800 | Hypoxia | 7050 (6860~7250) | 0.81 |

On day 5, graded X-ray doses were given to determine $TCD_{50}$. The "expected" surviving fraction after 5 administrations of 7.5 mg/kg Bleomycin, assuming additivity between divided doses, is 0.044.

Table III. Repair of Potentially Lethal Damage in C3H Mouse Mammary Carcinoma Cells Treated with X-rays and/or Bleomycin *in vivo*

| Treatment | | Time interval between treatment(s) and tumor removal(hr) | $TD_{50}$ (95% confidence limit) | Surviving fraction |
|---|---|---|---|---|
| Bleomycin (mg/kg) | X-ray (rads) | | | |
| — | — | — | $2.9(1.9\sim4.3)\times10^3$ | 1.0 |
| — | 2000 | 0 | $1.1(0.5\sim2.8)\times10^5$ | 0.026(0010~0.058) |
| 30 | — | 0.5 | $<1.5\times10^5$ | <0.02 |
| 30 | 2000 | 0 | $4.1(2.6\sim6.2)\times10^6$ | 0.00072(0.00047~0.0011) |
| — | 2000 | 6 | $1.2(0.9\sim1.5)\times10^4$ | 0.25 (0.19~0.32) |
| 30 | — | 6.5 | $1.6(1.2\sim2.3)\times10^4$ | 0.18 (0.13~0.24) |
| 30 | 2000 | 6 | $1.5(0.9\sim2.4)\times10^5$ | 0.020(0.012~0.032)[a] |

a) If combined treatments resulted in an additive effect, surviving fraction should be 0.044 (0.024~0.078), i.e., $0.25\times0.18$, which was not significantly different from data presented.

All the irradiations were given under hypoxic conditions and the time interval between Bleomycin and X-rays was 30 min.

bined treatment reduced the surviving fraction more effectively if irradiation was given under air condition than under hypoxic condition (see the last column of Table II).

The final series of study was designed to analyse the "less than additive" effect observed in all the combined treatments tested above. Animals were treated with X-ray alone, Bleomycin alone, or by both when transplants reached 8~9 mm in average diameter. Tumors were removed immediately or 6 hr after the treatment(s) and used for $TD_{50}$ assays. For convenience, following abbreviations will be used: $SF_0$ or $SF_6$ means surviving fraction of tumor cells excised immediately or 6 hr after treatment, respectively, and SF(X), SF(B), and SF(BX) indicate surviving fraction of tumor cells treated with X-rays, Bleomycin, and both, respectively. Results are presented in Table III together with surviving fractions calculated as a ratio of $TD_{50}$(untreated) to $TD_{50}$(treated). The surviving fraction of tumors exposed to 2000 rads and removed immediately thereafter, i.e., $SF(X)_0$, was 0.026 and was on the dose–cell survival curve previously obtained,[28] while that of tumors removed at 6 hr after 2000 rads, i.e., $SF(X)_6$, increased to 0.25, i.e., approximately 10-fold increase. This result implies that the potentially lethal radia-

tion damage could be repaired in 6 hr after irradiation. This increase in the surviving fraction was also observed in tumors treated with Bleomycin alone or with combined Bleomycin and X-rays, and was more extensive than in tumors treated with X-ray alone. Comparison of the surviving fraction after each treatment indicated that the combined therapy is found additive or slightly more than additive if $SF(X)_6$ and $SF(B)_6$ are used for comparison with $SF(BX)_6$. On the other hand, it might be analysed as "less than additive" if $SF(X)_0$ and $SF(B)_6$, $SF(X)_6$ and $SF(B)_0$, or $SF(X)_0$ and $SF(B)_0$ are employed for this comparison, as in the $TCD_{50}$ assays mentioned above.

## Discussion

It is now established that treatment with Bleomycin either *in vitro* or *in vivo* gives rise to a dose–cell survival curve which is concave upwards.[2,20,27] The initial sensitive portion of the survival curve appears to be exponential and is followed by a resistant tail. This appearance of the resistant component was interpreted according to the hypothesis termed a "binding-saturation model" in our previous paper.[27] The finding in the present study that the surviving fraction after a dose of 15~45 mg/kg was independent of the Bleomycin dose might possibly be explained by the upward-concave nature of the dose–cell survival curve.

One of the present results clearly indicated that the capability of tumor cells to repair potentially lethal damage was important for the assessment of the combined X-ray and Bleomycin treatments. It is now argued that tumor cells irradiated with X-rays are able to repair the potentially lethal damage if they were left in plateau phase culture or ascites, or in a solid tumor for several hours after irradiation.[9,12,29] However, this kind of repair is not involved in the dose–cell survival curve determined by a routine method, since tumor cells employed for its determination are those which had been irradiated *in vivo* and removed immediately thereafter or those which had been dispersed as a single cell suspension and then irradiated *in vitro*. Surviving fractions listed in Tables I and II were calculated by the use of a dose relation of such tumor cells, and accordingly, the capability of this kind of repair was not taken into account for the assessment of the combined treatment. Not only the potentially lethal damage induced by X-rays, but also that induced by Bleomycin is reported to be repaired *in vivo*,[11,17] and this finding was also consistently observed in the present study. It is also demonstrated that this kind of repair could be accomplished approximately within 6 hr after either Bleomycin or X-ray treatment.[12,18] These evidences indicate an important rôle of the repair of potentially lethal damage in the assessment of a given treatment regimen *in vivo*. In clinical radio- or chemo-therapy, tumors are usually treated with fractionated administrations and left *in situ* until the termination of the treatment. Therefore, tumor cells may be capable of repairing the potentially lethal damage after each treatment. It should be noted that, as indicated in the present study, collaboration of X-rays and Bleomycin could result in an additive effect, if the repair of potentially lethal damage is taken into account for evaluation of the treatment.

Stationary phase cultured cells have been reported to be less sensitive to some agents than log phase cells.[5,30] Data so far reported for Bleomycin are still conflicting.[3,7,21,22] However, of interest is a finding that tumor cells in a large tumor or in stationary phase are able to repair the potentially lethal damage more extensively than those in a small tumor or in exponential phase. This suggests that the repair of potentially lethal damage is one of the important and dominant factors for sensitivity of tumor cells to a given agent, in different size of tumors or different phase of growth. In addition, the combined treatment was found to reduce the surviving fraction more effectively if irradiation was given

under air condition than under hypoxic condition. This fact could be interpreted by an evidence that tumor cells irradiated under hypoxic condition were capable of repairing the potentially lethal damage more extensively than those treated under air condition.[25] This finding could also be interpreted by this kind of repair.

The mouse mammary carcinoma cells can accumulate and repair sublethal radiation damage. In the present study, a large recovery dose was observed if a dose of 2500 rads was followed by a 2nd dose 5 hr later. The recovery dose was independent of the administration of Bleomycin. This result suggests that the antibiotic did not affect the capability of the cells to repair sublethal damage, even though Bleomycin has been reported to produce strand breaks in cellular DNA.[19]

Age-dependent sensitivity of cultured mammalian cells has been reported to be similar for Bleomycin and X-rays.[18] Thus, the simultaneous administration of X-rays and the antibiotic might be expected to be less effective than Bleomycin followed by X-rays, say, 8 hr later, when the Bleomycin-resistant cells would have progressed into a Bleomycin-sensitive age which would also be sensitive to X-rays. In contrast to this prediction, the $TCD_{50}$ of tumors which had received Bleomycin and, 8 hr later, X-rays was only slightly, but not significantly, less than that of tumors treated with the same modalities 30 min apart. Several interpretations might be offered to account for this finding. First, the difference of sensitivity throughout the cell cycle might not be so great as that observed *in vitro*, or the synchronization might be very limited. Second, tumor cells might not progress as rapidly after Bleomycin treatment as predicted. Finally, desynchronization could take place promptly after each treatment. Some experimental observations suggest that cells are able to progress through the cell cycle rather rapidly after Bleomycin or X-rays.[2,15,32] After Bleomycin treatment, progression through the cell cycle is reported to be delayed principally in G2 phase,[2,31] and X-irradiated tumor cells have been demonstrated to be capable of rapid repopulation in a split-dose study.[15] Furthermore, it is noteworthy that the potentially lethal damage produced by chemotherapeutic agents can be repaired *in vivo*.[8] This repair, which might take place during each treatment interval, would reduce the effectiveness of a scheme designed to take advantage of age-dependent sensitivity factors.

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原　著

# ブレオマイシンのエールリッヒ腹水腫瘍細胞に対する致死効果およびその最適投与方法の提案

*千葉大学医学部第一内科
高部吉庸*　　渡辺道典*
**放射線医学総合研究所病院部
宮本忠昭**
***汐見ヶ丘病院
勝俣剛志***
****放射線医学総合研究所生理病理部
寺島東洋三****

**要旨**　Ehrlich 腹水腫瘍を使用し，*in vivo-in vitro* assay system で研究された Bleomycin の最適投与方法は持続注入法であった。この方法により potentially lethal damage の回復の影響を最少とし，最大の細胞不活化効果を得ることができる。

## はじめに

近年，組織培養法を利用して，細胞レベルで抗癌剤の投与方法を研究しようとする試みが行なわれるようになり，癌の化学療法を志ざす人々に新しい情報を提供しつつある。腫瘍治療の基本的原理は tumor cell sterilization であるという観点に立脚したこの試みは，薬剤と細胞との dose survival relationship および薬剤の細胞に対する time inactivation rate を明らかにすること，すなわち，薬剤の投与量あるいは投与時間と細胞の増殖力の不活化との相関関係を定量化することをもって出発点とする。人癌の化学療法にあたっては，*in vitro* レベルの実験条件と異なる種々の因子が癌細胞の不活化動態を修飾するので，その動態を知ることは抗癌剤の投与方法を考える上にさらに実際的な意義を加えるものと考えられる。

Bleomycin (BLM) の培養哺乳類細胞に対する致死効果は，1970年に寺島[1]等によって記載されて以来幾多の報告がなされた[2,3,4]。その致死曲線の特徴は，2相性で upward concavity を有することにある。この特徴的な2相性レスポンスの成因は，BLM 投与により細胞に BLM に対する抵抗性が誘導されるためであり，さらに，この抵抗性は BLM を除去することにより経時的に崩壊すること，BLM 投与の間隔を適当にとるならば抵抗性の誘導と崩壊は何度も繰り返えされうることが寺島，高部等によって明らかにされ，そして，適当な間隔の間歇投与によって最大の細胞不活化率の得られることが示唆された[3,4]。

その後，*in vitro* あるいは *in vivo* で BLM を作用させた場合，投与直後に assay された細胞の

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Lethal effect of bleomycin on Ehrlich ascites tumor cells and proposal for optimal schedule of bleomycin chemotherapy.

Yosinobu Takabe*, Michinori Watanabe*, Tadaaki Miyamoto**, Takeshi Katsumata***, and Toyozo Terasima****.
*First Dept. of Medicine, Chiba University School of Medicine. ** National Institute of Radiological Sciences, Hospital. ***Shiomigaoka Hospital. ****Division of Physiology and Pathology, National Institute of Radiological Sciences.

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生残率は，投与後数時間で assay された生残率より低いことが明らかとなり，BLM 投与により potentially lethal damage (PLD) の回復がひきおこされるためであると解釈された[5,6,7]。この知見は BLM の投与方法に関し新しい論理を与えた。われわれは，エールリッヒ腹水腫瘍 (EAT) 担癌マウスを使用し，*in vivo* に BLM を投与し *in vitro* で細胞生残率 (surviving fraction: S. F.) を検定する方法で，BLM の EAT 細胞に対する dose survival relationship, time inactivation rate を明らかにし，*in vivo-in vitro* assay system での BLM の最適投与方法を研究し，一つの結論を得るに至った。

なお，ホジキン病の1症例の BLM に対するレスポンスを血沈値を指標として観察し，実験的に決定した dose survival relationship の基本的性格がヒト腫瘍においても証明されうると考えられるので，併せてこれを報告する。

## 実験方法

1. 実験動物および細胞

4週令の ICR/JCL マウス（♂，体重20～25g）を使用（日本クレア産出）。EAT 細胞を $10^6$ ヶマウスの腹腔内に注射すると，4日目までは12時間の倍加時間で指数関数的に増殖するが，5日目以降定常増殖期に入り，移植後約12日で $10^9$ ヶに達しマウスは死亡する。すべての実験は移植後7日目に計画された。

2. BLM の投与

Bleomycin complex (Lot # F100AS4, 日本化薬）を使用。蒸溜水で溶解した BLM を凍結保存し，実験直前に融解し F10 メジウムで適当な濃度に稀釈し，マウスの体重1gあたり 0.01m*l* を背部皮下にマントー針で注射した。BLM 投与後の細胞生残率の経時的推移を求める実験を除いて，腹水腫瘍細胞の生残率は通例 BLM 投与後1時間に腹水をとり出し，稀釈した後に測定した。持続注入法は，ネンブタール麻酔したマウスの尾根部よりカテーテル（ATOM, INTRAVENOUS CATHETER FOR CUT DOWN, French Size 3 Fr.）を背部皮下に挿入し，注入速度 0.85m*l*/24hr の Infusion Pomp で6時間の持続注入を行なった。

3. *in vitro* survival assay

腹水をマントー針で採取後，10%の仔牛血清を加えた F10メジウムに浮遊し，浮遊細胞を coulter counter でカウントした後 F10メジウムで稀釈した。ひきつづいて，BLM の投与濃度に応じて500ヶから5000ヶの細胞を，生残率の1測定につき3枚の agar plate に播種した。plate は，37°C，5%炭酸ガス培養器にて10日間培養し，形成されたコロニーを弱拡大の光学顕微鏡で算定した。BLM 非投与動物における EAT 細胞のコロニー形成率 (plating efficiency: P. E.) は，全実験を通じて40～90%であった。BLM 投与動物における EAT 細胞の S. F. は S. F. =BLM 処理細胞の P. E. ÷ BLM 未処理細胞の P. E. の式から求められた。

4. agar plate の組成

plastic petri dish (60×15mm, Falcon Plastics, Calif.) に basal layer として 10% 仔牛血清と 0.5% の agar (Special Agar Noble, Difco, Detroit, Mich.) を混合した 6 m*l* の F10メジウムを固化し，その表面に seed layer として，20%仔牛血清と 0.3%の agar および適当数の細胞を混じた 3 m*l* の F10メジウムを重層した。なお，実験に使用したすべての F10メジウムには，ペニシリン 100単位 /m*l* とストレプトマイシン 100$\mu$g/m*l* が加えられた。

## 実験結果

(1) Dose Response Curve

0.01mg/kg から 5 mg/kg の BLM をマウスの皮下に注射し，1時間後（図1の黒丸印）および6時間後（図1の白丸印）に細胞を腹腔外にとり出して，S. F. を検定した。その生残率曲線は両者とも2相性の upward concave curve を示し，initial portion と terminal portion の変曲点は 0.25mg/kg から 0.5mg/kg の間に存在する。1hr exposure の曲線において，この変曲点以下の濃度での $D_{37}$ は 0.15mg/kg であるのに対し，変曲点以上の濃度での $D_{37}$ は 23mg/kg であった。また，6hr exposure の S. F. は 1hr exposure の S. F. より terminal portion で比較して 2.3倍から2.5倍上昇している。これは，BLM 投与により

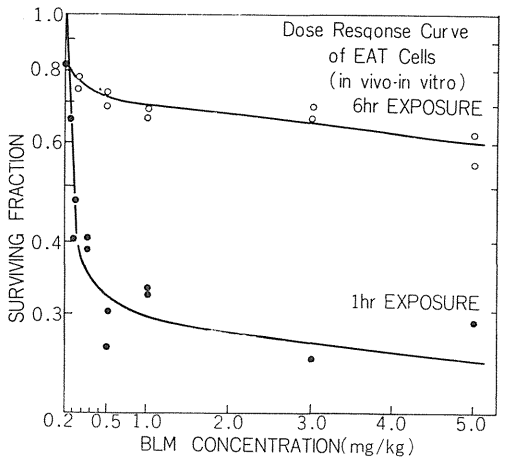


図 1　BLM による EAT 細胞の Dose Response Curve

EAT 細胞に与えられた損傷が，1時間から6時間までの間に回復することを示している。

(2)　Time Inactivation Curve

次に，BLM による損傷の回復の経時的推移および低濃度の投与と高濃度の投与とで，損傷の回復過程にどのような差があるかを調べた。図2は，0.1mg/kg，30mg/kg の BLM 投与後，経時的に S.F. を測定したものである。0.1mg/kg の投与でも，30mg/kg の投与の場合も，S.F. は1時間後に最低となり，3時間後までは急速に以後ゆるやかに上昇し7時間後にほぼ最大値に到達する。7時間後の S.F. の1時間値に対する増加率は，それぞれ1.5倍，3.5倍で，低濃度の BLM による損傷の回復の割合は，高濃度でのそれと比較して明らかに少ない。

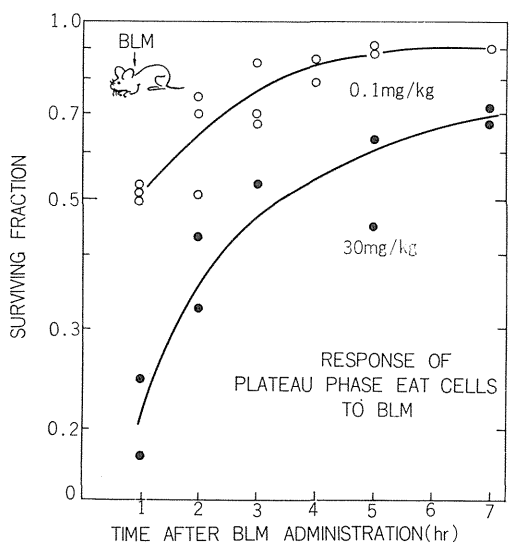


図 2　BLM による EAT 細胞の Time Inactivation Curve

(3)　BLM の分割投与による S.F. の経時的推移

図3の実験成績は，EAT 細胞に対して 0.1mg/kg，30mg/kg の BLM を1時間と3時間の2つの分割間隔で投与し，投与後1時間で S.F. を測定して得られた細胞不活化曲線を示している。横軸は BLM の第1回投与後の時間で，1時間後に得られた各測定値は薬剤投与の時刻にプロットされている。これによると，0.1mg/kg という低濃度では，1時間間隔の分割スケジュールの方が3時間間隔のそれより有効に細胞を不活化するようにみえる。

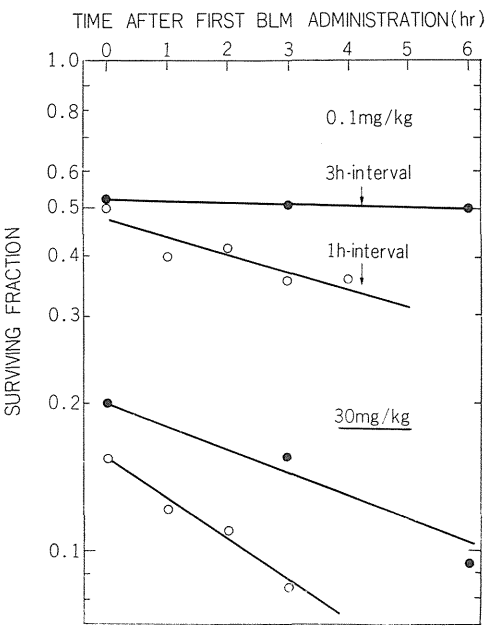


図 3　BLM の分割投与による S.F. の経時的推移

(4)　BLM 分割投与時の投与総量と S.F. の関係

一方，30mg/kg の投与の場合も 0.1mg/kg と同じ傾向に見えるが，横軸に投与総量をとって図を書き直してみると，図4のように 0.1mg/kg と

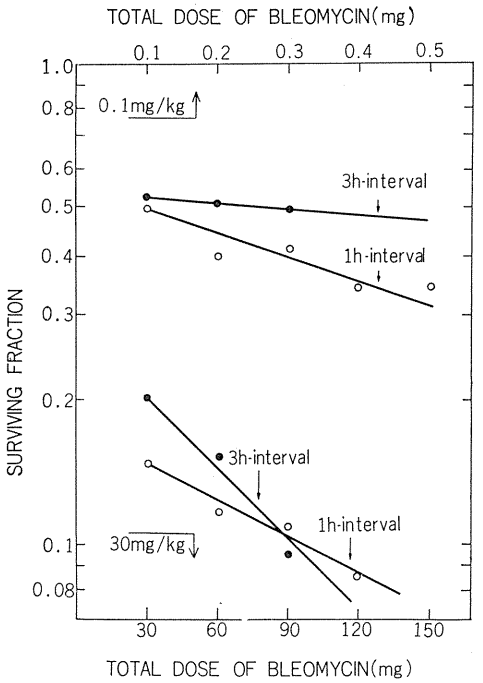


図 4 BLM 分割投与時の投与総量と S.F. の関係

は逆の関係，つまり3時間間隔の分割スケジュールの方が1時間間隔のそれより有効に細胞を不活化している。このような濃度あるいは投与間隔による細胞不活化率の相違は，BLM 投与によってひきおこされる損傷の回復の量と誘導される抵抗性の大小に左右されるが，詳細は項を改めて述べたい。

(5) BLM の持続注入法による S.F. の経時的推移

図3，図4の結果から，0.1mg/kg の濃度で1時間間隔の投与により最大の細胞不活化率の得られることが明らかになったが，この所見を拡張するならば，細胞の損傷の回復に時間を貸さない持続投与は一層効果的ではなかろうかと推測される。図5の実験は，担癌マウスに BLM を 0.1mg/kg/hr の速度で持続注入を行ない，1時間ごとに細胞を腹腔外にとり出し，6時間後までの S.F. の推移を観察したものである。比較のために，0.1mg/kg，1時間間隔投与の細胞不活化曲線（図4より）を破線で示した。投与開始直後には 0.1 mg/kg 分割投与は持続投与に比し急速な細胞不

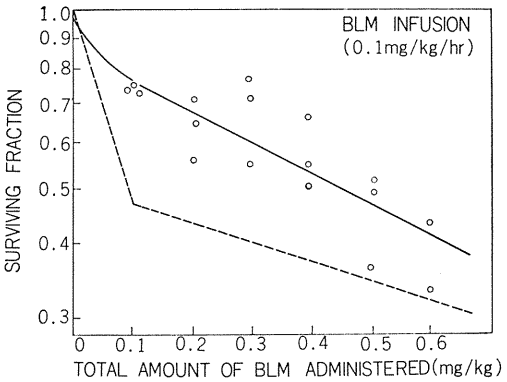


図 5 BLM の持続注入法による S.F. の経時的推移

活化を示すが，まもなく不活化速度は約1/10に低下する。持続投与の場合も本質的には同じ傾向のレスポンスを示すが，terminal slope は分割投与に比べて明らかにより速く細胞を不活化することを示している。

(6) ホジキン病の1症例の BLM に対するレスポンス

*in vitro*, *in vivo* の assay system から得られた BLM と細胞との dose survival relationship は BLM と人癌との間にも成り立つものであろうか。この問への一つの解答として，1972年より3

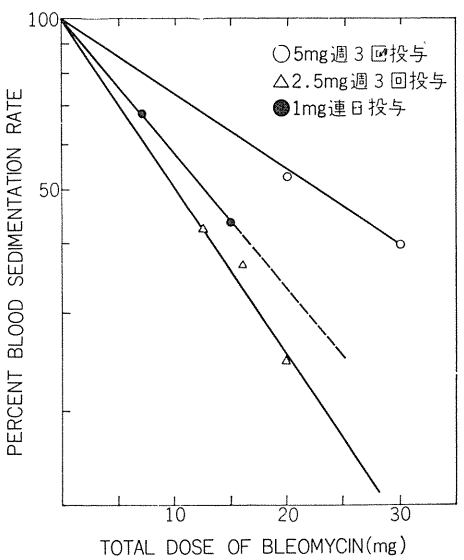


図 6 BLM によるホジキン病の治療
(a) 各投与法と血沈値の改善率との相関

年間にわたって BLM 単独治療を行なったホジキン病の1症例の BLM に対するレスポンスを示してみたい。患者は44才の男性で組織型は para-granulom type であった。本来，腫瘍の薬剤に対するレスポンスは腫瘍の縮小率を指標とすべきであるが，この患者においては，自覚症状および他覚的所見の軽快と増悪が血沈値の消長と著しく相関したので，次善の策として血沈値を指標とした。図6は，BLM の静注法で5mg 週3回，2.5mg 週3回，1mg 連日の投与を行なった際の投与総量に対する血沈値の改善率をみたものである。ただし，比較のため，治療前の血沈値を100に補正してある。それぞれの曲線の勾配を比べると，5mg の投与は 2.5mg，1mg の投与よりも血沈値の改善率が明らかに劣っている。次に，この関係を一層明白にするために，便宜的に各濃度を5回投与した時点での血沈の改善率を図6より求め，BLM の投与量を横軸にとって図を書きなおしてみると図7のような曲線が得られる。この曲線は，いわばこのホジキン病の BLM に対する dose

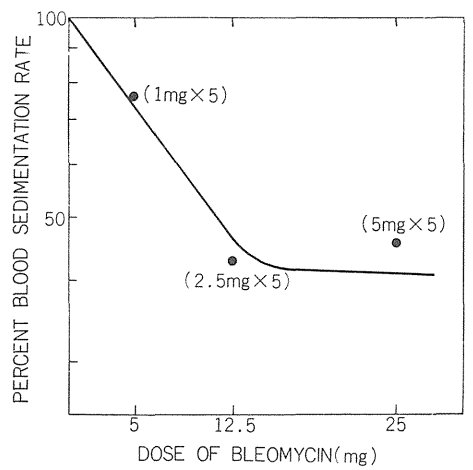


図7　BLM によるホジキン病の治療
(b)　血沈値を指標とした dose response curve

response curve というべきもので *in vitro*, *in vivo* の実験系で証明されたと同じ2相性のレスポンスを示している。変曲点は 2.5mg であった。

## 考　察

1969年以来，Little, Hahn らによって，放射線を照射された plateau phase の培養細胞における PLD の回復現象が研究されてきた[8,9]。その後 5-FU, Cyclophosphamide, BLM といった化学療法剤の投与後にも同一の現象の発生することが指摘された[5,6,7,10]。本報告の図1,2に示された，BLM 投与により EAT 細胞に与えられた損傷が経時的に回復するという現象を PLD と考えるには，結果的に同じような生残率の上昇をひきおこす2つの可能性を否定しなければならない。それは第1に，BLM 投与後，急速な細胞増殖により，腹水中の生細胞の割合が経時的に増加していく可能性であり，第2に，BLM によって致死的損傷を受けた細胞が経時的に除去されて，腹水中の生細胞の割合が死細胞より大きくなる可能性である。しかし，実験が行なわれた移植後7日目の EAT 細胞の population doubling time は約50時間であること，および，30mg/kg の BLM の投与後1時間から7時間までに1時間ごとに算定した細胞数は，$2.5\sim2.8\times10^8$ とほぼ一定であり，dye staining method によって同時に検定した死細胞の割合も2～5％と不動であることから，上記の2つの可能性は，この実験系において BLM 投与後に生残率の上昇をもたらすものではないと云うことができる。結局，BLM による EAT 細胞の損傷は PLD であり，この PLD は経時的に回復して細胞は再び増殖力を獲得すると説明できる。さらにつけ加えるならば，この回復は *in vivo* の条件下においてのみ起こり，*in vitro* の assay system にもちこむと PLD が致死損傷として固定されると云えよう。

PLD の回復は，tumor cell sterilization の立場から考えるとはなはだ不利な現象である。なぜなら，BLM によって細胞に与えられた障害が致死的ではないからである。このような PLD の回復現象は程度の差はあれ，人癌においても BLM の投与時に発生していると推測されるゆえに，PLD の回復を克服する方途を見出すならば，BLM による癌治療はさらに一層の効果を期待しうるであろう。PLD の回復を克服するには二つの道がある。一つは，BLM と他の agent との併用により PLD の回復を阻止すること。もう一つは，BLM 単独で PLD の回復の影響から可能な限り脱却し最大の致死効果をあげうるような投与方法を工夫

することである。前者については，BraunとHahnが chinese hamster cell (HA 1) に，*in vitro* で BLM を投与後 43°C という高温 (Hyperthermia) の条件下で細胞を1時間培養すると，PLD の回復が阻止されることを報告している[11]。しかし，Hyperthermia の臨床への応用は技術的に困難な面が多いので，BLM との併用により PLD の回復を阻止する化学物質の発見が俟たれるわけである。

この研究のめざすところは，第2の点の解決にある。EAT 担癌マウスを使用したこの実験系で，PLD の回復という細胞不活化の上で不利な現象をおさえこむために，いかなる投与濃度，投与間隔を選択したら良いであろうか。以下，実験結果に基づいて最適投与方法を考察してみたい。まず，図1で明らかなように，この assay system での does response curve は変曲点が 0.25～0.5mg/kg の upward concavity を有する2相性の曲線である。この曲線は，EAT 細胞の不活化には変曲点以上の濃度範囲の BLM を使用することが著しく不利であることを示している。ちなみに，図1の 1hr exposure の dose response curve において，変曲点以下の濃度範囲での $D_{37}$ は 0.15mg/kg であるのに対して，変曲点以上の濃度範囲でのそれは 23mg/kg であり，変曲点を境に著しい感受性の相違が認められる。このような BLM と細胞との dose survival relationship は，培養細胞系[1,2,3,4]，*in vivo* の実験[12,13]系を通じて普遍的なものであると考えられており，BLM に対する人癌のレスポンスにも布衍しうるものと思われる。その1例として，図7に BLM とホジキン病との dose response curve が2相性で upward concavity を有することを示した。その変曲点は 2.5mg であった。このことは，現在も広く行なわれている 15mg という1回投与量を再考慮する余地のあることを示唆している。今後，多くの症例で腫瘍の縮小を指標として BLM の人癌に対する dose response curve を描くことができれば，その変曲点以下の濃度を最適投与濃度と決定することができる。

次に投与間隔について考察したい。BLM の最適投与間隔を理論的に決定するにあたって考慮すべき2つの因子がある。それは，BLM 投与後の抵抗性の誘導と崩壊，および PLD の回復である。前者のみを考慮の対象として投与間隔を考えれば，誘導された抵抗性の崩壊を待って2度目の投与を行なうのが論理的である。一例として，培養L5細胞の場合，5 $\mu$g/m*l* の BLM-A5 投与後に誘導された抵抗性は，BLM の除去後4時間で崩壊することが明らかとなっている[3,4]。また，後者のみを考慮するならば，1度目の BLM 投与後，PLD 回復過程のなるべく早期に2度目の投与を行なうことにより，PLDの回復の影響を少なくして腫瘍細胞の不活化を増大させることが可能であろう。さて，EAT においては，BLM 投与後に誘導される抵抗性は 0.1mg/kg という低濃度では僅少であり，30mg/kg という高濃度では投与後1時間で強い抵抗性の誘導されることが実験的に証明された[14]。それゆえに，0.1mg/kg の分割投与で最大の細胞不活化率を得るための投与間隔は，PLD の回復の影響を考慮して可能な限り短時間が望ましく，逆に，30mg/kg の場合は抵抗性の崩壊を待って，つまり一定の間隔をおいて2度目の投与を行なうべきであると考えられる。この思考法の正当性は，図3,4の結果から証明された。すなわち，0.1mg/kg では，細胞不活化効果は1時間間隔の方が3時間間隔より大であり，30mg/kg ではその逆であった。次に，0.1mg/kg 投与における投与間隔をさらにつきつめて考えると，持続注入法によって PLD 回復の影響を最少とし，最大の細胞不活化効果の得られることが期待される。図5の結果は，0.1mg/kg の持続注入法の細胞不活率が，1時間間隔の分割投与のそれより大きいことを示している。EAT 細胞を使用したこの実験におけるこのような持続注入法の有効性の機序として，PLD 以外に BLM の cell cycle dependent な作用が関与しているのではないかという考え方もある。しかし，この考え方は，本実験においては6時間という cell cycle の関与する余地のない短かい持続注入時間で，細胞不活化率に明らかな差が認められることから否定されるであろう。

cancer chemotherapist にとって最大のテーマは，薬剤による副作用を可能な限り少なくしてよ

り大きな抗腫瘍効果をもたらすような投与方法を工夫することにある。また，BLM の致死的副作用である肺線維症の発生は，does dependent であることが知られており，この実験結果から導かれる低濃度，持続注入法は，その意にかなったものであると云えよう。近年，BLM の投与方法に関し，臨床あるいは動物実験のレベルで種々の試みがなされている。油性 BLM の開発，固型ブレオマイシンの局所投与[15]，あるいは術中 BLM・スポンゼル法[16]等がそれである。これらの方法の意義の一つは，BLM と腫瘍との接触時間が延長することによって抗腫瘍効果が高められることにあると考えられる。また，Cvitkovic 等[17]は，頭頸部の扁平上皮癌に BLM の持続注入法を行ない，17例中53%という高率のレスポンスを得たと報告している。Samuels[18]等は，stage III testicular neoplasia に vinblastin 投与後に BLM を持続注入した結果，23例中17例にレスポンスが見られ，その内9例は complete response であり，従来より彼等が行なってきた大量の vinblastin 投与と週2回の BLM 投与の組み合せよりすぐれた効果が認められたとしている。Samuels 等は，持続注入法の理論的基礎を BLM の cell cycle dependent な作用に求めている。彼等の引用する Drewinko 等の報告[19]によると，*in vitro* において human lymphoma cell に BLM を4時間投与した場合の percent S.F. は10% であるのに，35時間の連続投与により0.06% に下降する。そして，この理由は，$G_2$-M 期に感受性を示す BLM が，細胞の one cell cycle 以上の長時間の持続投与により $G_2$-M 期を通過する細胞に致死的な障害を与えるためであるとしている。しかし，臨床における BLM の持続注入法の有効性を BLM の cell cycle dependent な作用のみに帰するのは，EAT における6時間の持続注入の効果と，人癌細胞の cell cycle における $G_2$-M 期細胞の割合から考えても疑問がある。やはり，本報告に示されたPLD回復への抑制効果が最も大きな役割を果たしていると考えてよいであろう。

## おわりに

新しい抗癌剤は次々に開発されていく。その多くは数年を待たずに見捨てられ，生き残るものは僅かである。生き残った抗癌剤も，はたして，その最も良く働きうる投与方法で使用されているであろうか。抗癌剤の cell kinetics を，基礎あるいは臨床の分野から一歩一歩解明し，最適投与方法を追求することにより，癌の化学療法は今後一層の進歩発展を見るであろう。この研究から導かれた BLM の低濃度，持続注入法はその one step である。

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原　著

# Tumor Cell population kinetics にもとづく Futraful® の抗腫瘍作用の解析と単独・併用療法の投与諸原則

* 放射線医学総合研究所　病院部
宮本忠昭

** 千葉大学医学部第一内科
渡辺道典　高部吉庸

*** 放射線医学総合研究所　生理病理部
寺島東洋三

**要旨**　7例の転移腺癌の成長曲線と Futraful 投与中の縮小曲線との比較検討により，腫瘍の Futraful に対する感受性は成長速度に，反応の遅速はサイズに依存することがわかった。この結果，Futraful の単独・併用療法の原則を提案した。

## はじめに

腫瘍治療は，基本的には腫瘍成長の源をなす clonogenic cell[1] の sterilization を原理[2,3]とする。放射線と同様に，抗腫瘍剤の細胞致死作用は薬剤処理後の clonogenic cell の無限増殖能を *in vitro*, *in vivo* assay 系において測定すること[3]によって研究されてきた。その結果，抗腫瘍剤に対する細胞の感受性は，細胞の増殖動態等によって大きな修飾を受けることがわかってきた。たとえば，細胞周期依存性感受性変動，対数増殖期と定常期に見られる細胞の増殖相に依存する感受性の差異，Potential Lethal Damage (PLD) の回復，ブレオマイシン処理細胞で見られる cell drug interaction等[3]である。

当論文の症例に用いた Futraful (以下 FT と略す) は，5-Fluorouracil (5-FU) の masked compound であり，投与後生体内では，特に肝臓などで処理を受け，5-FU を放出する[4]。5-FUの細胞致死作用は，同調 DON 細胞を用いた実験で証明されたように，特定の細胞周期に強い依存性を示さない[5]。しかし，細胞の増殖相が違う場合は著しい感受性の差を示す。対数増殖期と定常期にある培養マウスL細胞[6]，あるいは endotoxin によって刺激した骨髄幹細胞と無刺激の幹細胞を用いた実験[7]によると，5-FU は，Non Proliferating (NP) 細胞に比べ，Proliferating (P) 細胞を特異的に不活化することが証明された。また，Hahn等[8]は，マウスの肉腫細胞が 5-FU 投与後，わずかに PLD の回復現象を示すことを報告した。

腫瘍の抗癌剤に対するレスポンスの最も直接的にして客観的な指標は，癌細胞の死に起因する腫瘍体積の減少であろう。当論文に記載する7例の転移腺癌は，FT 投与前に腫瘍の成長様式と成長速度を観察し得，投与後ひき続いて縮小様式と縮小速度を観察し得たものである。この観察により，腫瘍の FT に対する感受性は成長速度に依存し，腫瘍の縮小開始までの投与量は，腫瘍体積に依存

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Effect of Futraful on metastatic adenocarcinomas and proposal for some principle of single and combined chemotherapy.

Tadaaki Miyamoto*, Michinori Watanabe**, Yoshinobu Takabe**, and Toyozo Terasima***
* Division of Hospital, National Institute of Radiological Sciences.
** First Dept. of Medicine, Chiba University School of Medicine.
*** Division of physiology and Pathology, National Institute of Radiological Sciences.

癌と化学療法　3，1225-1235，1976

することがわかった。この観察結果に基づいて，後述するようなFTの投与諸原則が定式化された。

さて，細胞レベルで解明された5-FUの増殖相依存的細胞致死作用は，FTで得られた観察結果とどのように関係してくるであろうか。われわれは，この関係を解決する鍵が腫瘍成長における癌細胞集団の増殖動態を解明することにあるとの認識の上に立って，人癌のCell Kineticsに関するDataをできる限り集め，批判的に検討し，われわれの観察結果を最も合理的に説明し得る腫瘍の成長モデルの導入を試みた。この試みは大方成功したように思われる。さらに，このようにして仮説的な方法で導入された腫瘍の成長モデルは，思惟的なきらいはあるとはいえ，今後，合理的腫瘍治療を目差す者にとって研究すべき課題と方向を充分示唆しうるものと思われる。

## 1. 対象および方法

対象は過去3年間，放射線医学総合研究所：病院部において Futraful〔$N_1$-(2′-tetrahydrofuryl-5-fluorouracil〕で治療した約30名の患者より，腫瘍の成長とFT投与後の腫瘍の縮小を同時に観察し得た6例の転移腺癌と千葉県がんセンター大森医師の治療した[9]胃癌の肝転移の1症例を加えた計7例である。皮フ，表在性リンパ腺，肝臓への転移癌は経皮的に，肺転移癌は胸部X線写真上より，腫瘍の直径を経時的に計測し，球体と仮定して腫瘍体積を計算した。成長曲線 $mm^3$ の単位で，縮小曲線は比較体積値で表わした。

## 2. 症例

1) 症例-1 (case-1)：患者は55才の女性で，胃癌剔出後，約7カ月を経て皮フと表在性リンパ腺に多発転移癌を起し，引き続き黄疸と腹水貯溜を来たし重篤化した。この患者に，FT 1200mg／日を経口投与したところ，劇的に奏効し，約1カ月以内ですべての表在性転移腫瘍の消失と黄疸及び腹水の完全消退を見た。計測の対象とした腫瘍は表在性で多発性の皮フ及びリンパ腺転移癌である。腫瘍の成長曲線（図1）は横軸に観察開始後の日数をとり，縦軸にアトランダムに選んだ2ケの腫瘍の体積の増加を目盛った。この曲線から腫瘍が大きくなるにつれて成長速度が急激に減ずることが観察される。観察期間を便宜的に観察開始より5日（腫瘍直径3～6mm：サイズA），5日から14日（6～12mm：サイズB），14日以降（>12mm：サイズC）の3期に分けると，腫瘍体積倍加時間（Volume Doubling Time：VDT）はそれぞれ2日，4日，約12日となる。FT投与後の腫瘍体積の変化は，投与されたFTの総量あるいは薬剤投与開始後の日数に対して示された（図2）。腫瘍直径はサイズA. B. Cに当る大きさの特定の数個の腫瘍を選んで計測された。個々の腫瘍はその大きさに応じて指数関数的に，あるいはシグモイド型に縮小し，そのterminal slopeはほぼ同一の傾きであった。この指数関数部分の傾きは，FTに対する感受性を示しており，これをDo（体積縮小曲線の指数関数部分において体積を37％に減らすに要する薬剤の総量）で表わすと約6grである。一方，FT投与後，腫瘍体積が縮小を開始するまでの日数あるいはFTの投与量は腫瘍体積が大きくなるにつれて多くなった。これを，放射線による細胞のシグモイド型の生残曲線におけるshoulder部

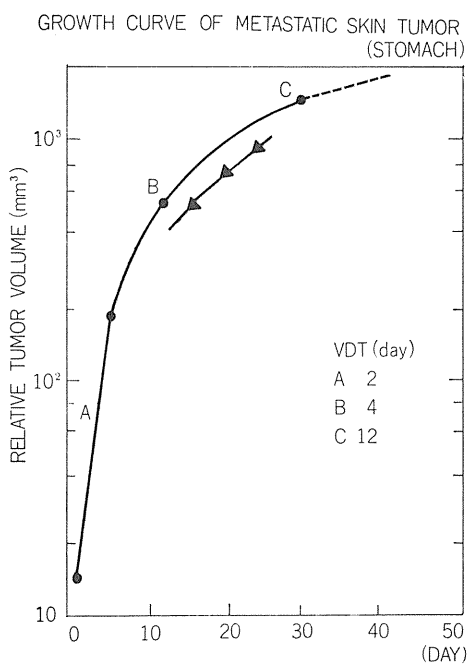


図1　Case-1の成長曲線。●は連続的に約30日間，成長を観察し得た腫瘍を示し，▲は途中より観察に加えた別個の腫瘍を示している。

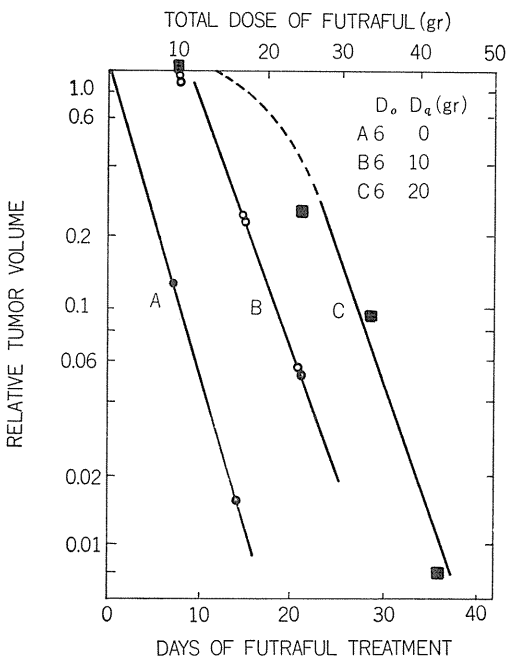


図 2　Case-1 の腫瘍縮小曲線。●は図1の成長曲線のAの部分に相当するサイズをもった腫瘍。○はBの部分に相当するもの，■はCの部分に相当する腫瘍をそれぞれ示している。

分の数的表現，Dq（曲線の指数関数部分の横軸への外挿値）で表わすと，A, B, C に相当するサイズの腫瘍は，0，10，20grの値をとり，それぞれ0，8，16日の遅れで縮小を開始した。

2)　症例—2（case-2）：患者は72才の女性で，回盲部大腸癌剔出後，約8カ月を経て血痰が喀出され多発性肺転移が発見された。入院精査によりさらに骨，肝への転移も明らかになった。この患者に，骨転移に対しては照射を併用しつつ，FT 1200mg/日の経口投与を行い，肺転移腫瘍の縮小の経過を観察した。FT投与前の成長曲線（図3）は，多発転移腫瘍の内より40日に渡って明っきりと計測し得た多少6ケのそれぞれの成長速度をもとめ，小さなものから大きなものへと順次ならべて合成し，1ケの腫瘍の成長として求めたものである[10]。この曲線より癌が成長するにつれて減速することが推察される。この曲線の指数関数的成長部分で計った VDTは48日であった。FT投与後は10ケの腫瘍について計測がなされた。それらの

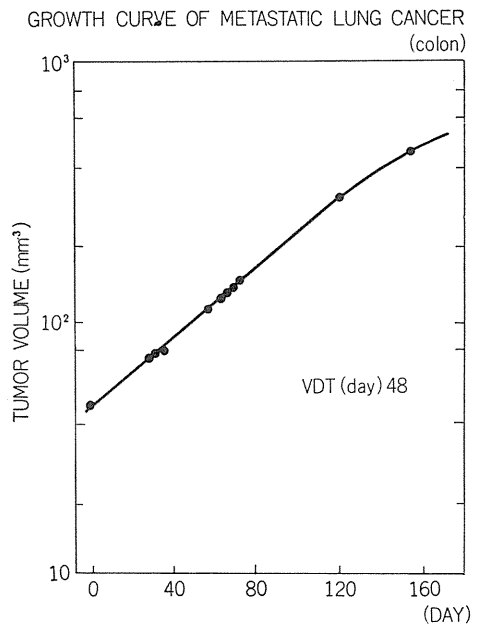


図 3　Case-2 の成長曲線。多発転移肺腫瘍の内より約40日にわたって明確に計測し得た大小6ケの腫瘍のそれぞれの成長を求め，小さいものから大きいものへと順次並べて合成し1ケの腫瘍の成長として表わしたものである。

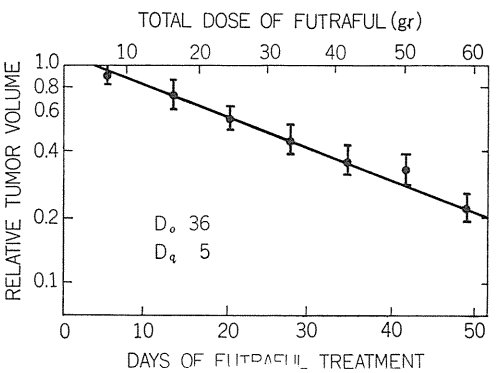


図 4　Case-2 の腫瘍縮小曲線。図は腫瘍直径 16±4mm の10ケの肺転移腫瘍の平均腫瘍縮小率を示している。

腫瘍直径は平均16±4mm であった。FT投与後の腫瘍体積はシグモイド型に縮小した（図4）。この縮小曲線の Do は 36gr であり，Dq は 5gr で，投与後4日して縮小を開始した。

3)　症例-3(Case-3)：患者は73才の乳癌の女性で，左乳房切断術後，約2年を経て多発肺転移が

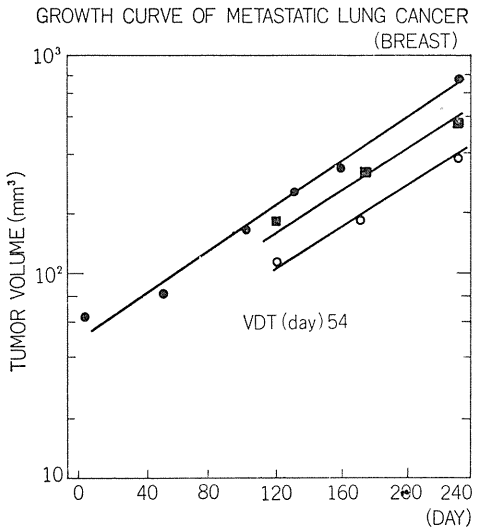


図 5　Case-3 の成長曲線。○，■，● は肺内に転移した三つの異った腫瘍を示している。

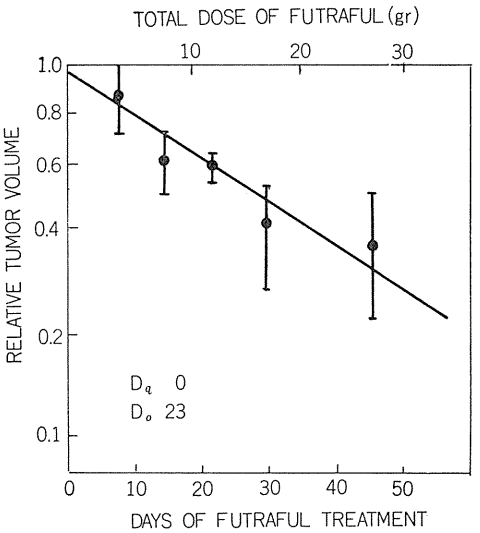


図 6　Case-3 の腫瘍縮小曲線。図は腫瘍直径 6.5±1.7 mm の 6 ケの肺転移腫瘍の平均腫瘍縮小率を示している。

発見された。全肺照射により一時腫瘍の縮小をみたが，半年後再び腫瘍が増大したため FT 600mg/日が投与された。腫瘍体積の増加は長期に渡って測定された 3 ケの肺転移腫瘍のそれぞれに対して求められた（図 5）。 3 ケの転移腫瘍はほぼ同一速度（VDT＝54日）で約 8 カ月の間，指数関数的に増大し続けた。FT 投与による腫瘍の縮小の経過は，腫瘍直径 6.5±1.7mm の 6 ケの腫瘍に対して観察された。各腫瘍は FT 投与後，直にほぼ指数関数的に縮小し，その Do の値は 23gr であった（図 6）。

4)　症例-4 (Case-4)：患者は 55 才の乳癌の女性で，右乳房切断術後，約 3 年を経て肺間，縦隔，頸部リンパ腺，皮フ，骨に多発転移を来たした。肺間，縦隔，骨には照射を併用しつつ，FT 1200 mg/日を服用させ，経時的に皮フおよび頸部リンパ腺の腫瘍の縮小を観察した。Ft投与前，観察を開始してから30日まで腫瘍は VDT＝28日の速度で指数関数的に成長したが，それ以降は成長速度が減じた（図 7）。FT投与直前に計測された 3 ケの腫瘍直径は 13.3±2.6mm であった。投与後，腫瘍はシグモイド型に縮小した（図 8）。Do は 26gr であり，Dq は 4gr で，投与後 3 日して腫瘍の縮小が認められた。

5)　症例-5 (Case-5)：患者は 51 才の子宮体部

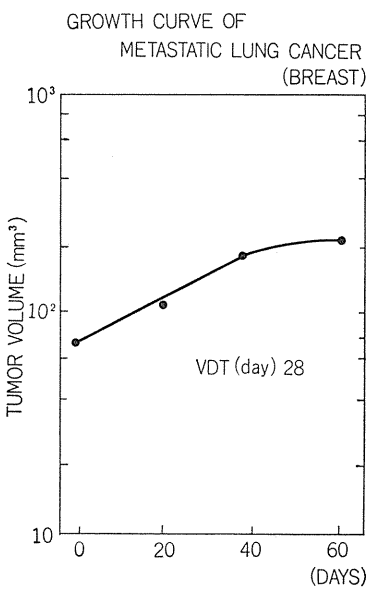


図 7　Case-4 の成長曲線

癌の女性である。子宮全剔術後，4 年を経て右肺野に孤立性の転移が認められた。腫瘍の成長は約 2 カ月の間，3 枚の XP で測定された。腫瘍の成長様式は明らかではなかったが，VDT は約 120 日であった。腫瘍はFT 800mg/日の経口投与後，直ちに指数関数的縮小を来たし，Doの値は 106gr

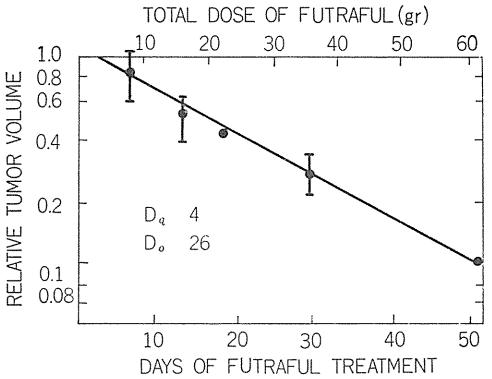


図 8　Case-4 の腫瘍縮小曲線。図は腫瘍直径 13.3±2.6 mm の3ケの皮フ，リンパ腺転移腫瘍の平均腫瘍縮小率を示している。

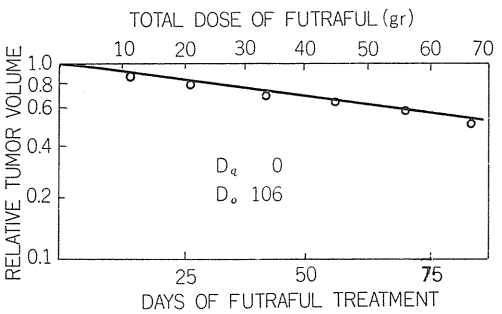


図 9　Case-5の腫瘍縮小曲線。1ケの肺転移腫瘍の腫瘍縮小率を示している。

であった（図9）。

6)　症例-6（Case-6）：患者は68才の男性で，ボールマン2型の胃癌である。肝臓に多発転移を起して心窩部下6横指に腫大したため経皮的に腫瘍の大きさを計測できた。成長観察期間は約1カ月と短かかったので成長様式の分析までできなかったが，測定し得た3つのポイントから VDT を求めると約10日であった。直径 30mm の腫瘍は FT 1200mg/日投与後，シグモイド型に縮小した。Do は 12gr であり，Dq は 9.6gr で，FT 投与開始後，8日経て腫瘍の縮小が開始した。

7)　症例-7（Case-7）：患者は50才の男性で，ボールマン2型の胃癌である。原発巣を切除後，7カ月を経て，直径80mmの肝転移が発見された。FT 800mg/日投与後，腫瘍は典型的なシグモイド型の曲線で縮小した（図10）。Do の値は 24gr で

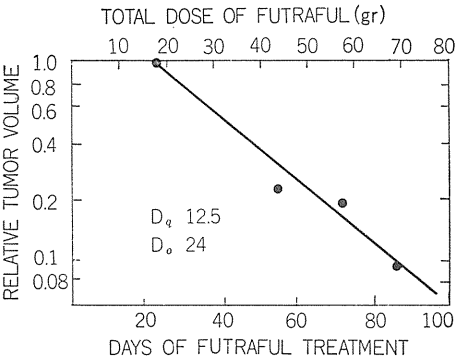


図 10　Case-7 の腫瘍縮小曲線。1ケの肝転移腫瘍の腫瘍縮小率を示している。

あり，Dq は 12.5gr で，FT投与後16日頃より縮小が始まった。FT投与前に腫瘍の成長は観察されていないので成長速度は求められなかった。しかし投与中起った再発腫瘍の成長速度は VDT で18日であったので，近似的なものとして代用した。

## 3. ま と め

以上7症例とその解析より得られた各転移腺癌の VDT，FT投与直前の腫瘍体積，Do，Dq および腫瘍縮小開始までに要した日数をまとめたのが表1と2である。表2にまとめた腫瘍の成長および縮小に関する各パラメータと図 1～10に示された腫瘍の成長および縮小曲線とによってもたらされた結果は，以下のようにまとめられる。

1)　転移腺癌の成長様式は基本的に指数関数的であるが，腫瘍体積が 500～1000mm$^3$ まで成長すると，その速度を減ずる。腫瘍の成長速度（VDT）は，それぞれの腫瘍に固有のものであり，同

表 1

| CASE | NAME | SEX&AGE | PRIMARY | METASTASIS |
|---|---|---|---|---|
| CASE-1 | T. Maezawa | ♀ 56 | Stomach | Skin, Ly. node Liver |
| -2 | Y. Sato | ♀ 72 | Colon | Lung, bone Liver |
| -3 | H. Nemoto | ♀ 72 | Breast | Lung |
| -4 | T. Ishii | ♀ 55 | Breast | Skin, Ly.node bone, Lung |
| -5 | M.Ishigami | ♀ 51 | Uterus | Lung |
| -6 | G. Ishidoya | ♂ 64 | Stomach | Liver |
| -7 | S. Kamagata | ♂ 50 | Stomach | Liver |

表 2

| CASE | VDT (day) | $D_o$ (gr) | Diameter (mm) | Volume ($mm^3$) | Lag time (day) | $D_q$ (gr) |
|---|---|---|---|---|---|---|
| CASE-1 | 2 | 6 | A 6 | 113 | 0 | 0 |
| | | | B 10 | 523 | 8 | 10 |
| | | | C 15 | 1767 | 16 | 20 |
| -2 | 48 | 36 | 15.9±3.9 | ＊2144 | 4 | 5 |
| -3 | 54 | 23 | 6.5±1.7 | ＊ 143 | 0 | 0 |
| -4 | 28 | 26 | 13.3±2.6 | ＊1231 | 3 | 4 |
| -5 | 120 | 106 | 10.5 | 606 | 0 | 0 |
| -6 | 10 | 15 | 30 | 14137 | 8 | 9.6 |
| -7 | (18) | 24 | 80 | 268082 | 16 | 12.5 |

＊Volume obtained from avearage diameter.

一症例における個々の転移腫瘍は同じ臓器内では同一速度で成長する（症例-3)。

2) FT 投与下における腫瘍は，体積が 500～1000$mm^3$以下の小さな場合には指数関数的に，それ以上の場合はシグモイド型に縮小する。

それぞれの腫瘍はFT に対し固有の感受性(Do)を持つ。この感受性は同一腫瘍ではその大きさに関係なく安定に保たれ，症例-1で典型的に示されたように，腫瘍が大きくなると腫瘍が縮小を開始するまでのFTの投与量 (Dq) は増えるが，いったん縮小が開始されるとその速度は腫瘍の大きさに関係なく同一である。

3) 腫瘍のサイズとFT投与後，この腫瘍が縮小を開始するまでに要した薬剤の量 (Dq) との関係を示したのが図11である。両者の間には正の相関が存在する。すなわち，同一腫瘍（症例-1）でも，異った腫瘍どうしでも共に，腫瘍体積が 500～1000$mm^3$ を越すあたりから腫瘍体積に比例して Dq が増加する。

4) 腫瘍の成長速度 (VDT) とFTに対する感受性 (Do) の関係を示したのが図12である。両者の間にはほぼ正の相関が存在し，成長の速い腫瘍はFTに対し高い感受性を示すことがわかる。

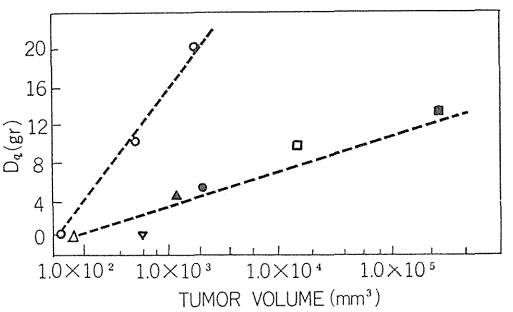


図 11 Case-1 (○) と Case-2(●), Case-3(△), Case-4 (▲) Case-5 (▽), Case-6 (□) Case-7(■)とでそれぞれ求められた腫瘍体積 ($mm^3$) と腫瘍体積縮小開始までに要したFTの投与量 (Dq) との関係。

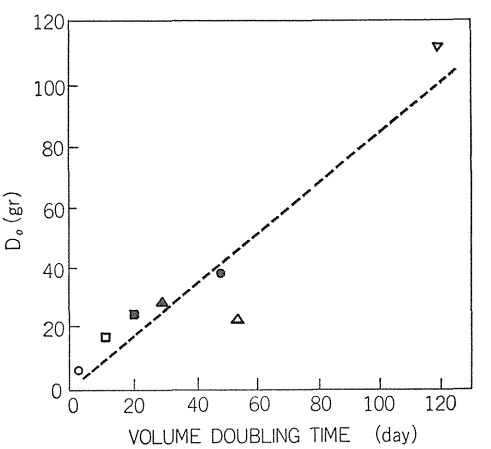


図 12 Case-1～7 までの体積倍加時間 (VDT) と体積を37％まで減らすに必要なFTの量 (Do) との関係。○ Case-1, ● Case-2, △ Case-3, ▲ Case-4, ▽ Case-5, □ Case-6, ■ Case-7

## 4. 考　按

人癌の成長様式と成長速度は腫瘍治療にたずさわる者にとって最も重要な研究テーマの一つである。すでに幾多の研究者が人癌の成長に関する報告[11～14]をしているが，これによると人癌は指数関数的に成長する。一方，腫瘍の成長様式に関する詳しい研究は，自然発生あるいは移植による小動物腫瘍を用いて行われた[14,15,16]。これによると，腫瘍がごく小さいうちはほぼ指数関数的に成

長するが，大きくなると減速し，上に凸の成長曲線を描く。当症例の転移腺癌は基本的に指数関数的成長をし，ある体積（500～1000mm³）に達すると次第に減速し（症例 1, 2, 4），上に凸の曲線を描いた。さらに，このような成長様式は子宮頸癌の多発肺転移例においてより一層はっきりと観察された[10]。これらの観察結果より推察すると，人癌と小動物腫瘍の成長様式は原則的に同一と考えられる。両者の成長曲線にみられる外見上の相違は，人癌においては指数関数的成長段階を主に観察するに対し，小動物腫瘍においては減速段階を観察したため生じたものと考えられる。これは両者の癌一担癌体重量比の大きな相違を考えると理解される。すなわち，50grのマウスにとって1grの癌は，すでに指数関数的成長期をすぎて著しく減速した段階であるが，50kgr の人間の 1kgr の癌に相当することを考えれば十分納得のゆく話である。このように腫瘍の成長様式は，担癌体の種差をとわず基本的に指数関数的であり，腫瘍がある大きさに達すると減速を開始するものと考えられる。従って，腫瘍の成長様式に関する研究の基本的課題は，指数関数的成長を可能にする細胞の増殖機構とこの成長からの減速を支配する機序である。

成長曲線の指数関数部分より計測した当症例のVDTは，表2に示すように2日から120日に渡り，その平均値は40日であった。charbit 等[17]は文献より集めた530例の腫瘍のVDTを病理組織型に従って整理した。これによると34例の原発腺癌の平均 VDT は166日，134例の転移腺癌のそれは88日であった。しかし，それぞれの癌における VDT のバラツキは大きく，遅いものは速いものの10倍余りにも達していた。このような長い VDT を持つ人癌はどのようにして指数関数的成長をするであろうか。$^3$H-TdR を全身的に投与し，経時的に腫瘍の一部を生検して得た標本にオートラジオグラフィー操作を施し Percent labeled Mitosis (PLM) の推移を求めると PLM 曲線が得られる。倫理上の問題は別にして，人癌の cell population kinetics の解析はほとんどこの方法で行われてきた。この方法で解析された人癌はすでにかなりの数に達するが[18,19,20,21]，そのすべてにおいてPLM曲線の最初の波は明確に検出し得る。そしてこの波の下降脚（ほぼM＋$G_2$＋S 時間に相当）までの時間は30時間以内におさまることがわかった。このことは人癌細胞がその種類に関係なくM＋$G_2$＋S 期を比較的短い一定のCell Cycle Time で進行することを示している。ところが第2波は，全標本で明確に検出されず，さらに時間を追うにつれて PLM の値は一定のところに収束する。 Mendelsohn[22]は $C_3H$ マウス乳癌において，この収束値が同一標本の％Labeled Cell より高い値を示すことに着目して，Cell Cycle にない細胞，すなわち Non-Proliferative (NP) 細胞の存在する可能性を指摘し，Growth Fraction (GF＝P/NP＋P) なる概念を提唱した。この概念はその後 NP 細胞に対する実証をともなわないままに，PLM 曲線より Computer によって種々の Cell Cycle Parameter を求めるための基本的仮定の一つとして採用され，人癌の PLM 曲線の解析にも応用されるようになった。一方，数は少ないが，Cumulative Labeling 法を用いて解析した人癌のデータ[23,24]は，Labeled Cell が時間の経過とともに一定の割合で増加し，遂に 100％に達することを示した。この実験結果は，NP 細胞の存在を否定する異った学説を生み出した。すなわち，すべての人癌細胞は Proliferate (P) している。しかし，人癌細胞集団を構成する個々の細胞の $G_1$ 期は大きなバラッキを持つため，PLM 曲線では第2波が検出し得ないとするものである。あとで詳しく考察するが，NP 細胞の存在に関してはすでに指摘した腫瘍の成長段階の相違を考慮することにより，この問題のポイントが一層明らかにされるであろう。

さて，PLM 曲線を GF 等の仮定をおいて Computer を用いて解析する方法でも Cumulative Labeling 法より得られた Labeled Cell の蓄積曲線からでも，腫瘍細胞集団の倍加時間 (Population Doubling Time：PDT) が求められる。人癌における PDT は数日から十数日と計算されているが，この値は実測の VDT よりはるかに短い。この VDT と PDT の大きな相違は，成長にともなって腫瘍から失われる細胞 (Cell loss) がかなり大きいことによって説明されるようになった。 Steel 等[25]は，この Cell loss の量を1日に産生される細胞に対する失われる細胞の割合として，1-PDT

/VDT の式より求め，Cell loss factor ($\varphi$) として数式化した。この方法により，彼等[26]は14日以上の VDT を有する人癌は 50% 以上の Cell loss があると推定した。一方，藤田[27] は Cumulative Labeling法を施した人癌の標本より，支質細胞が時間とともに Label される割合を求め，Labeled 100%に達する時間を求めると，この時間がほぼ Cell が VDT に一致し，同様の方法で求めた実質細胞の値 (PDT) と大きな時間的ずれのあることを発見し，この相違が Cell loss にもとづくものと推定した。このような細胞の増殖機構をもった人癌が，指数関数的に成長するには，実質細胞集団の Cell Cycle Distribotion が一定に保たれつつ qopulation の倍加が起り，この過程で一定の割合で Cell loss が起り，その結果，実質と支質の割合も変ることなく維持されることが必要である。このようなバランスを保ちつつ体積を増してゆく腫瘍の最小成長単位を今かりに Growth Unit (GU) と命名すると，その形態学的表現は一つの毛細血管をとりまく半径50～100$\mu$m 以内の小腫瘍塊となろう[28] (図13)。

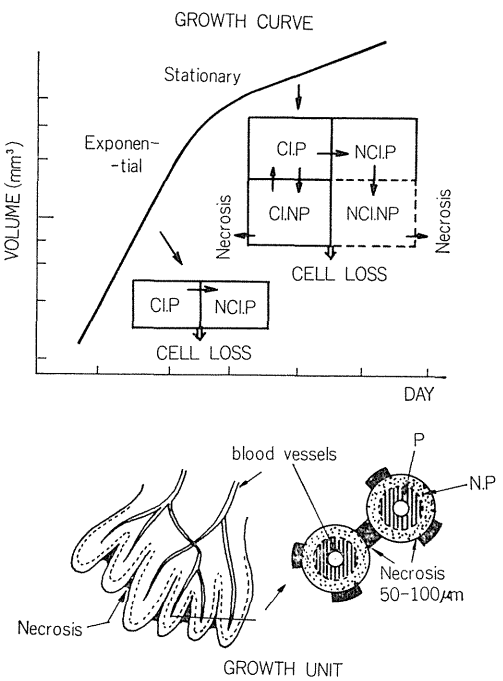


**図 13** Growth Unit (GU) 仮説にもとづく腫瘍成長モデル。上図は腫瘍の各成長段階 (Exponential と stationary) における GU 内の細胞集団の動力学的変化を示している。P：Proliferating Cells, NP:Non-Proliferating Cells, Cl. P:Clonogenic Protiferating Cells, Cl. NP:Clonogenic Non-Proliferating Cells. 下図は GU の形態学的モデル。

ここでCell loss についてさらに詳しく検討する。Cell loss とは具体的には，分裂に際してある確率で生ずる細胞の散発的な死，分化による Exfoliation，遊走，浸潤，転移による移動，そして壊死から成る。この内壊死は，腫瘍の栄養障害によって起る集団的細胞死と考えられるものであり，前3者のCell loss とは異なるものと思われる。すなわち，腫瘍に壊死を生ずる栄養障害は，実質細胞の増殖に支質の血管の発育が追いつけなくなりもたらされるか，腫瘍の全身播種による担癌体の栄養低下によってもたらされるかであろう。このような腫瘍成長にとって病的な環境は，腫瘍がかなりの大きさに成長したときに起るに違いないし，これによって腫瘍の成長は減速をよぎなくされるであろう。Steel等[29]は，指数関数的に成長する動物腫瘍と上に凸の成長曲線を描く，減速段階にある動物腫瘍に，それぞれ $^3$H-TdR をパルスと持続的に投与して，PLM 曲線および Labeled Cell の蓄積曲線を求めた。これによると，PLM 曲線より求め得た腫瘍細胞の世代時間は両腫瘍間では大差はなかったが，蓄積曲線では，前者では Labeled Cell は時間とともに一定の割合で増加し，遂に100%に達するのに対して，後者では 40～50% ぐらいまで増加し，それ以降は plateau に達した。この実験結果は，同一腫瘍の違った成長段階で比較検討したものではないという不十分さはあるが，少なくとも腫瘍の成長が減速過程に入ると NP 細胞が生ずることを示している。指数関数的成長を続けてきた腫瘍が，ある大きさに達すると，支質血管の発育不良か，担癌体の栄養低下かによって腫瘍細胞は栄養障害に陥る。その結果，一部のP細胞は NP 細胞になり，NP 細胞の一部は壊死に陥る。このような過程によって腫瘍成長の減速が起ると考えられる。図13は以上検討してきた腫瘍の成長の機構と減速の機序をわかり易く模式化したものである。

ここで，さらに FT 投与下で観察された腫瘍の指数関数的縮小とシグモイド型縮小の機序の検討

にすすもう。腫瘍の指数関数的縮小は，当症例のみならず，放射線による規則的分割照射を受けた腫瘍でも観察されている[30]。人癌はすでに検討したようにGUの指数関数的増加によって指数関数的成長が可能となると考えられる。そして，このGUの増加は，GUを構成するすべての細胞ではなく，自己再生能力を持つClonogenic (Cl) Cellの増加が源となるに違いない。とすれば，VDTは，GUの増加率，さらにはCl. Cellの増加率と一致するはずである。支質の血管の発育を支えるangiogenetic Factor[31]をCl. Cellが造っている証拠が示されてきていることを考慮に入れるならば，Cl. Cellが指数関数的に不活化されるとき，それに支えられた血管が同様に吸収され，GUも指数関数的に減少すると考えられる。その結果，腫瘍体積も指数関数的に減少することになる。このようにGUの概念を導入することによって，Cl. Cellの増加率，減少率が腫瘍体積の増加率，減少率と相関する機序が明白になった。

以上の検討にもとづいて，図12に示した腫瘍の成長速度とFTに対する腫瘍の感受性，および図11に示した腫瘍体積と腫瘍縮小開始までのFTの投与量との相関関係を説明してみたい。

1) FTに対する腫瘍感受性(Do)はどのようにして腫瘍の成長速度（VDT）に依存するか。VDTはPDTとCell lossの量とによって規定される。Cell lossの量はFTの投与と全く関係がないので，図11に示されたDoとVDTの相関関係は，FTとPDTとの間に存在すると容易に推測される。指数関数的成長段階にある腫瘍の実質細胞はすべてCell Cycleにあると考えられるので，FTに対する感受性は単位時間内にFTによって致死的Damageをうける P 細胞の割合の多少に依存する。単位時間内に生み出される P 細胞の割合は腫瘍の世代時間あるいはVDTに依存するから，単位時間内に多くの P 細胞を産生する速い成長をする腫瘍はその逆の遅い成長をする腫瘍に比べてFTに高感受性を示すことになる。

2) 腫瘍体積がある大きさ以上に達すると，その大きさに依存して腫瘍縮小開始までのFTの投与量（Dq）が増加するのはなぜか。図11にDqで示される腫瘍縮小までの遅滞期（Lag）は，腫瘍体積が500～1000mm$^3$に達してから初めて検出されるので，この体積になると成長速度が減少することと深く係り合いがあるい思われる。すなわち，腫瘍の成長速度の減少は，栄養障害によって壊死とともにNP細胞が出現し増加することによって起る。この成長段階にある腫瘍は，血管近傍にある P 細胞がFTによって優先的に損傷をうけpycnosisを起して除去される。これによって血液に十分環流されるようになったNP細胞は，供給された栄養刺激により recruitment[32]を起し P 細胞に変わる。この P 細胞はFTの作用によって直ちに殺されるが，このような機序で P 細胞の補給が続く限り血管の吸収は起らず，血管を支柱とした腫瘍の基本構造(GU)は維持される。しかし，ひとたびNP細胞が消耗し P 細胞だけとなれば，Shoulderのない指数関数的成長段階にある小腫瘍において観察されたように，P細胞の死滅の割合に応じて血管自体の吸収が起り，それに一致した率でGUの減少をもたらす。その結果，腫瘍体積は指数関数的に縮小する。このように，FT投与後に見られたシグモイド型の腫瘍縮小の機序をわかり易く図解したのが図14である。症例-1は他の症例と異なり，腫瘍体積の増加の割合に比べDqの増加の割合が大きいが，これはこの腫瘍の成長が著しく速いため血管の発育が追いつけず，必然的にNP細胞の占める割合が体積に比して多くなるためであろう。これに対して，VDTの比較的長い他の腫瘍（症例-2. 4, 5, 6）は，血管の発育もある程度付随でき，症例-1に比べ体積の割には栄養障害の程度も軽く，NP細胞の増加する割合も少いため，Dqも小さい傾向にあると考えられる。

FTによる腫瘍体積の指数関数的縮小率はGUの指数関数的減少率，すなわちCl. Cellの不活化率と一致すると考えられる。細胞の不活化は$N=Noe^{-KD}$の式で表わすことができる。Nは生残細胞数，Noは治療前の細胞数，Dは薬剤の投与総量，Kは細胞の不活化定数である。仮説によれば腫瘍縮小の指数関数部分の速度は，Cl. Cellの不活化率と一致するので，Doの逆数として求められるKは，この腫瘍のCl. CellのFTに対する感受性値でもある。今，症例-1を例にとると Do=6gr となる。治療前のこの患者の担癌細胞総数を

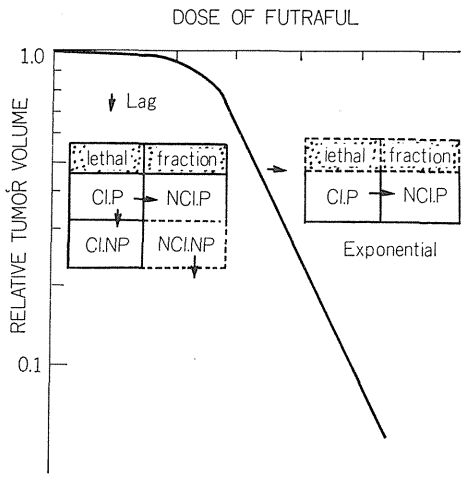


**図 14**　Growth Unit (GU) 仮説にもとづくFT投与後の腫瘍縮小モデル。P細胞を選択的に殺すFTを投与された減速段階 (stationary) にある腫瘍では，まずP細胞が致死的 Damage をうけ除去され，続いて NP 細胞がP細胞に移行 (recruit) を起し，ひき続きFTによる Damage をうける。NP細胞がすべてP細胞に移行したのちは Damage をうけたP細胞の割合に応じて血管が吸収され腫瘍体積の縮小が起る。

$10^{12}$と仮定すれば，生残率 $N/No=10^{-13}$，つまり90％治癒をもたらすのに必要な FT の総投与量は約 180gr と計算される。結局，この症例は，FT投与中に再発を起こし死亡した。死亡までに投与したFTの量は100grに達した。剖検時生体をむしばんでいた腫瘍は，FT投与前に生検で採取した皮フの FT 感受性腫瘍とは形態的に一目で異なるより未分化な像を示した。100gr のTF投与ののち，もし感受性腫瘍が残存するとすれば約 $10^6$ 個程度である。剖検材料はほとんどすべてが未分化腺癌より成っていたが，詳細な検索の結果，副腎の転移巣に，未分化腺癌のなかに感受性腫瘍集団の組織形態と一致する小腫瘍塊が散在していた。この結果は，FT感受性腫瘍は計算どうりFTによって減少しつづけたが，FT耐性細胞がFTに抗して成長しつづけ，FT感受性腫瘍と交代し，ついには耐性腫瘍が患者を殺すところまで成長したと説明される[33]。このように症例-1ほどの高感受性腫瘍でも治癒を達成するには 1200mg/日投与で180日余りの日数を要する。従って，図12に示すように，症例 2, 3, 4, 5, 6, 7 例は症例-1に比べ Do で5-7倍大きいので，約2.5年から3.5年 (180×5～7) に渡る連続投与が治癒を期待するため必要であろう。

以上の考察の結果，FTによる治療に当って次の投与原則が示唆される。

1)　成長の速い腫瘍は，たとえ大きなものであってもFT単独療法で寛解に導くことができる(症例-1) ばかりか，投与量いかんでは治癒させうる可能性もある。

2)　成長の遅い腫瘍では，副作用が少なく長期に渡って十分な量が投与できれば，単独でも寛解に導くことができる。しかし治癒を期待するには年余に渡る投与が必要である。さらに，腫瘍体積の大きいものは，腫瘍の縮小を引き起すまで大量のFTの投与が必要なので，この場合にはFTによる副作用を減らすため大きい腫瘍に対しては，手術による剔出，放射線療法の併用 (症例-2, 4)，寛解導入剤として有効な Mitomycin C[10] との併用 (症例-6) などを同時に採用し，積極的に腫瘍体積を減少させ，すなわち NP 細胞の量を減少させ，FT が有効に働くP細胞の多い小腫瘍にすべきである。

最後に，今後，FT での治療に当っては症例-1で経験したようなFT耐性腫瘍の発生，および年余に渡る FT 投与によってもたらされる新たな副作用の出現等の問題に対処することが大きな問題としてクロズアップされてくることを指摘したい。

稿を終るに当って，当症例の治療に御協力をして下さった栗栖明病院部長をはじめとする病院部医師，臨床研究部医師，および図の作成に御協力下さった松田和子殿に感謝いたします。

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# Effect of Bleomycin on Cell Survival and Some Implications for Tumor Therapy

T. TERASIMA, Y. TAKABE, M. WATANABE and T. KATSUMATA

Division of Physiology and Pathology, National Institute of Radiological Sciences, and First Department of Medicine, Chiba University School of Medicine, Inohana, Chiba-si

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Survival response and proliferative kinetics data of tumor and normal cells after exposure to chemotherapeutic agents have proved to be useful in constructing the rational design of tumor therapy. In this communication, however, several features of survival kinetics of cultured and transplantable tumor cells after bleomycin treatment are briefly mentioned on the basis of data of our colleagues. In view of these findings some principles of chemotherapy by bleomycin will be proposed.

## *I. Dose-Survival Response Relation*

The upward-concave nature of the dose-survival response has generally been found among mammalian cells in culture [1]. Figure 1 represents the survivals obtained after 1 h treatment of four kinds of cultured cells with various concentrations of bleomycin. The cell lines were derived from mouse fibroblast (L5), Green monkey kidney (Vero), trans-

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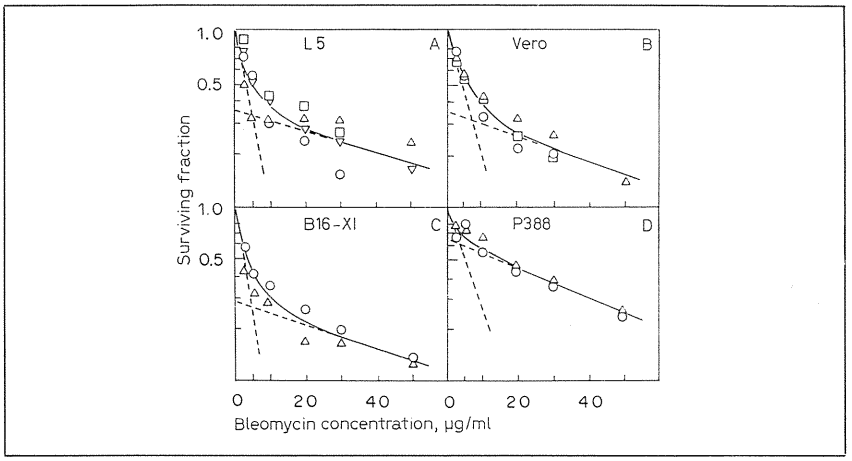


*Fig. 1.* Dose-survival response of cultured mammalian cells [1].

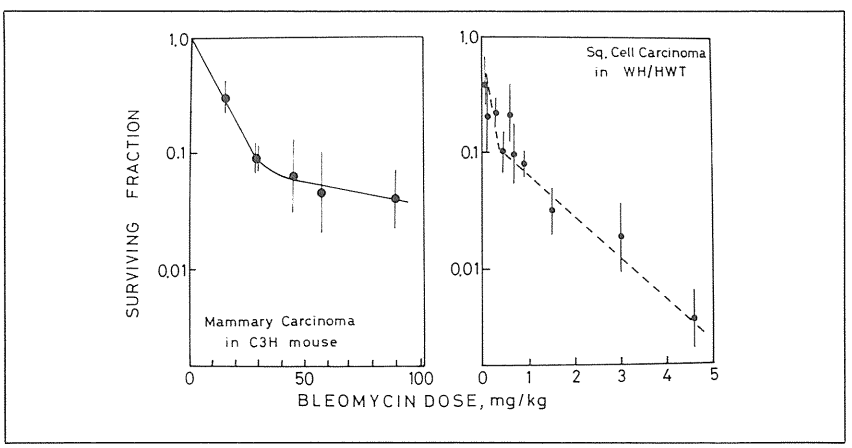


*Fig. 2.* Dose-survival response of transplantable tumor cells [2].

plantable mouse melanoma (B16-XI) and mouse lymphatic leukemia (P388). None of these show sigmoidal inactivation just as found after radiation. Thus, the drug does not appear to produce any sublethal damage in mammalian cells. The slope of initial portion, which stands for the sensitivity of cells, as well as the fraction of initial and terminal component, are not consistent among cell lines, but B16 melanoma cells, epithelial origin, appear to have a higher sensitivity than others.

Figure 2A also shows the upward-concavity of the dose-survival relation of C3H mouse mammary carcinoma cells as determined by $TD_{50}$ method by URANO *et al.* [2] (NIRS). Parallel tumor-bearing mice were treated with graded doses of bleomycin. The tumor was excised 1 h after administration and the single cell suspension obtained by trypsinization was injected into groups of mice after appropriate dilution. Figure 2B again shows the same type of survival response of squamous cell carcinoma of mice obtained by the same $TD_{50}$ assay method. The data were communicated by K. SAKAMOTO (University of Tokyo). Comparison of the doses giving 10 % survival in these data reveals that the squamous carcinoma cells are roughly 10 times more sensitive than mammary carcinoma cells.

Figure 3 is probably the earliest data showing the upward-concave response which was reported by UMEZAWA *et al.* [3], then drawn by Y. TAKABE (Chiba University). The solid form of Ehrlich carcinoma was treated with 10 fractions of graded concentrations and the tumor weight was measured at day 15.

The upward-concavity cannot be attributed to the presence of resistant mutants in the cell population as well as to the differential sensitivity of cells during the cell cycle, because the progeny of the survival at tail portion of the curve gave the identical response to the drug and the synchronous population of L5 cells. Burkitt lymphoma cells (Miyamoto, Chiba University) or Chinese hamster cells [4] also exhibited a similar curve shape. The reason for this particular response is partly due to the induction of resistance which will be demonstrated in the following section.

### *II. Time-Survival Kinetics*

When the survival of mouse L cells was determined as a function of time after bleomycin addition, the resultant inactivation curve revealed an involvement of two components. This is shown with four different concentrations of the drug in figure 4. The observed bending is not due to a heat inactivation of the drug, because the renewal of bleomycin-containing growth medium made at 1 h or later did not improve the inactivation rate of terminal component of the curve. Thus, it may imply that the cells acquired the less sensitivity by bleomycin addition. In other words, the drug may induce a relative resistance to bleomycin *per se* in cells. If the

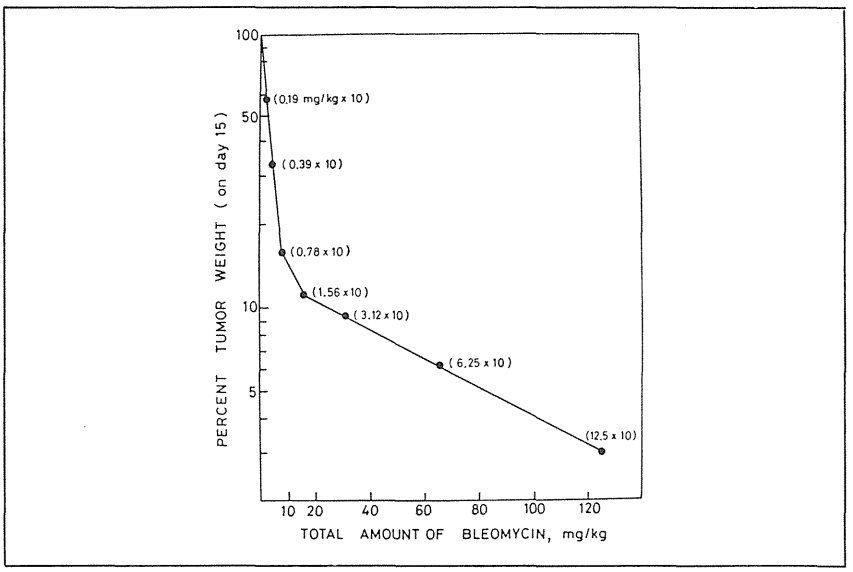


*Fig. 3.* Response of Ehrlich carcinoma to fractional treatment with bleomycin [3].

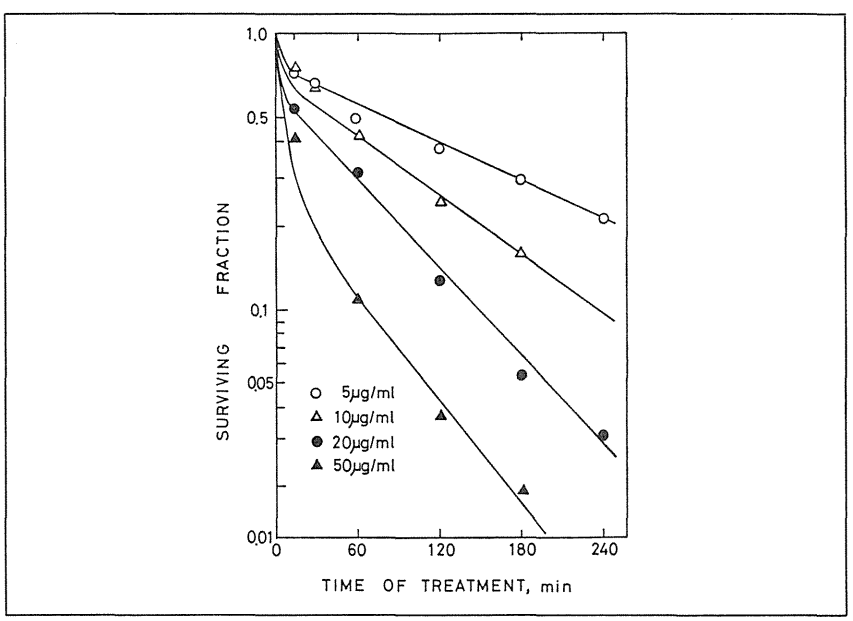


*Fig. 4.* Time-survival curve of mouse L cells to different concentrations of bleomycin [1].

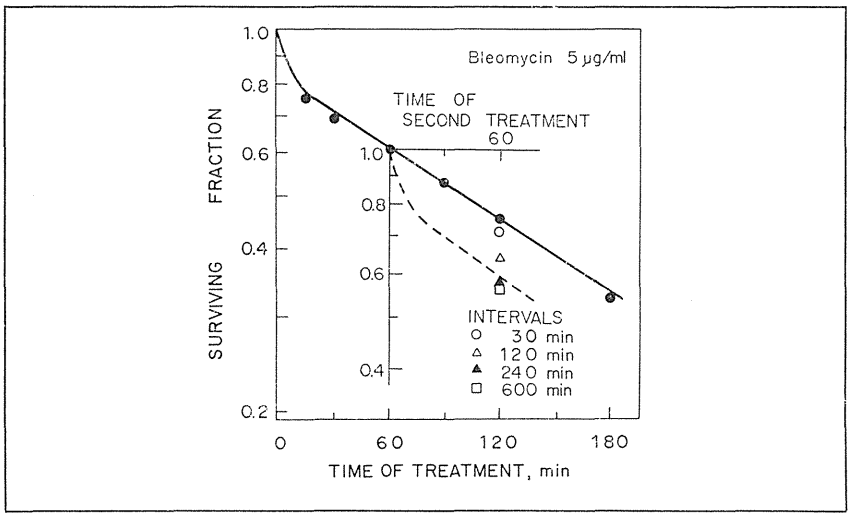


*Fig. 5.* Survival of L cells after two fractionated doses given at different intervals [1].

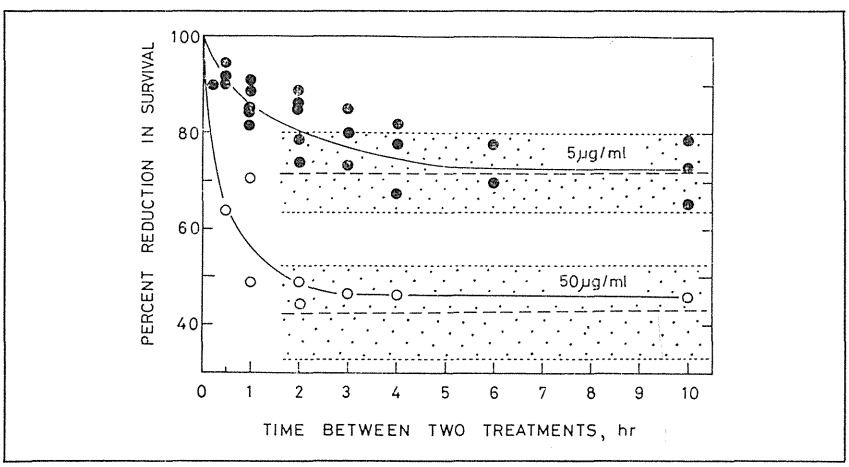


*Fig. 6.* Decay of induced resistance as revealed by two-dose fractionation [1].

hypothesis is true, the resumption of the original sensitivity could be expected after removal of the drug. This can be tested by the second bleomycin treatment [1].

As shown in figure 5, the cultures incubated with 5 μg/ml bleomycin were washed to remove the drug at 60 min and added with fresh ordinary

medium. After various incubation times as indicated in the figure, the same 5 $\mu$g/ml bleomycin medium was added for exactly 60 min and the survivals were examined. Two-dose survivals in the inset scale were decreased as a function of time interval between two doses. The results obtained from repetition of the same type of experiment were summarized in figure 6 in which each two-dose survival was taken as a fraction of 0 h-interval value.

The figure shows that the two-dose survival reduced with increase of the time interval and finally reached the level of broken line with shaded area, which represents the average value of survival and standard deviation expected when the reversion to the original sensitivity was completed. A similar experiment was carried out with two doses of 50 $\mu$g/ml. From figure 6, it can be seen that the reversion of sensitivity, i. e. the decay of induced resistance, normally completes 2–4 h after removal of the drug irrespective of the concentrations used. Induction and decay of the resistance demonstrated here was repeatable for the same culture if adequate intervals were posed between successive doses.

### *III. Theoretical Tumor Cell Sterilization*

The terminal portions of the time-survival curve shown in figure 4 exhibited the different slopes which appeared to be concentration-dependent. If the inactivation constant of the terminal portions was measured and plotted as a function of the drug concentration, the upward convex relation was found. It reveals that higher concentrations are less efficient or more wasteful than lower concentrations. This can partly explain why the upward concavity was found in the dose-survival response curves.

On the basis of these findings, the way of the efficient tumor cell sterilization can be considered [1, 5]. As shown in figure 7, the time inactivation of tumor cells is described by two component equation. If the treatment of hypothetical tumor cells was carried out continuously at 5 and 50 $\mu$g/ml concentrations, and if the drug concentration after a single administration is assumed to be constant only for 1 h and to be nil thereafter, the total amount of drug needed to sterilize $10^8$ cells would be approximately 300 and 900 $\mu$g, respectively. It indicates that the use of higher concentration is wasteful just as mentioned previously. More efficient treatment can be effected by the fractionation schedule which

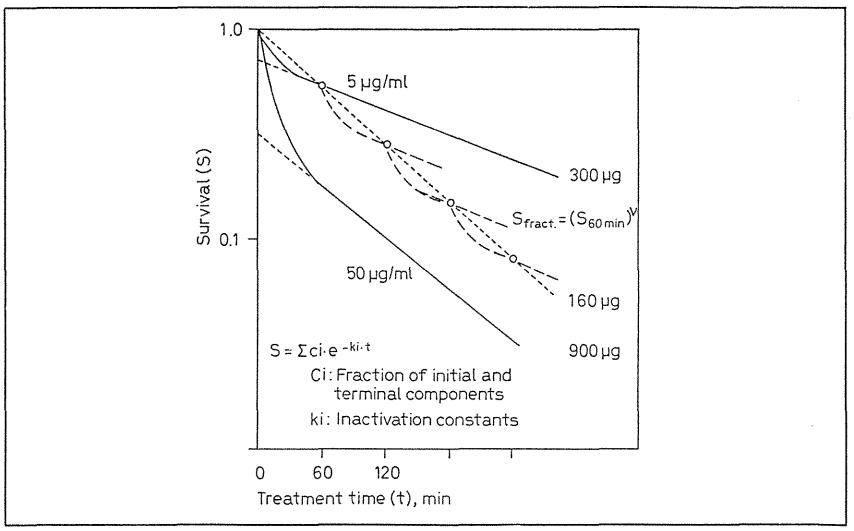


*Fig. 7.* Schematic presentation of continuous and fractional treatments.

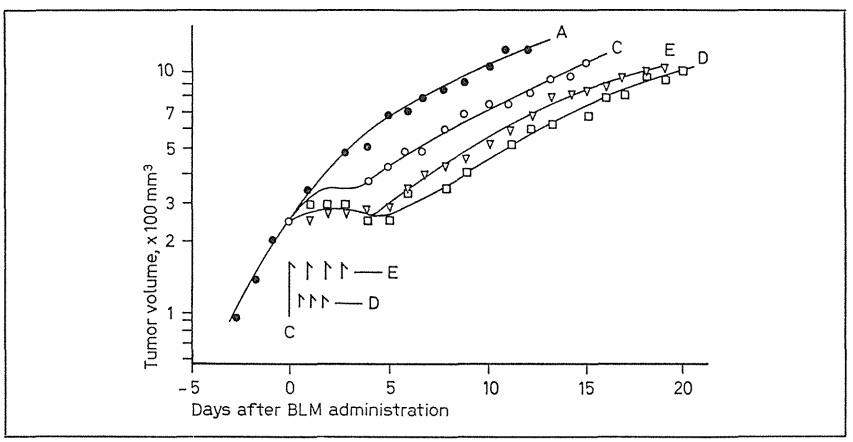


*Fig. 8.* Effect of single and fractional treatments on growth of C3H mammary carcinoma [2].

permits to obviate the induced resistance. If 1 h treatment at 5 $\mu$g/ml is given repeatedly at an adequate interval, the cell sterilization would follow the broken line and finally reach $10^{-8}$ with about 50 % amount of the drug of continuous treatment. Therefore, the present conclusion would be that as far as the proliferating tumor cells are concerned, the fractional treat-

ment at the lowest practicable concentration of bleomycin results in the most efficient sterilization.

This principle was tested by URANO *et al.* [2] (NIRS) by using C3H mammary carcinoma. Figure 8 shows the change in tumor volume with the time after the initiation of treatment. Curve A represents the control growth of tumor. A single shot of 100 mg/kg at 0 time produced the delay of growth as shown by curve C, whereas the same amount fractioned into four shots at 12- and 24-hour intervals gave an appreciable delay in the tumor growth. The result is apparently in favor of fractions.

## *IV.* In vivo *Studies*

In an attempt to extend cell culture findings to an *in vivo* experimental system, Ehrlich ascites tumor cells were chosen, because: (1) the cells grow in dispersed form and, therefore, trypsinization can be skipped for assay of proliferative potential, and (2) the cells are clonogenic in soft agar medium with a plating efficiency of 60 %.

The dose-survival response relation can be obtained by removing ascites tumor cells exactly 1 h after injection of bleomycin and by assaying for survival. Tumor cells grown 7 days in the abdominal cavity (i. e. plateau phase) normally give survivals of 40–50 % after a 0.1-mg/kg dose, 20 to

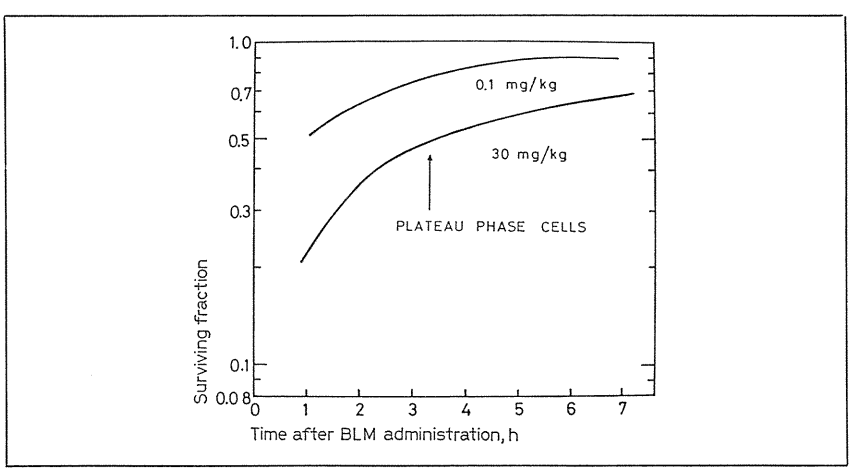


*Fig. 9.* Change in survival of Ehrlich ascites tumor cells following single administration of bleomycin.

30 % after a 30-mg/kg dose. However, these survival values remarkably increased if removal and assay of the tumor cells were made several hours later [7]. This is presented in figure 9. The drug in amounts of 0.1 and 30 mg/kg was injected at 0 time. From 1 h onward, tumor cells were removed at intervals and survivals were measured as a function of time after the drug injection. The result showed about a 2-fold increase in survival for 0.1 mg/kg, and a 4-fold increase for 30 mg/kg within 7 h. The increment seemed larger when the cell inactivation was greater. On the contrary, 3-day grown (log-phase) cells did not seem to exhibit immediate increase in survival.

Similar findings have been obtained by LITTLE [6] with cultured mammalian cells in plateau phase after exposure to radiation, and referred to as 'a repair of potentially lethal damage (PLD)'. The present increase of survival would be explained as follows: The tumor cells are given PLD by exposure to bleomycin. In plateau phase cells, PLD undergoes repair from time to time. But if cells were removed from the abdominal cavity and put to the assay condition, the remaining PLD is converted to lethal damage. In other words, the damage is fixed.

If PLD repair develops in plateau phase tumor cells, which presumably constitute a portion of growing and non-growing fraction of tumor, the therapeutic results would be strikingly detracted. For instance, the fractional treatment given at 7-hour or 1-day intervals would only give tumor cells an opportunity for a significant amount of repair to develop. Then, it follows that the continuous treatment provides the best result in view of tumor cell sterilization. On the other hand, the resistance induced by the first dose would not have a chance to decay when the continuous treatment is given. The final consequence of such contradictory effects was examined by fractional treatments at different intervals. The result revealed that the fractional treatment given at 1-hour intervals inactivated tumor cells at higher rate than does the fraction given at 3-hour intervals. Extrapolation of the results is that the greatest inacivation rate is effected by the continuous treatment.

*Summary*

Tumor is normally composed of cells growing and non-growing and, therefore, of cells having different responses to exogenous agents. Hence, it is not possible to treat a tumor with an unitary principle. As far as the

tumor cell sterilization by bleomycin is concerned, the growing fraction of tumor should be managed by fractional schedule, while plateau phase cells by continuous infusion. Based on the cell sterilization kinetics, further improvement of chemotherapy by bleomycin must be sought. The investigation will duly involve the studies on the nature of resistance induction and PLD repair.

## *Acknowledgments*

The authors acknowledge Drs. H. UMEZAWA (Institute of Microbial Chemistry), T. MIYAMOTO (Chiba University), M. URANO (Natn. Inst. Rad. Sci.) and K. SAKAMOTO (University of Tokyo) for their kind permission to use data and for their helpful discussions. Bleomycins (A5 and A2 complex) were generously supplied by Nippon Kayaku Co. Ltd. throughout these studies.

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Dr. T. TERASIMA, Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, *Chiba-si* (Japan)

# ACTION OF BLEOMYCIN ON PROLIFERATIVE CAPACITY OF MAMMALIAN CELLS

Toyozo TERASIMA,*1 Michinori WATANABE,*2 Yoshinobu TAKABE,*2 and Tadaaki MIYAMOTO*2

*Division of Physiology and Pathology, National Institute of Radiological Sciences,*1 and First Department of Medicine, Chiba University School of Medicine*2*

Various cellular effects of bleomycin (BLM) were studied with cultured and transplanted mammalian cells. Cinemicrographic observation revealed that most cells treated with BLM were killed transmitotically. The antibiotic induced strand scission in cellular DNA as evidenced by the sucrose gradient sedimentation method. The DNA lesion was susceptible to repair reaction as was the damage resulting from X-ray.

The dose-survival relationship of all mammalian cells showed an upward concavity. Kinetic analysis of the survival response revealed that the resistance of cells to BLM was induced soon after addition of the antibiotic and disappeared a few hours after its removal. Consideration of these kinetic findings may provide a principle for an efficient therapeutic schedule.

Another remarkable property of BLM action was a production and repair of potentially lethal damage in plateau phase tumor cells. The information obtained from the plateau phase cells *in vitro* or *in vivo* could be relevant to the understanding of the response of the noncycling, clonogenic fraction of tumors. Also, it can be emphasized that the cellular aspect of normal tissue damage will be of great importance for further improvement of therapeutic efficacy.

Studies on the survival response of tumor cells to exogenous agents in relation to growth status and cell cycle phases, as well as studies on cell cycle kinetics following exposure to agents, will provide basic principles for efficient tumor therapy (*44*). Recent results of cellular level studies regarding bleomycin (BLM) action have, in fact, introduced some useful information into the chemotherapy field.

The present review will mainly concern our results except for the cell cycle-related data and will discuss some implications for clinical tumor therapy.

## *Response of Macromolecular Syntheses to BLM*

The effect of BLM on macromolecular syntheses of mammalian cells was

*1 Anagawa 4-9-1, Chiba 280, Japan (寺島東洋三).

*2 Inohana 1-8-1, Chiba 280, Japan (渡辺道典, 高部吉庸, 宮本忠昭).



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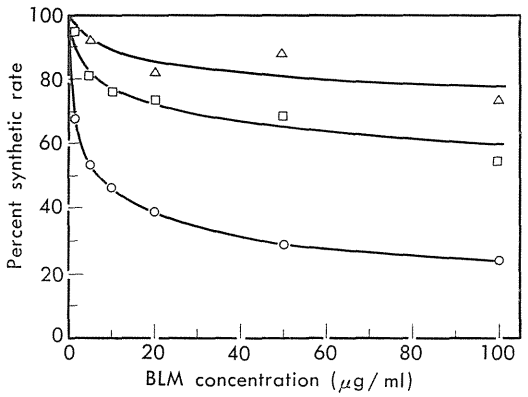


FIG. 1. Dose-response relationship of macromolecular syntheses of mouse L5 cells

Incorporation of labeled precursors was examined after incubation of cells with varying concentrations of BLM for 60 min. ○ DNA; □ protein; △ RNA. (From Watanabe *et al.* (*55*) by permission from the publisher of *J. Antibiot.*)

first examined by Kunimoto *et al.* (*16*) and later by Suzuki *et al.* (*33*). Their results showed that the inhibition of DNA precursor incorporation by the antibiotic observed in a few mammalian cell lines was moderate and consistent, whereas the incorporation of labeled uridine and leucine was reduced slightly, if any, after exposure for several hours. Similar findings have also been obtained by Tobey for Chinese hamster ovary (CHO) cells (*47*).

More critical measurements have been undertaken by Watanabe *et al.* using precursor-depleted cells (*55*). The dose-response relationship obtained for mouse L5 cells is shown in Fig. 1. DNA synthesis was affected most at every concentrations tested, while protein and RNA syntheses were less affected in that order. Suppression of DNA and RNA syntheses may be attributed to the template damage as mentioned in the following section. The inhibition of DNA synthesis was quickly reversed after removal of the antibiotic. Such inhibition of cellular syntheses may have some effects on the traversal of the cell cycle (*56*). The upward-concave response curve of these syntheses deserves attention and will be discussed later.

### *Damage and Repair in Cellular DNA*

It was expected that BLM would induce DNA damage *in vivo*, since a reduction of melting temperature and a formation of athyminic nucleic acids after incubation of DNA molecule with BLM have been reported by several workers (*10*, *24*, *25*). This was tested by determining the sedimentation profile in sucrose gradient of DNA isolated by sodium dodecyl sulfate treatment (SDS-DNA) (*38*, *42*).

HeLa cells prelabeled with radioactive thymidine were treated with BLM for 30 min at 37° before isolating DNA. Figure 2 represents the sedimentation

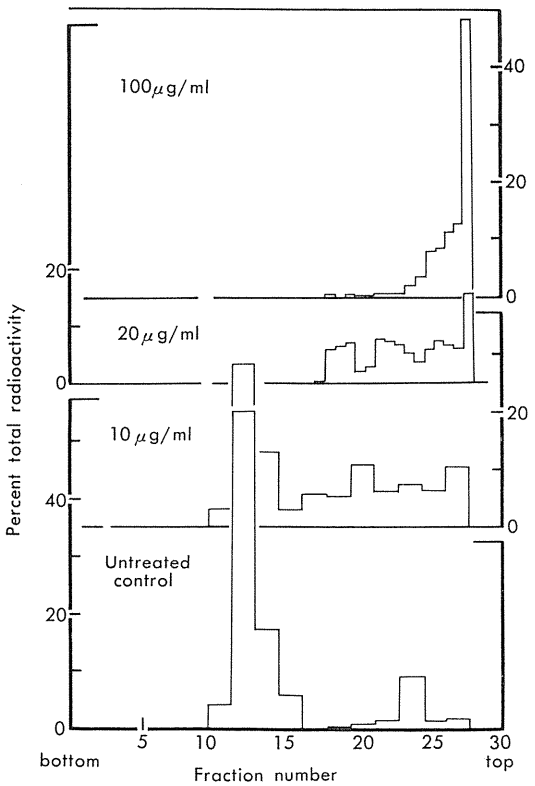


FIG. 2. Sedimentation profiles of DNA recovered from BLM-treated cells
HeLa cells treated with BLM for 30 min were lysed in 2% SDS layer placed on top of sucrose gradient. After ultracentrifugation, amount of DNA in each fraction was determined by radioactivity measurement. (From Terasima *et al.* (*40*) by permission from the publisher of *Gann*)

profiles of native SDS-DNA obtained after exposure to graded concentrations of BLM (*40*). It indicates that 10 μg/ml of BLM was the minimum effective concentration for producing double-strand breaks of SDS-DNA. Similarly, single-strand breaks of alkaline SDS-DNA were demonstrated at the concentrations of more than 1 μg/ml. Extensive breaks were produced after 10 μg/ml treatment as shown in the middle panel of Fig. 3. Similar result has been reported earlier by Suzuki *et al.* (*34*) and later by Fujiwara and Kondo (*10*) and by Saito and Andoh (*30*). The latter authors have proposed that pronase-sensitive linkage in DNA is preferentially cleaved by low concentrations of the drug.

Further incubation of the culture after removal of BLM allowed the broken pieces to rejoin as evidenced by reappearance of the prominent peak in the upper panel of Fig. 3 (*40*). Although it is not certain whether a full return of the profile to the original level can be expected after a longer incubation period, it obviously demonstrates that the rejoining of broken single-strand pieces occurred just as rapidly as is found in X-ray irradiated cells (*48*). Hence, it can be assumed

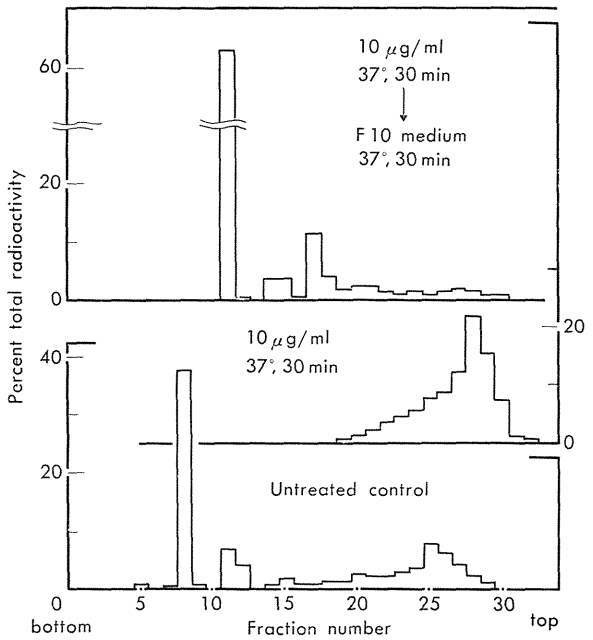


FIG. 3. Breakdown and repair of DNA of HeLa cells after BLM treatment (From Terasima *et al.* (*40*) by permission from the publisher of *Gann*)

that the action of BLM on cellular DNA leads mainly to the development of clean breaks which are susceptible to the operation of repair enzymes. In this connection, Fujiwara and Kondo have demonstrated that the antibiotic provoked unscheduled incorporation of $^{3}$H-thymidine into non-S phase HeLa cells, indicating that a portion of single-strand breaks were readily repaired (*10*).

*Cinemicrographic Analysis of BLM-treated Individual Cells*

Katsumata *et al.* (*14*) have carried out experiments in which mouse L5 cells grown synchronously were treated pulsewise (1 hr) with BLM at the $G_1$-S transition phase and were followed individually by time-lapse cinemicrography. By analyzing the recorded films the posttreatment behavior of treated individual cells was classified into five different patterns (A–E) as shown in Table I. The fraction of each pattern was determined for cells treated with two different concentrations of BLM and for those untreated.

Pattern A, in which cells were killed without division, increased depending on the increase of BLM concentration. Similarly, fraction of cells which were neither killed nor divided by the termination of observation (Pattern B) increased with increasing concentration. It implies that the higher concentration of the antibiotic induced extraordinary delay of division and ultimately caused cells to die before they reach mitosis (Pattern A). A notable finding was that the majority of treated cells were killed after the posttreatment division (Patterns C and D). It indicates that the potential damage either in DNA or in the extra-DNA com-

TABLE I. Pattern of Clonal Growth of BLM-treated Cells

| Pattern of clonal growth | Fraction of clones (%) | | |
|---|---|---|---|
| | Untreated control | BLM concentration ($\mu$g/ml) | |
| | | 20 | 100 |
| A —— × | 0 | 7 | 21 |
| B —— ○ | 0 | 0 | 25 |
| C —┬ × × | 0 | 60 | 45 |
| D —┬┬ × | 0 | 13 | 0 |
| E —┬┬ | 100 | 20 | 8 |
| Number of cells examined | 22 | 15 | 24 |

Crosses represent death of individual cells. In pattern B, the cells were neither killed nor divided within 140 hr of observation. From Katsumata *et al.* (*14*).

ponent given at the $G_1$-S transition phase must be transmitted through mitosis before it becomes lethal (transmitotic death).

The cumulative frequency of division after treatment was determined as a function of time after mitosis as shown in Fig. 4. The curves represent the first posttreatment division for treated and untreated cells, since the cultures had been mitotically synchronized at zero time. The median generation time of treated cells was 1.5 and 4.3 times greater than the value of untreated control (22 hr). The growth curve obtained after treatment with 20 $\mu$g/ml seems to indicate that there are two fractions differing in the cumulative frequency distribution of division, as shown by the steep and less steep slopes. It suggests that cells sustain two different kinds of damage as related to the division delay.

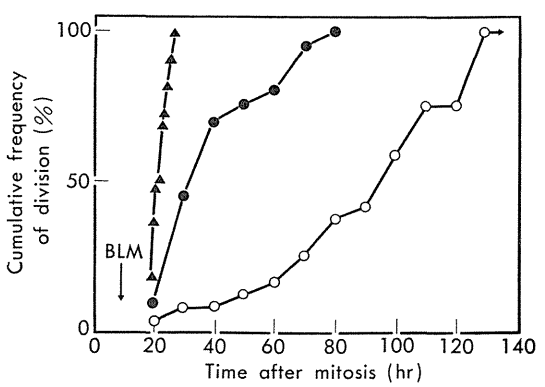


FIG. 4. Posttreatment division of synchronous L5 cells treated with BLM
Mitotically synchronous L5 cells at 0 hr were treated with BLM for 1 hr at $G_1$-S transition (8 hr), then followed by time-lapse cinematography. ▲ untreated; ● 20 $\mu$g/ml; ○ 100 $\mu$g/ml. (From Katsumata *et al.* (*14*) by permission from the publisher of *Gann*)

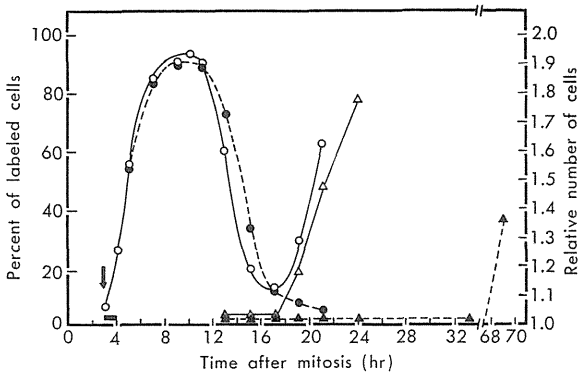


FIG. 5. Progression of synchronous L5 cells pulsely treated with BLM at $G_1$ phase

○ percent of DNA-synthesizing cells; △ relative number of cells. ○△ control; ●▲ 100 μg/ml. (From Watanabe *et al.* (*56*) by permission from the publisher of *Cancer Res.*)

The latter damage leading to severe prolongation and greater distribution of the generation time predominated after 100 μg/ml treatment.

Watanabe *et al.* (*56*) have investigated whether such a considerable delay can be attributed to the block at a given stage during the cell cycle. Figure 5 shows the result of an experiment in which the total number of cells and fraction of DNA-synthesizing cells were observed for synchronous L5 cells treated similarly with 100 μg/ml of BLM. It revealed that the delay of division was almost entirely due to the prolongation of the $G_2$ period. It is noted that a certain damage given to cells at the $G_1$-S transition is not repaired until it becomes apparent at $G_2$. Recently, Hittelman and Rao (*12*) have demonstrated by premature condensation method that BLM induced chromosomal damage at different cell cycle stages. They have emphasized that unrepaired damage induced in chromosomes may affect the progression through the cell cycle. It has also been demonstrated that the progression through DNA synthesis period can be affected in the presence of the drug (*56*).

The demonstration of damage in chromosome or in DNA appears to be in favor of DNA-target theory. However, the role of a possible membrane lesion in the cell killing by BLM cannot be dismissed. Fujimoto (*9*) has presented evidence that radioactive BLM molecules are predominantly located at outer vicinity of the nuclear membrane of C3H mouse ascites tumor cells, although the biological significance of this is not known.

A remarkable morphological change of the treated cells was the increase in size and volume, and the occurrence of perinuclear granules, and marked cytoplasmic projections (Photo 1). Most of the cells with prolonged generation time were about 5 times greater in cross section than the untreated control (*14*).

*Survival Response Studies*

The survival response of various cultured mammalian cells to BLM has been studied by several investigators (*2*, *39*, *41*). The dose-survival relationship for mouse fibroblast cells (L5) and mouse lymphatic leukemia cells (P388) is shown in Fig. 6, where cells were treated with varying concentrations of the drug for 1 hr. As seen in Fig. 6, the survival response curve shows an upward-concave curvature which consists of a steep (initial) and less steep (terminal) portion. Later, this feature has been generally found among various mammalian cells ranging from cultured or transplantable tumor cells to normal somatic cells (Table II). The reason for this has been discussed by Terasima *et al.* (*41*). The resistant, terminal component cannot be interpreted either by differential sensitivity through the cell cycle or by the presence of genetically determined, resistant cells, since synchronous $G_1$ cells (3.5 hr after mitosis; open symbols in Fig. 6) or a clone derived from a BLM survival again have shown a similar upward-concave response curve (Katsumata, unpublished). The evidence that the upward-concave or biphasic survival response curve was exhibited by cells in all stages of the cell cycle has been reported by Barranco and Humphrey (*2*) and by Drewinko *et al.* (*8*). This also agrees with the above theory (the cell cycle dependence of sensitivity will be mentioned in the other review).

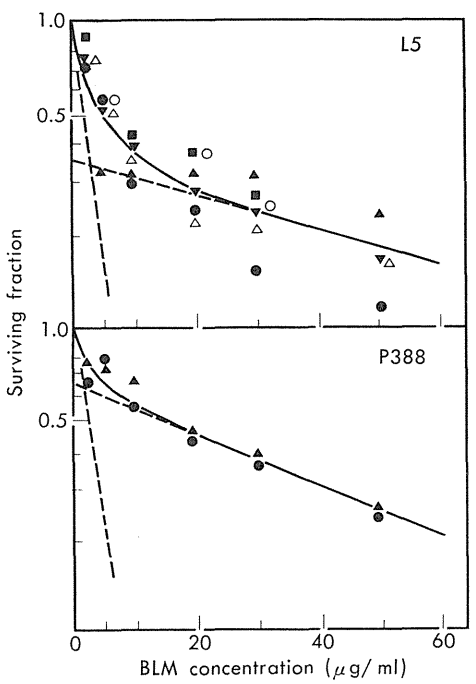


FIG. 6. Dose-survival relationship of BLM-treated mammalian cells
Different closed symbols denote separate experiments. Open symbols in the upper panel are survivals of $G_1$ population. (Redrawn from Terasima *et al.* (*41*))

TABLE II. Sensitivity of Various Mammalian Cells to BLM

| | Cells | Sensitivity to BLM[a] ($D_{37}$: μg/ml) | | Inflexion point |
|---|---|---|---|---|
| | | Initial portion | Terminal portion | |
| Cultured cells | | | | |
| 1 | L5 (mouse fibroblast) (*41*) | 2.5 | 65 | *ca.* 5 |
| 2 | Vero (green monkey kidney) (*41*) | 4 | 67 | *ca.* 8 |
| 3 | CHO (Chinese hamster ovary) (*2*) | 8 | 123 | *ca.* 10 |
| 4 | V79 (Chinese hamster lung) (*22*) | — | 16 | |
| 5 | B16-XI (mouse melanoma) (*41*) | 3 | 70 | *ca.* 5 |
| 6 | P388 (mouse leukemia) (*41*) | 2.5 | 49 | *ca.* 3 |
| 7 | HeLa S3 (human cervical carcinoma) (*39*) | — | 45 | |
| 8 | P3HR-1 (Burkitt's lymphoma)[b] | 2 | 27 | *ca.* 5 |
| 8′ | P3HR-1 (Burkitt's lymphoma)[b] | 6 | 97 | |
| 9 | EMT6/M/CC (mouse mammary tumor) (*50*) | — | 25 | *ca.* 10 |
| 9′ | EMT6/M/CC (mouse mammary tumor) (*50*) | — | 42 | |
| Transplantable tumors | | | | |
| 10 | Mammary carcinoma (mouse) (*54*) | 13 mg/kg | 85 mg/kg | 35 mg/kg |
| 11 | Squamous cell carcinoma (mouse) (*29*) | 0.1 mg/kg | 2.7 mg/kg | 0.2, 3 mg/kg |
| 12 | Ehrlich ascites carcinoma (mouse)[b] | | | <0.5 mg/kg |
| Normal somatic cells | | | | |
| 13 | Plaque-forming cells (mouse) (*26*) | Insensitive | | |
| 14 | Lymphoblast (PHA[c]-induced, human) (*26*) | | | 10 |
| 14′ | Lymphoblast (PHA[c]-induced, human) (*46*) | | | <10 |
| 15 | Bone marrow CFU-S[d] (mouse) (*49*) | — | *ca.* 180 | |
| 15′ | Bone marrow CFU-S[d] (mouse) (*49*) | — | *ca.* 180 | <50 |

[a] Sensitivity was determined after 1-hr incubation with BLM, unless stated in the following remarks: 3, 30-min incubation; 8, exponential; 8′, stationary; 9, exponential, 2-hr incubation; 9′, stationary, 2-hr incubation; 10 and 11, $TD_{50}$ assay; 12, agar colony assay; 14 and 14′, *in vitro* treatment; 15, noncycling, *in vitro* treatment (30 min); 15′, endotoxin-stimulated, *in vitro* treatment (30 min).

[b] 8 and 8′ by T. Miyamoto, unpublished; 12 by Y. Takabe, unpublished.

[c] PHA: phytohemagglutinin.

[d] CFU-S: colony-forming unit in spleen.

Terasima *et al.* (*41*) assumed that the resistant tail in the dose-survival curve resulted from the induction of resistance by the antibiotic. This was demonstrated by two-dose fractionation in the time-survival curve. The closed circles in Fig. 7 represent the time-survival curve where cultures with BLM added were rinsed to remove the antibiotic at specified intervals and incubated with the ordinary growth medium for survival assay. The surviving fraction was reduced quickly after addition of BLM and then at the slower rate from 30 min onward. For two-dose fractionation the treatment was terminated at 120 min by replacing the antibiotic with ordinary growth medium. After incubation of BLM-free cultures for 60 min, the time-survival curve was again determined by adding BLM, as shown in the inset of Fig. 7. From the result in which the parallel reduction of survival level was demonstrated after the second exposure, the following can be pointed out: (1) The resistance induced by the first treatment disappeared when the antibiotic was removed, although a complete reversion to

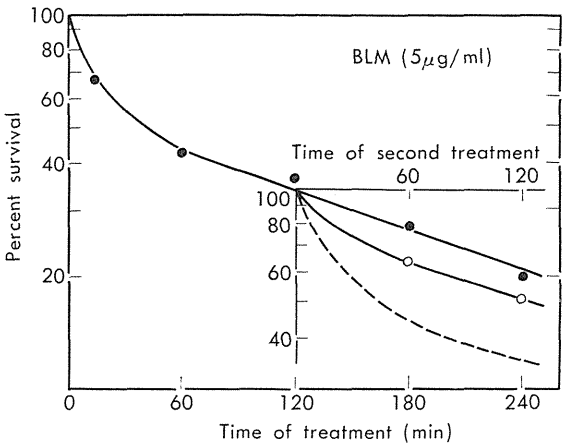


FIG. 7. Two-dose fractionation experiment

Closed symbols show time-survival curve obtained from incubation of L5 cells with 5 μg/ml BLM. Open symbols represent time-survival obtained from the second treatment which was carried out 1 hr after removal of the drug. Broken line is the survival level expected from complete reversion to the original sensitivity. (Reprinted from Terasima *et al.* (*41*))

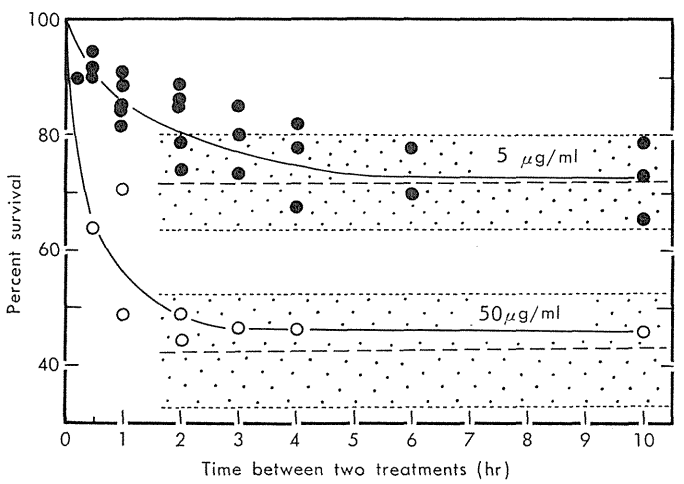


FIG. 8. Disappearance of induced resistance as a function of time between two treatments

Decay of induced resistance was followed by two 60-min treatments with 5 and 50 μg/ml of BLM. Broken line with dotted band represents the average level and standard deviation of survival of original population to single exposure. (Reprinted from Terasima *et al.* (*41*))

the original sensitivity was not obtained during 1 hr, and (2) cells were again susceptible to resistance induction by the second treatment.

Figure 8 represents the decay of induced resistance as determined by two 60-min dose fractionations at 5 and 50 μg/ml concentrations. The percent of

two-dose survivals decreased as a function of time between two exposures and finally reached the level expected from reversion to the original sensitivity. Hence, it was concluded that the resistance induced by BLM, whatever the concentration used, disappeared approximately 4 hr after its removal.

No material basis has yet been found for the nature of resistance. However, the induction and the decay of resistance were similarly observed for the inhibitory effect of BLM on macromolecular syntheses (*55*). The general occurrence of this particular response leads us to assume that the same mechanism operates for the induction of resistance in various effects.

BLM-inactivating enzyme has been found in various mammalian tissues by Umezawa *et al.* (*53*). The enzyme in cultured mammalian cells may be activated by the addition of BLM, thereby reducing the BLM activity as seen in the time-survival curve (Fig. 7). Otherwise, activation of DNA polymerase may be one of the interpretations, since it can repair DNA breaks induced primarily by the drug. Alteration of permeability by BLM may be another possible mechanism. However, as far as a preliminary experiment using $^{14}$C-BLM is concerned, the association rate of antibiotic with cells as shown by radioactivity measurement was not altered appreciably for 2 hr after addition of BLM (M. Watanabe, unpublished).

Urano *et al.* (*54*) have proposed a "binding-saturation model" by which the upward-concave response curve was kinetically interpreted. Nevertheless, it could not specify any mechanism which underlies the response.

Relevance of "resistance induction and decay kinetics" to tumor therapy will be mentioned later.

*Growth Phase Dependence of Sensitivity*

Although the growth phase dependence of sensitivity to cytotoxic agents has been reported early by Madoc-Jones and Bruce with 5-fluorouracil (*19*) and by Shuve and Rauth with phleomycin (*32*), Barranco *et al.* (*3*) were the first to have shown this with BLM. Later, Mauro *et al.* (*21*) studied the response of plateau-phase cells and obtained results inconsistent with those of former authors. For Chinese hamster-derived CHO cells used by Barranco *et al.* the sensitivity of the plateau phase was 10 times higher than log phase cells, whereas another hamster-derived cell line (V 79) showed less sensitivity in plateau phase than in the exponentially growing phase. Similarly, our Burkitt's lymphoma cells in exponentially growing phase were 3 to 4 times more sensitive than plateau phase cells, as shown in Fig. 9. The discrepancy between Barranco's and others' data has not been solved.

With respect to the observed difference in sensitivity, either of several factors are thought to be responsible; namely, (1) difference in capability of repair of potentially lethal damage (PLD) given by BLM, (2) difference in membrane permeability, (3) cell cycle-dependent sensitivity difference exaggerated by a compartmental shift in the plateau phase population, and (4) difference in target sensitivity. In the case of Burkitt's lymphoma cells, it has been found that the

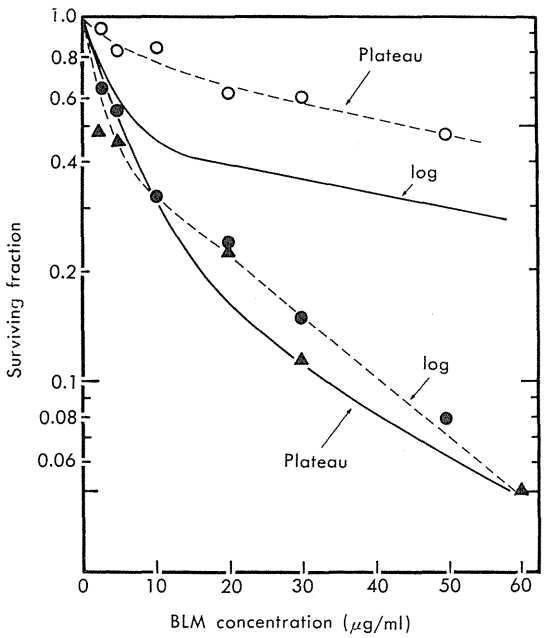


FIG. 9. Dose-survival curve of log and plateau phase cells
Broken lines with experimental points, Burkitt's lymphoma P3HR-1 cells (T. Miyamoto, unpublished); solid lines, Chinese hamster ovary cells (redrawn from Barranco *et al.* (*3*) by permission from the publisher of *Cancer Res.*).

plateau phase population was predominantly $G_1$ cells which were least sensitive to the drug (T. Miyamoto, unpublished).

In view of the importance of plateau-phase cells as an *in vitro* model for nongrowing fraction of tumor cells, the observed low sensitivity and its underlying mechanism must be a great concern for those who attempt tumor control. A clue will be given in the next section.

### *Production and Repair of PLD*

The radiation survival of mammalian cells is greatly influenced by post-irradiation conditions which favor either the expression of, or the repair of, PLD induced by radiation (*27*). A remarkable condition was found by Little (*17*, *18*) with cultured human cells: If cells were allowed to remain in the plateau phase of growth for some time after exposure to radiation, the survival measured after dispersion of the culture increases with time during which PLD is repaired. The discovery, which had a great relevance to radiotherapy, also had an impact on the chemotherapy field, and it has been seriously questioned whether similar type of damage repair develops after drug treatment. In recent years several groups investigating BLM action have demonstrated the repair of PLD in plateau-phase cells in culture (*4*, *28*) and in tumor (*36*, *51*) (G. M. Hahn, personal communication).

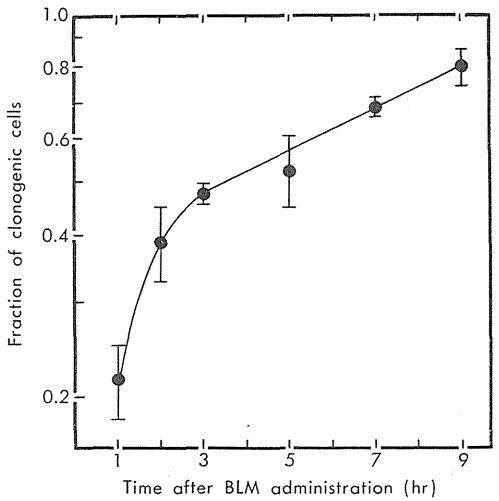


FIG. 10. Repair of potentially lethal damage given by BLM in Ehrlich ascites tumor cells

Tumor cells grown 7 days in ascites were colony-assayed at intervals after a single dose (30 mg/kg) of BLM. (From Takabe *et al.* (*36*) by permission from the publisher of *Gann*)

Figure 10 represents one of PLD repair data now available to us. Mice bearing 7-day grown Ehrlich ascites tumor cells (early plateau phase) received a single shot of 30 mg/kg dose of BLM through a subcutaneous route. At intervals the ascites was removed and assayed for the number of clonogenic cells by plating a known number of cells into soft agar layer. As seen in Fig. 10, the number of colony formers increased rapidly from 1 to 3 hr, then, slowly from 3 hr on, and reached the level roughly 4 times greater than 1-hr value at 9 hr. The increment of survival appeared to be related to the amount of damage given primarily. The result indicates that the damage was repaired with time during which the tumor cells remained *in vivo*, but the unrepaired cells were converted to a lethal state when cells were put into the assay condition. Other experiments revealed that exponentially growing ascites tumor cells were also susceptible to a production of PLD, but repaired the damage with a delay of several hours.

Based on the demonstration of PLD repair in various biological systems, it is now obvious that an exact dose-effect relation of noncycling (plateau phase-like) cells of certain tumors is not readily determined. Using EMT-6 mammary tumor, Twentyman (*51*) showed that, in sharp contrast to the dose-survival relationship determined immediately after drug administration, the 24-hr determination gave only a negligible response.

### *Cellular Basis of Tumor Therapy by BLM*

Findings obtained from studies mentioned above are extremely useful for understanding the cellular basis of BLM chemotherapy. This section concerns

four problems which are thought to be relevant to designing therapeutic schedules.

*Survival curve consideration*

In view of the generality of the upward-concave response curve among various mammalian cell systems (see Table II), a consideration of the response should provide useful principles for tumor therapy.

Firstly, it can be seen from Fig. 6 that the low concentration range of BLM inactivates cells more efficiently than the higher concentration range. Therefore, a determination of the inflection point in the dose-survival relationship for each tumor would be critically important, in order to avoid excess amount of the antibiotic which may affect normal tissues. Studies on time-survival kinetics by two-dose fractionation showed that inefficient cell killing in higher concentrations was possibly due to the induction of resistance by the antibiotic.

The second important finding is that the resistance is induced within 30 min after addition of BLM and entirely disappears 4 hr after its removal (Fig. 8). As shown in Fig. 11, induction and decay of resistance were repeatable if adequate intervals were posed between repeated exposures to BLM (*35*, *41*).

These findings appear to be greatly relevant to a construction of the therapeutic design. As discussed previously (*41*, *45*), it is obviously profitable (1) to adopt fractionation scheme by which the disadvantage resulting from a development of resistance will be avoided, and (2) to use small doses which fall within the effective concentration range. The prediction based on survival response data of cultured cells was beautifully verified by experiments of Urano *et al.* (*53*).

Experiment illustrated in Fig. 12 shows the change in volume of C3H mouse mammary tumor with time after initiation of BLM administration. Inhibition of tumor growth was greater when 100 mg/kg dose was divided into 4

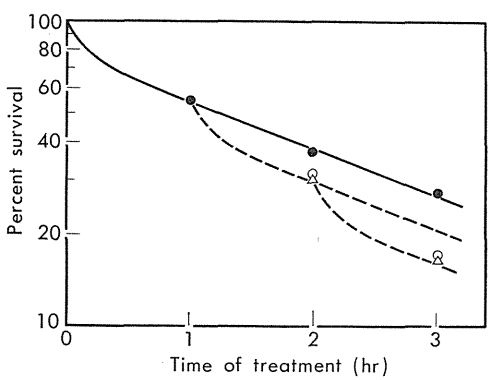


FIG. 11. Survival response of L5 cells after fractional treatment

One-hr treatment of L5 culture with 5 $\mu$g/ml BLM was repeated at intervals of 4 hr (○) and 10 hr (△). Closed symbols show survival after continuous treatment. Broken lines represent the survival response expected when a decay of resistance was completed during the interval between fractions. (Reprinted from Terasima *et al.* (*41*))

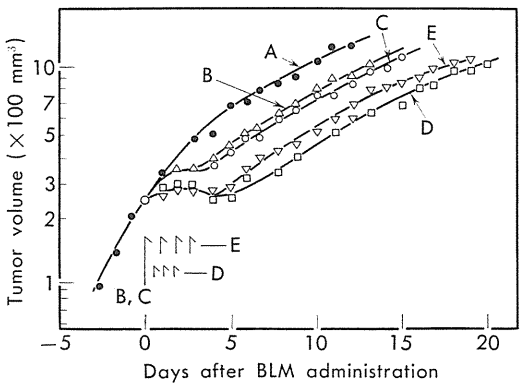


FIG. 12. Change in volume of C3H mouse mammary carcinoma after BLM treatment

A, untreated; B, 50 mg/kg × single shot; C, 100 mg/kg × single shot; D, 25 mg/kg × 4 fractions at 12-hr intervals; E, 25 mg/kg × 4 fractions at 24-hr intervals. (Redrawn from Urano *et al.* (*54*) by permission from the publisher of *Cancer Res.*)

shots (E and D) than when the same total dose was given as a single shot (C). Less effect of 24-hr interval fractionation may be due to a repopulation of tumor cells. Optimum interval would be shorter than 12 hr, being compatible with the decay time measured *in vitro*. In the meantime, the difference in effect between 50 mg/kg dose (B) and 100 mg/kg dose (C) was disproportionately slight. This can be expected from the dose-survival curve (not shown here) in which the effect of both concentrations was beyond the inflection point.

However, a 25 mg/kg dose was small enough to exert more efficient cell killing than a 50 or 100 mg/kg dose. This may be another reason why the four-fractionation schedule gave greater inhibition.

The principles for therapy mentioned above would be the case regardless of whether the tumor cells are cycling or noncycling, although the effect will be considerably diminished in noncycling, possibly plateau phase-like, cells in which PLD repair occurs.

The elucidation of the nature of resistance will be of particular importance since it would improve therapeutic efficacy by providing means to control induction and decay processes.

*Response of noncycling fraction*

Noncycling, clonogenic cells are a subject of great interest to chemotherapists, since these cells, constituting a significant and sometimes predominant fraction of tumors, are relatively insensitive to chemotherapeutic agents. In recent years particular emphasis has been placed on studies of plateau-phase cells in culture, because it was elucidated that the plateau-phase cells have several properties in common with noncycling tumor cells (*11*) and, in addition, they manifest a remarkable capability of posttreatment repair (*17*).

BLM is perhaps the first antibiotic which is known to produce PLD in plateau-phase cells in culture and tumor cells. The repair of PLD sets in soon

after BLM administration and develops quickly for the first several hours. Hence, it can be expected that fractional treatment given at shorter intervals allows smaller amount of PLD to be repaired, resulting in greater cell sterilization. On the other hand, resistance induced by the first dose will not have a chance to decay if the antibiotic is continually present. This would give higher survival. The consequence of such contradictory effects was examined by fractional treatments of Ehrlich ascites tumor cells with different concentrations at different intervals. The result of limited experiments seems to show that the greatest inactivation rate is obtained by continuous treatment if the dose is small enough (*45*).

*Inhibition of cell cycle traverse*

The effect of BLM on cell cycle traverse may be referred to the other review in this volume. The most remarkable hindrance of cell progression caused by this antibiotic is an accumulation of cells in $G_2$ phase (*2*, *47*). In this connection, an interesting result is that the $G_2$ block can be induced even when the cells were exposed pulsely to BLM in $G_1$ phase (*56*). The blocked population will then be efficiently inactivated by the second shot of "X-ray type agents" which specifically kill $G_2$ cells (*43*). The practicality of the combination schedule would depend on whether conditioning method which can collect more $G_2$ cells and timing for the second shot can be successfully developed.

It has been reported that BLM induces partial synchrony in proliferating populations (*5*). This may give another chance for scheduling combination therapy.

*Determination of normal tissue damage*

Clinical studies with BLM have revealed that the antibiotic exerts no important effect on gastrointestinal tract, hematopoietic, and immune systems (*6*, *31*, *52*, *57*). In comparison with severity of damage in proliferative systems caused by other antitumor agents, this finding was rather unusual. Umezawa *et al.* (*53*) have explained it on the basis of distribution of BLM-inactivating enzyme which was found among all tissues except for skin and lung.

The sensitivity of bone marrow stem cells in mice was measured by spleen-colony assay method (*22*, *49*). Like effects of other cytotoxic agents, noncycling stem cells were found relatively insensitive to BLM, while stem cells moved into cell cycle following endotoxin stimulation manifested the dose-survival response almost equal to those of cultured CHO or mouse leukemia cells (P388).

Most cytotoxic agents studied cause immunosuppression, presumably inhibiting proliferating lymphocytes (*20*). However, BLM appears not to exert a remarkable alteration of immunocapacity. Response of immunocompetent cells was studied in mice and man (*26*, *46*). As shown in Fig. 13, plaque-forming cells (PFC) of C57BL mice were extremely insensitive regardless of the time of administration of BLM in relation to antigen injection. Phytohemagglutinin (PHA)-induced blastogenesis of human peripheral lymphocytes was moderately affected just as found in cultured mammalian cells. Also, cellular immunity against L1210 leukemia cells and allograft remained essentially unaffected (*1*, *7*).

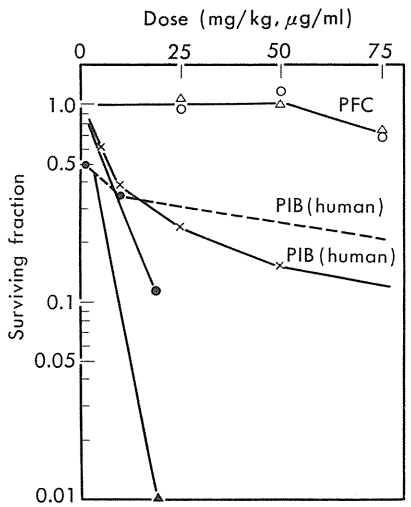


FIG. 13. Effect of BLM on immune response in mouse and human lymphocytes

Open symbols represent the response of PFC to BLM and closed symbols are response to daunomycin. The agent was given 24 hr before (○) and 48 hr after (△) antigen injection. Crosses represent response of human blood lymphocytes in terms of PHA-induced blastogenesis (PIB). Data were obtained from Ohno *et al.* (*26*) by permission from the publisher of *Gann*. Broken line was taken from Tisman *et al.* (*46*).

Relatively weak action of BLM on immunocapacity, although appreciable by a certain *in vitro* test, should be of immense benefit for clinical tumor therapy.

Clinical experience has indicated that the lung and skin are the critical tissues which limit the therapeutic gain of chemotherapy (*13*, *15*, *23*, *37*, *57*). It would be necessary to investigate the responses of these tissues in a quantitative or even in a qualitative manner for further improvement of therapeutic efficacy.

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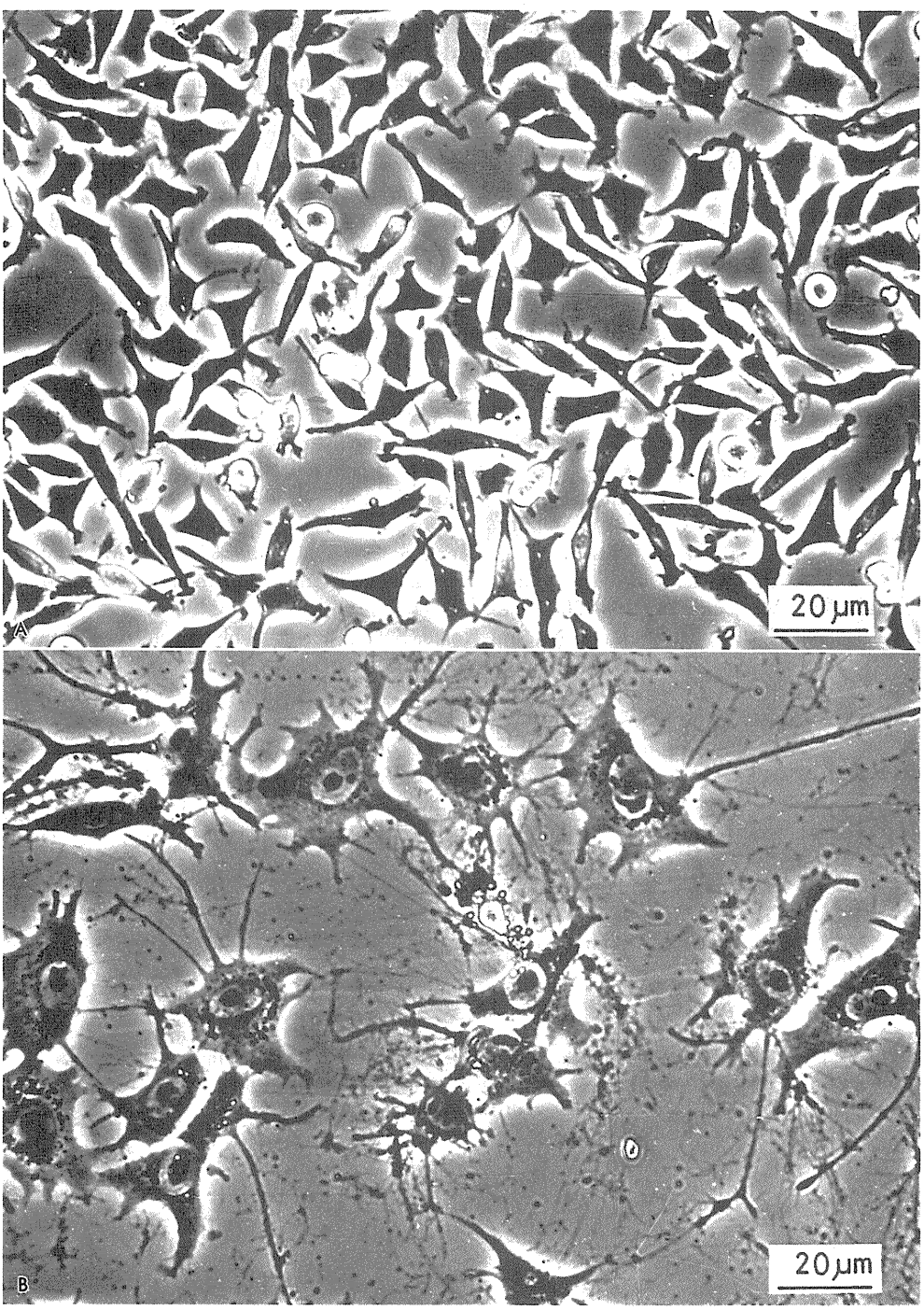
## EXPLANATION OF PHOTO

PHOTO 1. Morphological change of mouse L5 cells after BLM treatment
A: Untreated cells grown in F10HI medium supplemented with 5% calf serum. B: Cells 36 hr after treatment with 100 μg/ml of BLM for 1 hr.


20 µm
A
20 µm
B

原　著

# ブレオマイシンとマイトマイシンの連続的併用（B-M）療法による末期子宮頸癌の治療成績について

放射線医学総合研究所・病院部
宮本　忠昭*

千葉大学第一内科
高部　吉庸**　渡辺　道典**

放射線医学総合研究所・生理病理部
寺島　東洋三***

**要旨**　肺転移を主体とする末期子宮頸癌に対し，Bleomycin と Mitomycin-C の連続的併用（B-M）療法を施し，80％に完全寛解という著効を得た。この療法のスケジュール，寛解の仕方および維持療法等治療成績について報告する。

## はじめに

Ⅰ期，Ⅱ期の子宮頸癌は手術でも，放射線でも癒し易い癌とされる。さらにⅢ期，Ⅳ期に進展しても骨盤内に限局していれば放射線により30％近い5年治癒率が得られる[1]。再発癌はより難治であるが，範囲が限局していれば同療法により20％近い5年治癒率が得られる[2]。しかし，この癌もひとたび放射線治療の適応範囲を越えて広がると，極端に治療成績が低下し，遠隔転移を伴う症例はすべてが2年以内[1]か3年以内[3]に死亡している。その内，文献より著者が集めた肺転移を含む遠隔転移群28例の肺転移発見後の平均生存率は約100日であり，一年以上生存する例はほとんどなかった。放射線による姑息的治療が効を奏する骨転移を主体とする群も全例が一年半以内に死亡している[4]。このような段階まで進行した子宮頸癌を仮に末期（late stage）とすれば，これは予後において，肺癌．胃癌等の進行癌と大差はないといえる。

末期子宮頸癌の根治療法は理論的には化学療法をおいて他にない。このような認識にもとづいて種々の抗癌剤が，その開発の歴史とともに試みられた。単剤では，Cyclophosphamide, Methotrexate, Vincristine[5] 5-FU, 6-MP, Chlorambucil, Mitomycin[6] 等が次々と試みられたが良くて20～30％の効果率という悲観的結果に終った。扁平上皮癌に特異的効果を示すといわれた Bleomycin が開発され，期待されたが単独では臨床的に意味のある反応は示さないとするもの[7]や，20％に効果があるとするもの[8]等，期待はずれの結果となった。また最近では，固型癌一般に広い感

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Drastic remission effect of a sequential combination of Bleomycin and Mitomycin-C (B-M) on advanced cervical cancer.

Tadaaki Miyamoto*
Yosinobu Takabe, Michinori Watanabe**
Toyozo Terasima***
*Division of Hospital, National Institute of Radiological Science.
**First Department of Medicine, Chiba University School of Medicine.
***Division of Physiology and Pathology, National Institute of Radiological Science.

癌と化学療法　4，273-291，1977

受性を持つ Adriamycin で治療した結果，5例中3例に効果があったと報告されている[9]。一方，これらの薬剤の併用も試みられた。Cyclophosphamide と Vinclistine の併用は，単剤それぞれより若干よい寛解率となり[10]，Adriamycin と Bleomycin の併用では Adriamycin の寛解率と同じであった[9]。また Bleomycin と Methotrexate の併用療法では，完全寛解は一例もなかったが，60％（12/20）に50％以上の体積縮小を示す例があり，この効果が平均7．5カ月にわたって維持されたという[6]。さらに他の進行癌と同様に多剤併用療法も試みられているが，その内，肺転移を主体とする43例の子宮頸癌に施行された太田等の提唱による METT，MFC，METVFC 療法の結果によると，最も効果率のよかった METVFC による著効率（腫瘍直径が50％以上縮小したもの）は33.3％に達し，縮小効果持続期間は平均で4カ月であり[11]，それに応じて平均生存率は無効例に比べて3～4カ月延長されたとされる[12]。このように，末期子宮頸癌に対する化学療法は，薬剤の開発とその組合せに，日進月歩のあとが見られるが，現在，なお末期の生存期間を大幅に延長するところまで行かず，他の進行固型癌とともに新しい抗癌剤の開発を待っている状態である。

臨床がこのような歴史を歩んでいる一方で，癌の化学療法の基礎理論は，組織培養法の技術の進歩と細胞周期の知識の拡大によって，細胞致死作用等の諸種のカイネテックスを中心に，治療のデザインを組みたてる基本的情報を豊富に提供してきた。Bleomycin（BLM）は，市川等[13]により，臨床的研究が発表されて以来，15mg/日で週2回の投与方法が定式化されてきた。しかし，1971年以来，主として組織培養細胞で，BLM の Dose Response Curve（DRC）が求められ，2相性の不活化様式を示すことが明らかにされており[14,15]，これにもとづいてより効果的な投与方法が提案されてきた。すなわち，高濃度を一度に投与するよりも2相性の DRC の変曲点以下の濃度を分割して投与する方が，同じ総投与量でより大きな効果が期待できることである。特に，BLM 感受性腫瘍におけるこの変曲点は低く，動物腫瘍でも，人癌でも 0.1mg/kg とされている[16,17]。この値は50kgr の人間では5 mg に相当する。BLM による Time Inactivation Curve（TIC）によると，培養細胞は BLM との接触とともに抵抗性を獲得し，2相性となる。しかし，この抵抗性は，BLM をとり除くと崩壊し，接触前の感受性をとりもどす[15]。この事実より，分割して投与する方がより一層効率よく細胞を殺すことがわかった[18]。しかし，*in vivo* 腫瘍では，BLM 投与後，一旦生じた細胞死は数時間以内に回復される。すなわち Potentially Lethal Damage（PLD）の repair を起すことが発見された[9]。この結果，BLMにより誘導された抵抗性による細胞致死効果率の低下を分割投与によって防ぐより，BLM 投与後に発動する PLD の回復を持続投与法によって防ぐ方がより大きな細胞致死率をあげることが明らかになった[17]。Mitomycin-C（MMC）は，臨床的に小量頻回がよいか，大量間歇がよいか長く議論されてきた[20]が，最近では大量間歇投与に向っている。MMC の DRC からすると一定の shoulder を持つことから[21,22]，比較的大量を1回に投与する方が効率よく細胞死を起す。人間における MMC 投与後の血清濃度変化と血液障害[23]からの回復動態[24]を加味して1回8 mg から16mg を2～3週おきに投与することが適当と判断される。以上の基礎的データにもとづいて，著者は実際の BLM の投与法としては，15mg. 週2回投与より5 mg・毎日投与の方がよく，その投与方法も点滴に入れて持続的に注入すべきであると考えている。一方，MMC は，体重，血液障害の有無を考慮して若干投与量を工夫するが，原則として10mg を管注又は静注する方法を採用している。

## 治療対象

対象は昭和49年7月以降，放射線医学総合研究所・病院部において BLM と MMC の連続的併用（B-M）療法をうけた肺転移を主体とする末期子宮頸癌15例と肺・肝・骨に多発転移を起した子宮体部癌の1例の計16症例である。症例2を除くすべての例は stage Ⅱ か Ⅲ に属し，根治的放射線療法を受けている。症例2は，照射時すでに多発肺転移を併発し，stage Ⅳ とされた。また，転移発生時，原発の局所再発を伴っていたのは症例

6のみである。組織は症例16の腺癌（子宮体部癌）を除いて，すべて非角化性の扁平上皮癌であった。

## 化学療法

1)．寛解導入療法

薬剤の投与方法は，ブレオマイシン（日本化薬）5mg を1週間点滴静注する。その翌日，マイトマイシン-C（協和発酵）10mgを20％ブドウ糖に溶解して静注する。これを1コースとして，1週間の休薬期間を設け，これを5コース行なうのを原則とした（図1）。症例1と2の投与方法は原則と多少異なるが，これはこの投与法を確立するための予備的な試みとして行われたためである。以降の症例においても実際に施行したコースの回数は，症例の肺所見，血液所見に応じ副作用を考慮して減じてある。症例8は静脈の確保が困難なため BLM の投与はすべて筋注で行った。また，症例11～16例は，血流改善を目的としてウロナーゼ6,000単位から18,000単位を点滴に入れた。

Schedule of B-M Therapy

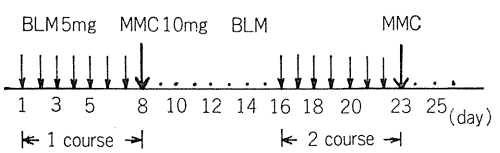



図 1.

2)．強化療法

強化療法は，症例1と他の4例の再発例に対して行われた。方法は表2に示すように，B-M コース，MMC の追加，また再発巣が限局しているときは放射線療法を追加した。実際には，これらの療法を同時にあるいは連続的に併用した。

3)．維持療法

維持療法は寛解導入後の再発防止を目的として，カルボコン(C. Q) 0.5mg を毎日，朝夕2回に分けて服用させ，6カ月間続けることを目標とした。

4)．腫瘍効果の評価と定量的解析の方法

a)．完全寛解（Complete Remission: CR）は，腫瘍による自覚，他覚症状の完全消失，および，触知しうる腫瘍，X線写真，シンチグラムに写った腫瘍体積が90％以上縮小したものとした。部分寛解（Partitial Remission: PR）は，完全寛解の条件の内，どの一つでも満足しないものとした。腫瘍体積の減少が25％以下のものは無寛解（No Remission: NR）とした。

b)．B-M 療法による腫瘍縮小効果を定量化するため，症例1～9の肺転移腫瘍を対象として，治療開始前，治療中および治療終了後の腫瘍体積を経時的に計測し，治療前値に対する比較体積値の変化として，症例ごとに腫瘍縮小曲線を求めた。胸部X線写真（単純および断層）上より，球形の腫瘍は，腫瘍の2方向の腫瘍直径を計り，球体として体積を算出した[25]。不整形の腫瘍は，その形を厚紙に写しとり，その重さを計り，3/2乗をかけて腫瘍体積を比較する基数とした。

## 成　績

当療法の臨床成績を総めて表1と表2に示した。

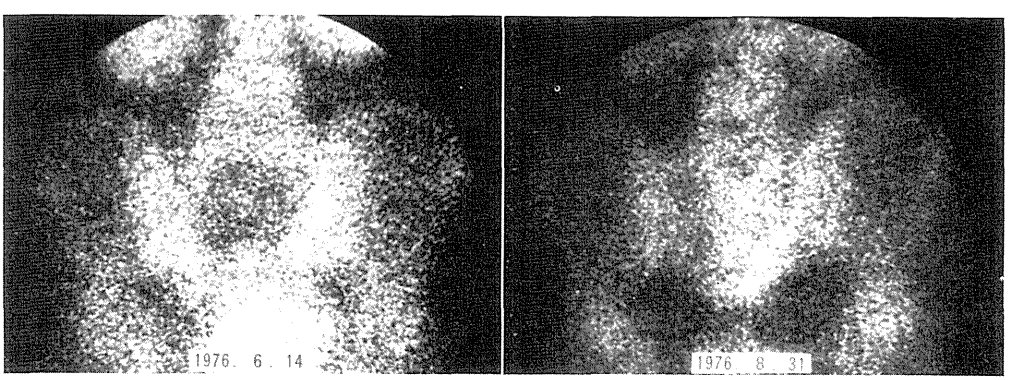
1)．腫瘍効果について

子宮頸癌（扁平上皮癌）は15例中12例（80％）が完全寛解，2例（13％）が部分寛解，1例（7％）が無寛解であった。寛解例の自覚，他覚症状の消失を総めてみると表3となる。腫瘍にもとづくこれらの症状は，すべて3コース以内に，平均すると2コース以内に消失した。ことに症例12は，神経麻痺の回復と仙骨の石灰化という劇的寛解効果を示したので，この症例の治療経過を紹介する。症例は40才の主婦である。昭和50年9月，性器出血があり，10月に子宮頸癌Ⅲ期と診断された。すでに手術の適応なく，同年11月から12月にかけて放射線療法をうけた。照射による反応は腫瘍の大きさの割によく予後良好と判定されたが，翌51年3月より腰痛が出現し，次第に増強し，6月に入ると下肢痛および下肢の知覚鈍麻が加わり歩行が出来なくなった。同月入院したときは尿，便失禁が加わり，骨盤写真で，第5腰椎と仙骨が完全に破壊されていた。骨スキャン像（写真12a）を見ると仙骨部は全く $^{32}$Pのとり込みがない。入院後，直ちに同部に照射が開始され4,000radまで達し若干の疼痛の緩和を見たが，麻痺は全く改善されずB-M療法の適応となった。当療法が1コース終ると，疼痛は激減し，2コース終了後は，尿・便

表 1

| Patient | Age | Metastatic sites | First course of treatment | | | | | Interval between 1st & 2nd course (month) |
|---|---|---|---|---|---|---|---|---|
| | | | Induction treatment | | | Length of CR (month) | Relapse | |
| | | | Regimen | Dose (mg) | | Response | | |
| | | | | BLM | MMC | | | |
| 1. Y.I. | 64 | lung | B−M+M+M+… | 70 | 50 | CR | | − | 2 |
| 2. A.N. | 69 | lungs | B−M+M+M+… | 30 | 26 | CR | 4 | + | 4 |
| 3. S.O. | 43 | lung | B−M+B−M+… | 175 | 50 | CR | 5 | + | 6 |
| 4. Ki.K. | 39 | lung | B−M+B−M+… | 175 | 50 | CR | | − | 3 |
| 5. A.K. | 55 | lung, mediastinum abdominal node | B−M+B−M | 70 | 20 | CR | 2 | + | |
| 6. N.S. | 53 | lungs, (local relapse) | B−M+B−M+… | 140 | 40 | CR | | | |
| 7. Ka.K. | 53 | lungs, bones | B−M+B−M+… | 140 | 40 | CR | 2 | + | 1 |
| 8. Y.M. | 49 | lungs, liver, bones | B−M+B−M+… | 175 | 50 | CR | | | |
| 9. I.U. | 43 | lungs, inguinal node | B−M+B−M+… | 105 | 40 | CR | > 5 | | |
| 10. S.Y. | 73 | pelvic and abdominal nodes, bone | B−M+B−M (Radiotherapy) | 70 | 20 | CR | | | |
| 11. M.I. | 51 | liver, bone, pelvic node | B−M+B−M+… | 105 | 30 | PR | | + | 1 |
| 12. K.Y. | 41 | pelvic and abdominal nodes, sacrar bone | B−M+B−M+… (Radiotherapy) | 105 | 30 | CR | | | |
| 13. H.K. | 43 | neck node, lungs | B−M+B−M | 70 | 20 | NR | | | |
| 14. T.N. | 67 | bone, liver, lungs, skin, neck node | B−M+B−M+… | 130 | 40 | PR | | | |
| 15. T.B. | 43 | bone, skin, neck and inguinal nodes | B−M+B−M+… | 140 | 40 | CR | | | |
| 16. F.S. | 53 | bone, lung, liver | B−M+B−M | 70 | 20 | NR | | | |

CR：完全寛解、PR：部分寛解、NR：無寛解



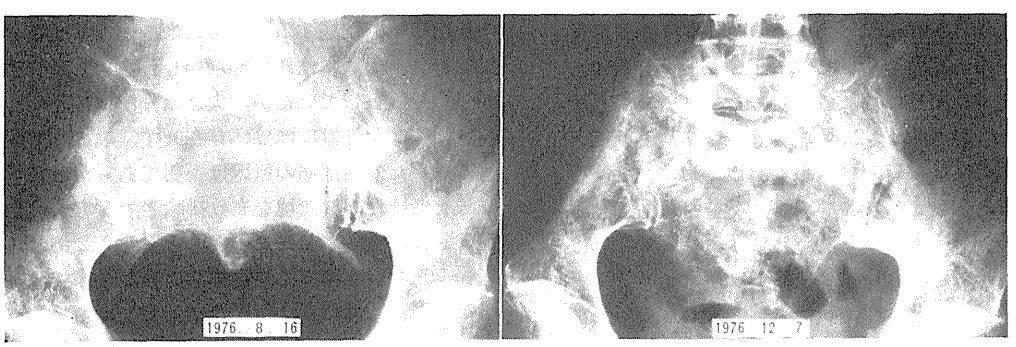

a　　b

写真 12　(症例12)

表 2

| Patient | Second course of treatment | | | | | | Survival (month) | Cause of death |
|---|---|---|---|---|---|---|---|---|
| | Regimen | Consolidation | | | Maintenance | | | |
| | | Dose (mg) | | Radiotherapy (rad) | Duration (month) | Total dose of CQ (mg) | | |
| | | BLM | MMC | | | | | |
| 1 | M＋M | | 20 | | | | ＞ 30 | |
| 2 | B－M＋M＋M | 17.5 | 18 | | | | 13 | relapse |
| 3 | B－M | 35 | 10 | 4000 | 4.5 | 67.5 | ＞ 20 | |
| 4 | | | | | 6.0 | 90 | ＞ 18 | |
| 5 | | | | | | | 4 | oesophageal perforation |
| 6 | | | | | | | 3 | lung fibrosis |
| 7 | B－M | 35 | 10 | 4000 | 4.0 | 32 | 7 | relapse |
| 8 | | | | | 2.0 | 30 | ＞ 11 | |
| 9 | | | | | | | ＞ 5 | |
| 10 | | | | | 5.0 | 75 | ＞ 12 | |
| 11 | | | | | 0.5 | 8.5 | 4 | necrosis of liver meta, relapse |
| 12 | | | | | ＞ 3.0 | ＞ 45 | ＞ 5 | |
| 13 | | | | | | FT－207 CQ | ＞ 5 | |
| 14 | | | | | | | ＞ 4 | |
| 15 | | | | | | | ＞ 3 | |
| 16 | | | | | | FT－207 CQ | 5 | progression |

CQ：カルボコン（カルバジノキノン）、FT－207：フトラフール




c　　d

写真 12　（症例12）

表 3

| Patient (CR) | Symptom and signs before treatment | No. of B-M course until the symptom and sign disappear |
|---|---|---|
| 1 | cough | 1 |
| 2 | cough | 1 |
| 3 | bloody sputum, cough | 2 |
| 4 | mild dyspnea, | 2 |
| 5 | cough, dysphagia, lumbago | 2 |
| 6 | cough, dyspnea | 1 − 2 |
| 7 | bloody sputum, cough, bone pain | 2 − 3 |
| 8 | cough | 1 |
| 9 | cough | 1 |
| 10 | lumbago | 2 |
| 12 | paralysis of legs incontinece | 3 |
| | leg pain, lumbago | 2 |
| 14 | lumbago, chest pain | 2 |
| 15 | leg and thigh pain | 2 − 3 |
| | average | 2 |

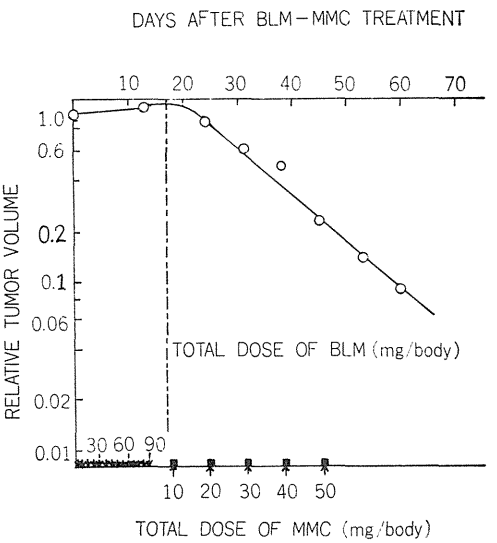



図 2 （症例1）

失禁がとれ，3コース終了後は遂に下肢の知覚鈍麻もなくなり，つえを使って歩行が出来るようになった。4コース終了後の骨スキャン像（写真12b）では，仙骨部は周囲の骨盤と同様にアイソトープをとり込んでいた。しかし，この時点では同部の石灰化の所見（写真12c）は見られなかった。9月に入り，コルセットを着用し，カルボコンを服用しつつ退院した。12月現在，骨盤写真上，明らかに化骨が起り（写真12d），元気で家事に勤んでいる。完全寛解の有様を示すため，症例1から9までの胸部写真，骨写真，シンチグラムを供覧する。（写真1，2，3，4，5，6，7，8，9），部分寛解は症例11と14である。症例14は左脇腹皮下の巨大転移と，肝，肺，骨，リンパ腺をまきこむ全身転移である。3コース終了後，巨大転移は消失したが，胸部写真上，肝・骨スキャン上，腫瘍が残存し，リンパ腺転移巣も触知しうるので部分寛解とした。症例11は，55才の主婦で，右外陰部，大腿内側部痛と季肋部痛を主訴として入院した再発癌である。スキャンの結果，右坐骨と肝への巨大転移が明らかになった。B-M療法を3コース施行すると，右季肋部痛は消失したが外陰部痛は残った。肝シンチグラム上腫瘍陰影の縮小は起らなかったが，形体は著しく変形した。この頃から39°台に達する原因不明の間歇熱が出没し，患者の消耗が著しくなった。再び右季肋部痛を伴うようになったので，肝転移壊死巣の膿瘍化を疑い肝穿刺を行い，ドレナージを行った。排泄物は化膿しておらず，細胞診で壊死塊，肝細胞，白血球が検出されたが悪性細胞はなかった。その結果，下熱したが，ドレーンがつまると発熱し，これをくり返えすうちに右胸水が出現し，心不全を起して死亡した。剖検すると肝実質の8割近くが壊死塊であり，周辺には新しく増殖したと思われる小転移巣が散在していたが，大きな癌塊は見当らなかった。また骨盤には癌は存在せず，最後まで頑固に残った外陰部痛の原因は不明であった。化学療法を継続し，壊死塊を充分排除させる方法が工夫できれば延命させ得た症例であった。2例の無寛解のうち，症例16は子宮体部由来の腺癌であり，他の一例（症例13）は，原発は扁平上皮癌である。共にB-M2コースを施行したが，腫瘍の成長は一時止まったのみであり，MMC単独の効果と判定して，FT-207を含む他の療法に変更した。

2). 再発と強化療法

完全寛解後の再発は4例ある。症例2は寛解導

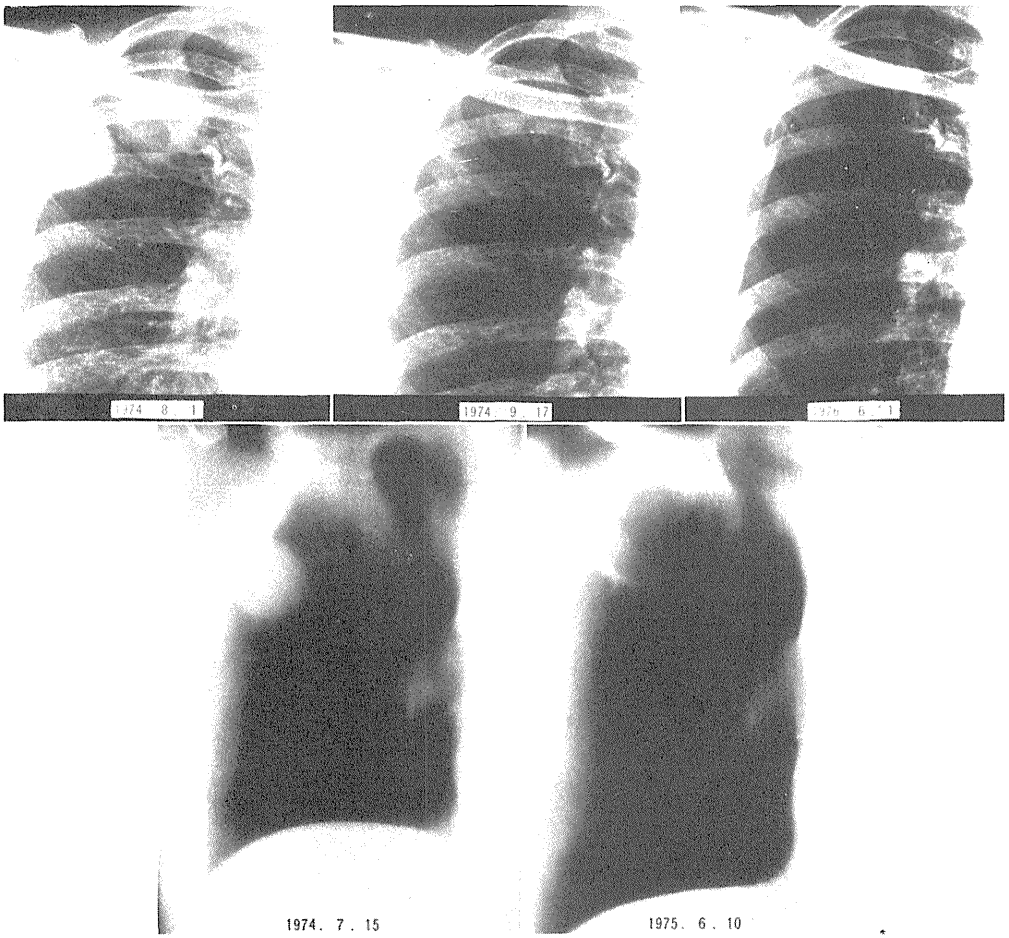



写真 1 （症例 1）

入後，4カ月のちに再発した。再発時は，坐骨転移による鼠径部痛を伴った。BLM を2.5mg/日とMMC を6.0mg に減量してB-M療法を1コース行い，続いてMMC を6mg 2週おきに追加すると鼠径部痛は消失し，初回と同様に肺転移腫瘍も縮小した。その縮小の有様を図3の右図に示した。この図より，90%腫瘍縮小を起すMMCの量を求め初回治療のそれと比較するとレスポンスは同程度であった。しかし，BLM による薬疹が強く，当療法の継続が不可能になった。結局，乳び腹水が生じ全身衰弱で死亡した。症例3は寛解導入後，約5カ月して再発の徴が見られたので，さらに1コースを追加し，続いてリニヤックで4,000radを照射した。その後，維持療法としてカルボコンを4カ月服用させた。治療開始後，18カ月経た現在，再発なく生存している。症例5は，原発に照射後3年，ウイルヒョウ，食道を巻き込む縦隔転移を次々と起し，その都度，照射を行い切り抜けたが，遂に肺転移を生じB-M療法の適応となった。当療法を2コース施行すると肺腫瘍は消失（写真5 a. b. c）し，激しい腰痛，嚥下障害もとれた。しかし，血小板減少と食道潰瘍の誘発によりコースの継続を見合せている間に再発の徴が現われた。以上3症例は，コースの回数の不足や維持療法を行わなかったための再発であり，再発癌もB-M療法に感受性であった。3症例の平均寛解持続期間は約4，5カ月である。これらの再発に対して症例7は，肺転移，骨転移（写真7）が4コース施行後，一旦，消失あるいは瘢痕化したあと，肺転移の十数ケの転移巣の内より，3ケの腫瘍の増殖

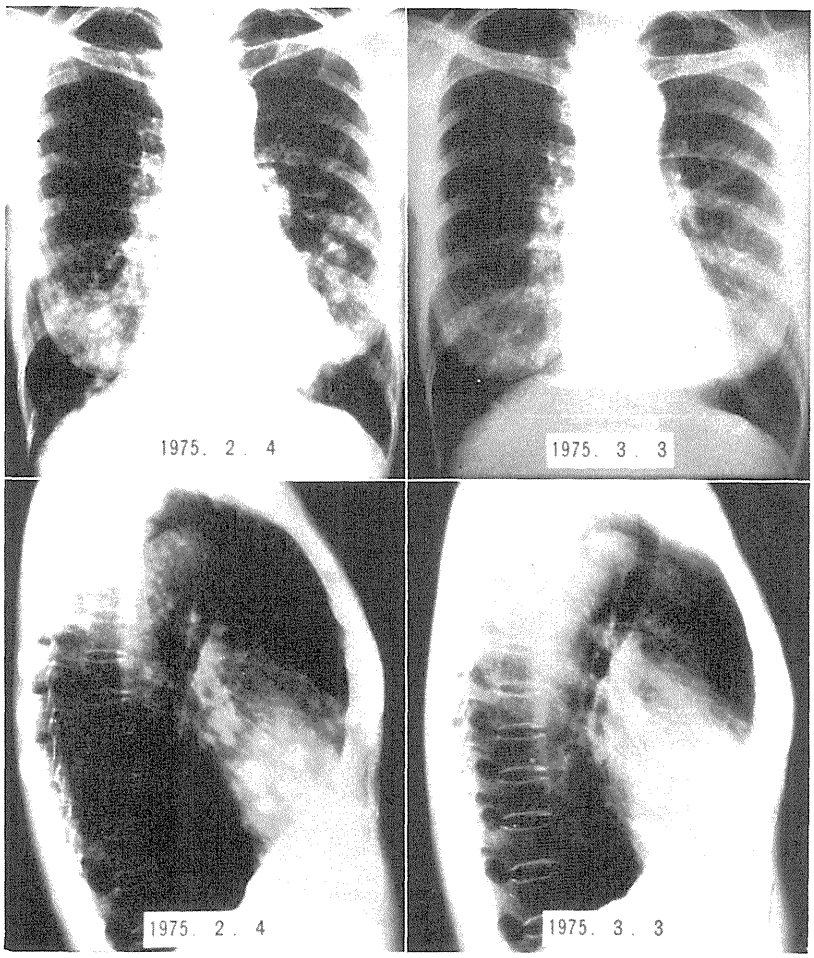



写真 2 （症例2）

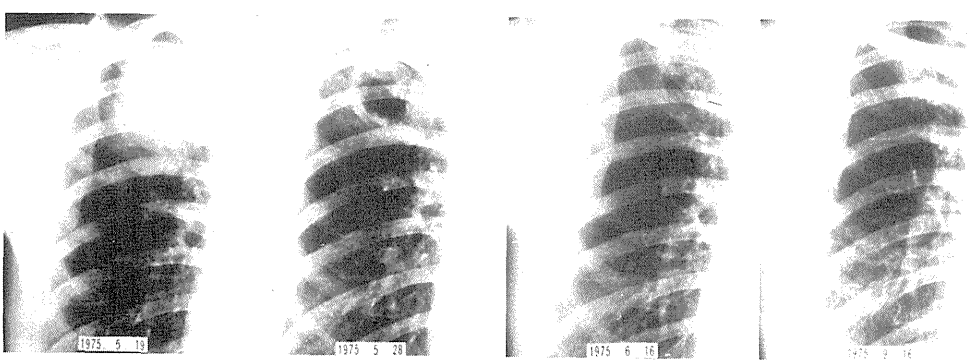



写真 3 （症例3）

が5クール目を施行中に生じた。これは，明らかにB-M療法に耐性の腫瘍の出現であった。当患者はそののち，照射，カルボコンの追加等行ったが，この再発をえ抑きれず死亡した。

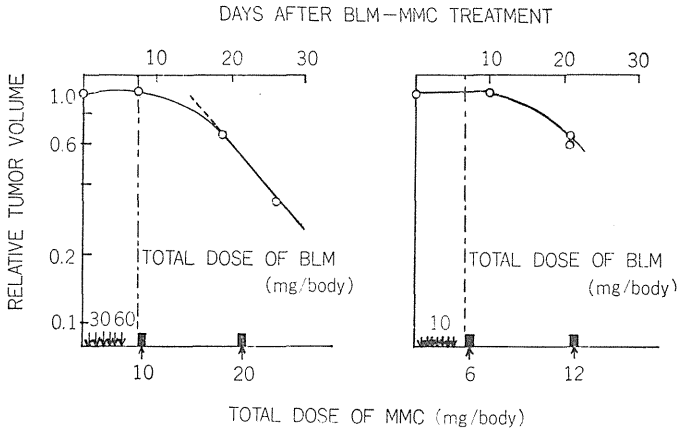



図 3 （症例2）　左図　初回治療，右図　再発治療

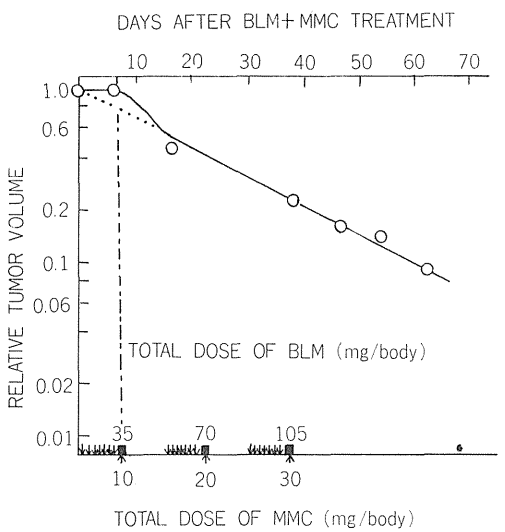



図 4 （症例3）

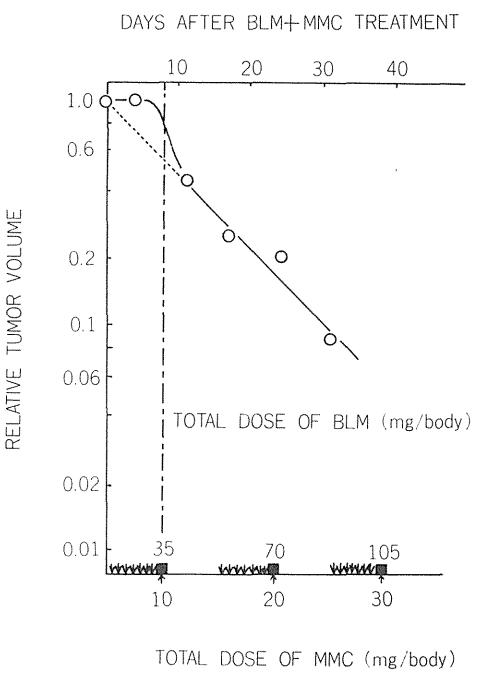



図 5 （症例4）

3）．維持療法

カルボコンによる維持療法は，完全寛解導入後の再発の経験にもとづき，これを防ぐ目的で開始された。維持療法を行った，あるいは行っている患者は7例である。この内，維持療法を終えた症例は5例で，目的どおり6カ月間，問題なく服用し終ったのはたった一例であった。他の4例は，肺

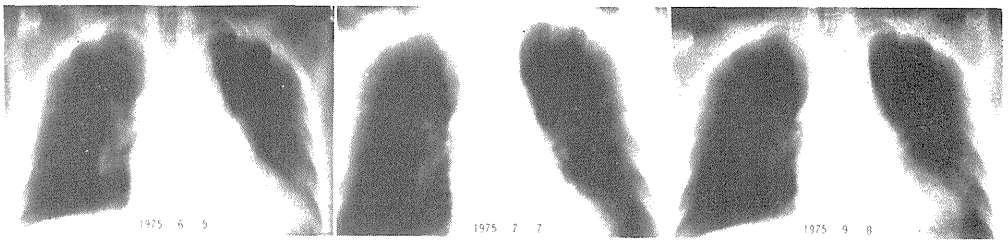


写真 4 （症例 4）

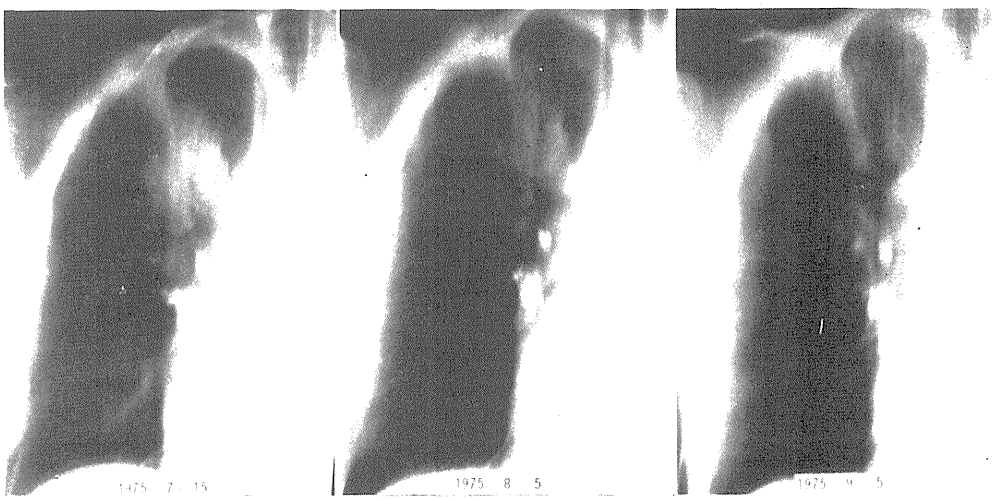


写真 5 （症例 5）左から a b c

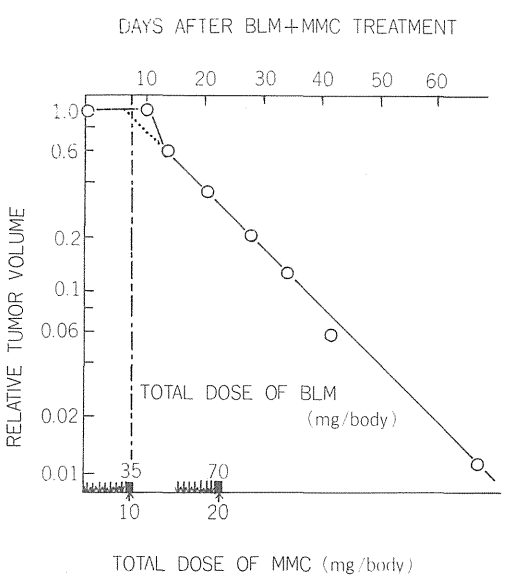



図 6 （症例 5）

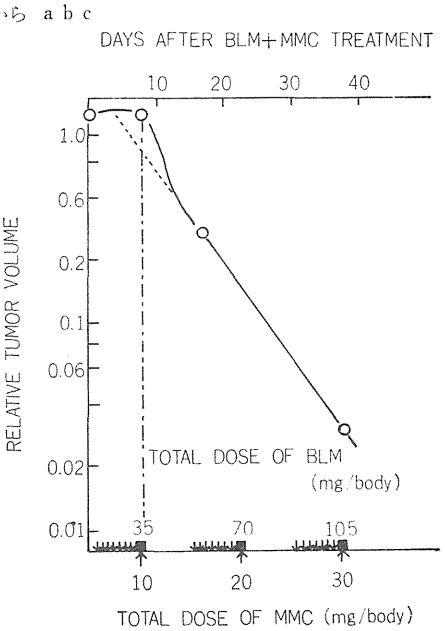



図 7 （症例 6）

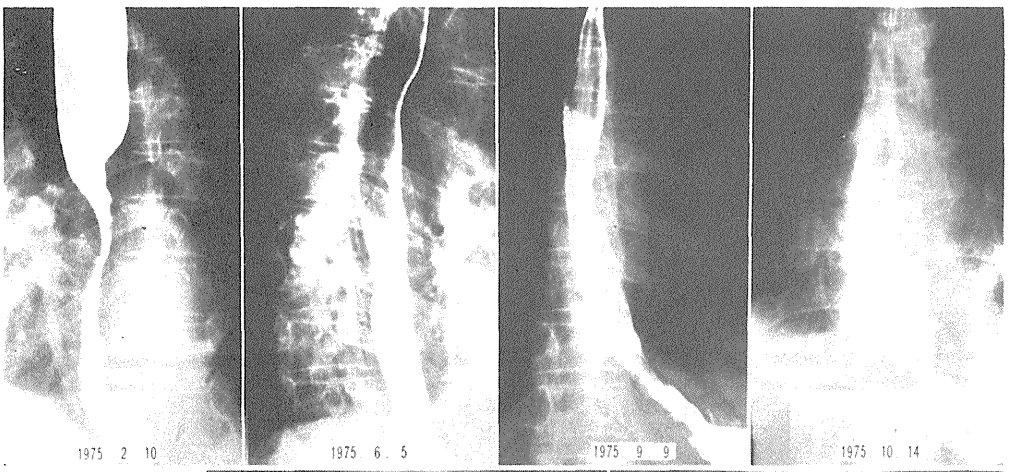
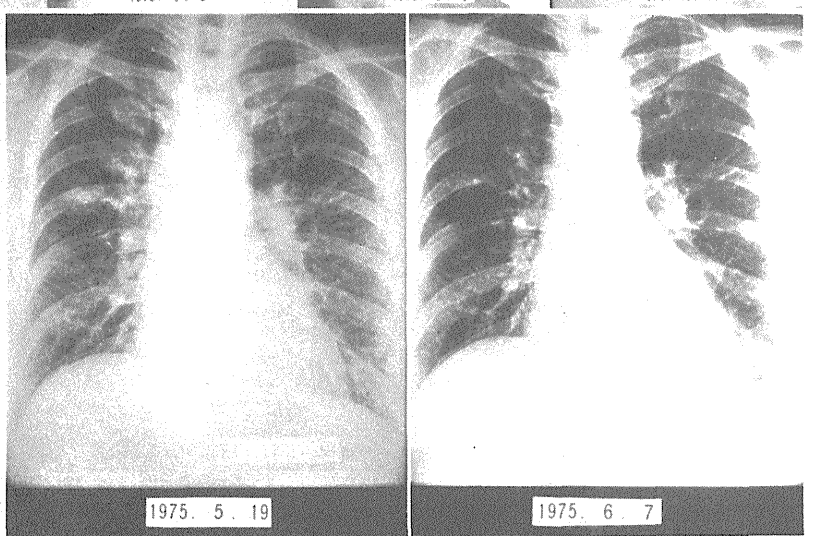



写真 5　(症例5)
左からｄ ｅ ｆ ｇ



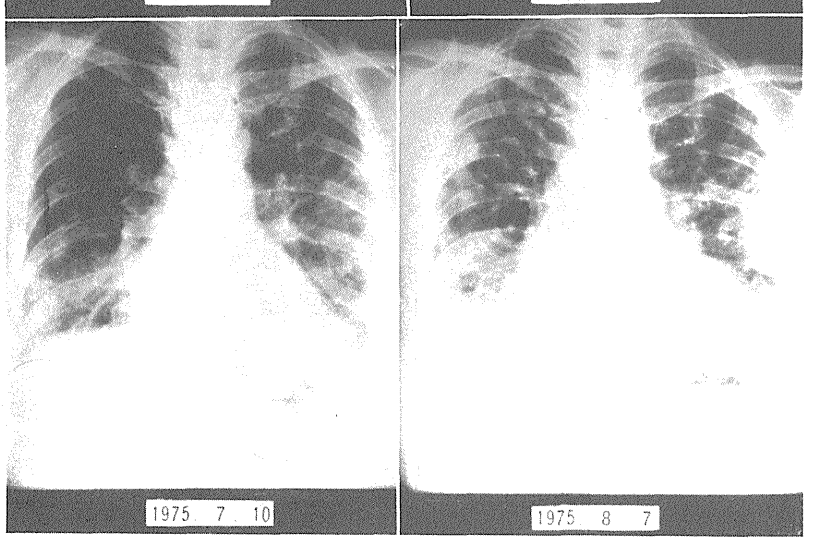
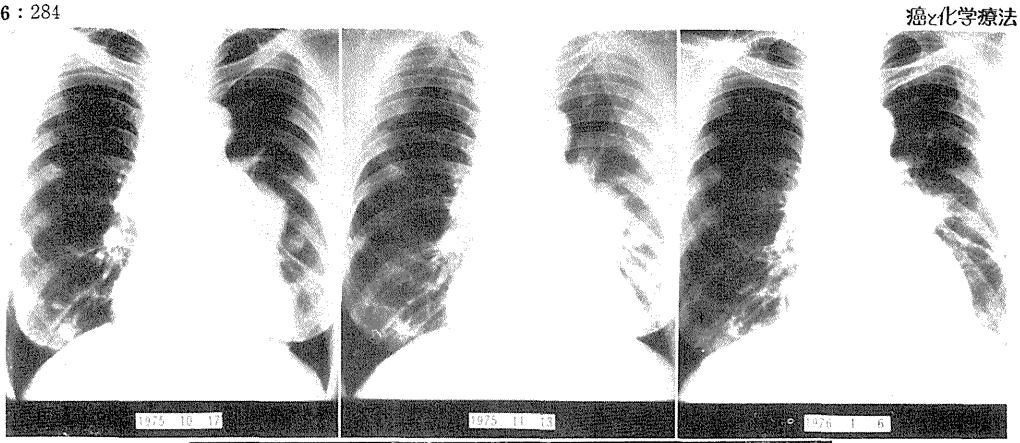
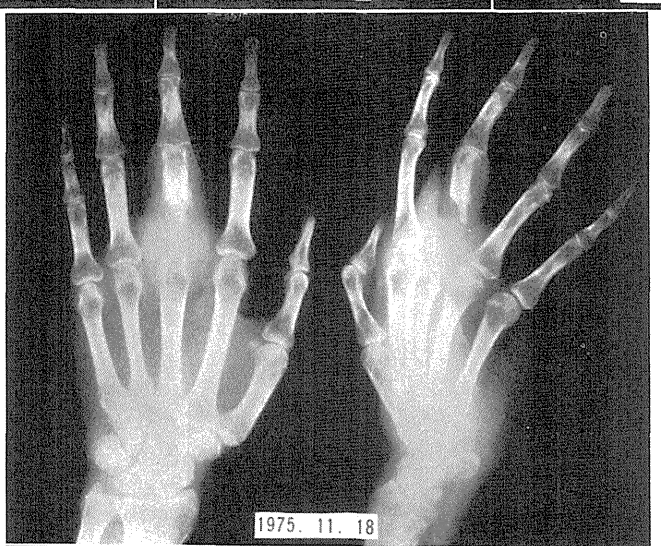




写真 6　(症例6)
上段左：a　右：b
下段左：c　右：d





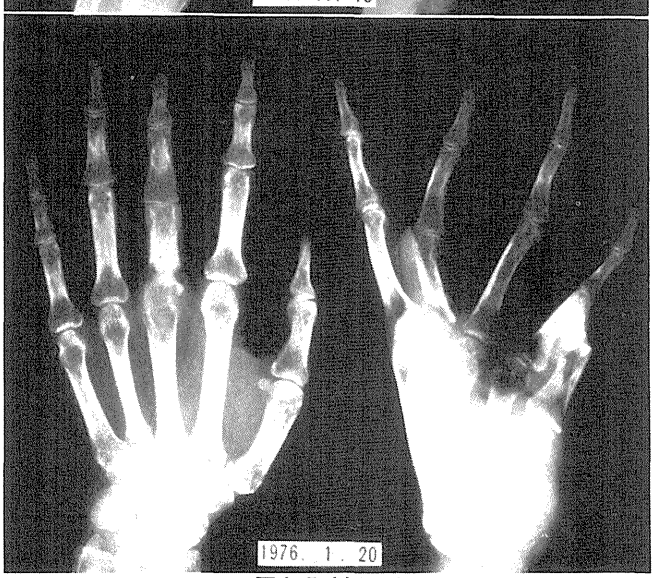




写真 7（症例 7）

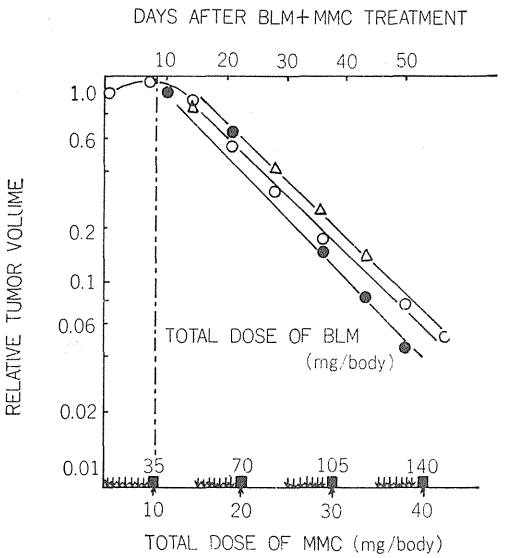



図 8（症例7）

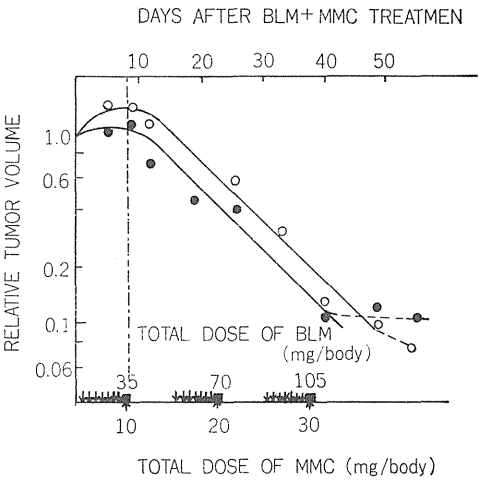



図 9（症例8）

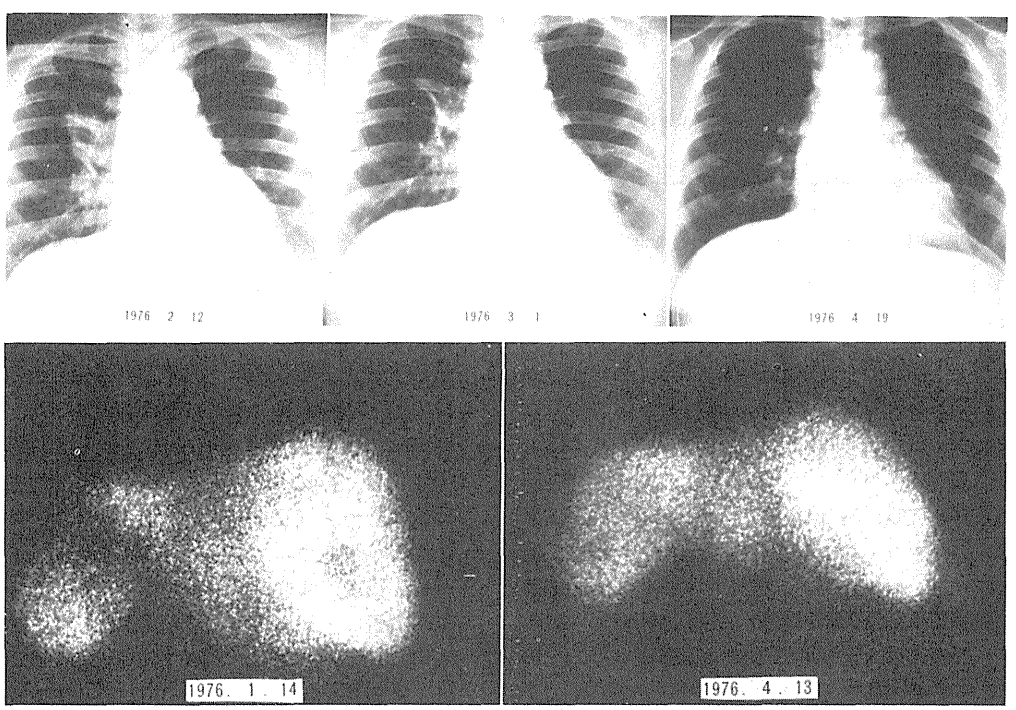



写真 8（症例8）

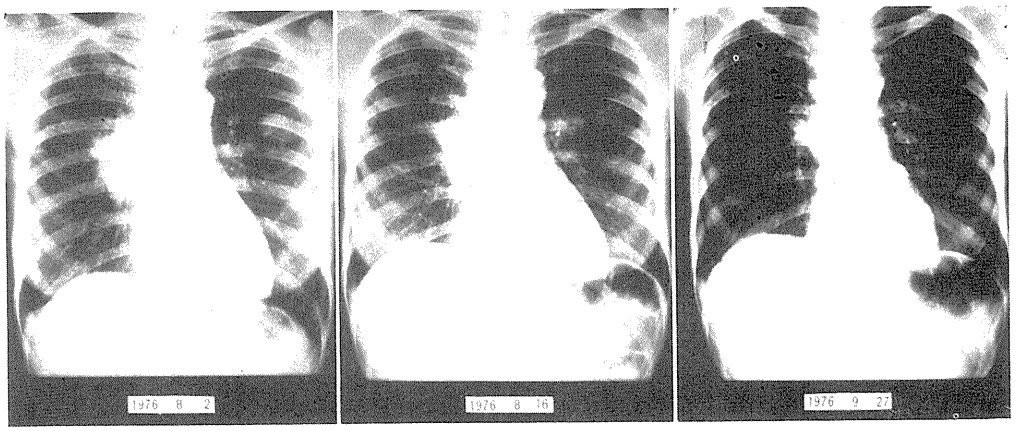



写真 9　（症例９）

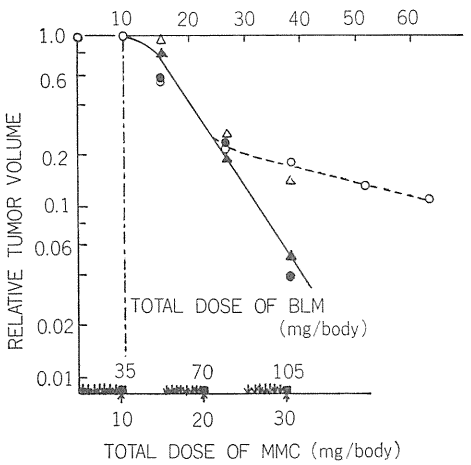



図 10　（症例９）

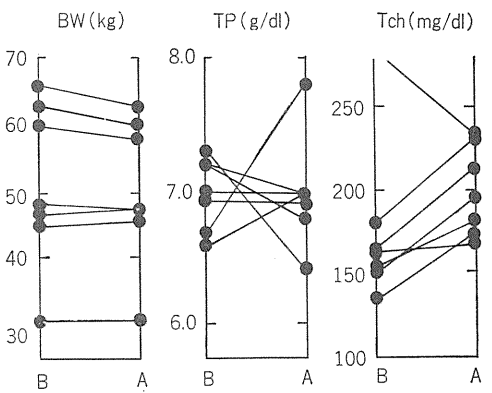



図 11　B：コース施行前，A：４コース施行後，BW：体重，TP：総蛋白，Tch：総コレステロール

線維症の続発，重篤な帯状疱疹の合併，胆のう炎の発生，高度の貧血等の理由で，カルボコンの継続服用を中止した。しかし，維持療法を行った症例の中から一例の再発もなく全例が生存している。

4)．副作用

寛解導入時，B-M療法が一般状態に与える影響を，４コース以上施行した７症例について，前後の体重，血清総蛋白，総コレステロールの変動で評価してみた。（図11）これらの指数でみるかぎり，全身の栄養状態に与える影響は少ない，BLM に由来すると思われる副作用（表４）は，平均総投与量が120mg と比較的少量のため，全体に軽度である。しかし，２例に肺線維症が出現した。

１例は寛解導入時に出現した症例６である。当例は55才の女性であり，局所の再発と咳嗽・呼吸困難を主訴とする癌性リンパ管炎（写真６ａ）で入院した。局所再発巣に対しては照射を併用しつつ，肺転移にB-M療法を施行した。１コース施行後，症状は全くとれ，２コース施行中に肺野の転移巣は消失した。（写真６ｂ）しかし，３コース施行中に線維化の傾向が見られ（写真６ｃ），４コース終了後は，肺野は蜂窩状となり，高熱を発し，呼吸困難にて死亡した。他の１例は症例８である。当例は49才の女性で，入院時，肺，骨，肝（写真８）に転移を起していた。B-M療法５コース施行，完全寛解に入ったので，カルボコンを服用させ退院させた。退院後，２カ月して突然肺機能が

表 4 BLEOMYCIN TOXICITY

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | Incidence (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total dose of bleomycin (mg) | 70 | 30 | 175 | 175 | 70 | 140 | 175 | 175 | 105 | 70 | 105 | 140 | 70 | 130 | 105 | 70 | Average dose 120 |
| fever > 38°C | + | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | 13 |
| anorexia, neusea | − | − | − | − | − | + | − | ‡ | − | − | − | − | − | − | − | − | 13 |
| fatigue | + | − | + | + | − | + | − | + | + | − | − | − | − | − | − | − | 33 |
| stomatitis | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | 6 |
| piqmentation (skin and nail) | − | − | − | + | − | + | − | + | − | − | − | − | − | − | − | − | 20 |
| alopecia | − | − | − | − | − | − | ‡ | − | ‡ | ‡ | − | − | − | − | − | − | 20 |
| lung fibrosis | − | − | − | − | − | ‡ | − | ‡ | − | − | − | − | − | − | − | − | 13 |
| hepatotoxicity | − | + | − | − | − | − | − | − | + | − | − | − | − | − | − | − | 6 |
| proteinuria | − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | 6 |
| drug eruption | − | ‡ | − | − | − | − | − | − | − | − | − | − | − | − | − | − | 6 |

* + mild ‡ severe

表 5. MITOMYCIN-C TOXICITY

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | Incidence (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total dose of MMC (mg) | 50 | 26 | 50 | 50 | 20 | 40 | 50 | 50 | 40 | 20 | 30 | 40 | 20 | 30 | 20 | 20 | Avverage dose 37 |
| min. WBC ($mm^3$) | 1000 | 1700 | 3300 | 2600 | 1200 | 3200 | 2200 | 1900 | 5400 | 2500 | 7500 | 3300 | 3600 | 1900 | 5700 | 4100 | 33 (3100) |
| min. platlet ($\times 10^4/mm^3$) | 6.5 | 6.5 | 13.5 | 8.0 | 3.0 | 10.5 | 9.5 | 11.0 | 5.5 | 10.0 | 22.5 | 17.5 | 20.5 | 19.5 | 34.0 | 41.0 | 40 (16.0) |
| fatique | + | − | + | − | − | − | − | + | + | + | − | − | − | + | − | − | 33 |
| anorexia | + | − | + | − | − | − | − | − | + | + | − | − | − | − | − | − | 20 |
| proteinuria | − | − | − | − | − | − | − | − | + | + | − | − | − | − | − | − | 6 |
| hepatotoxiciity | − | − | − | − | ‡ | − | − | − | − | − | − | − | − | − | − | − | 6 |
| drug eruption | + | − | − | − | + | − | − | − | − | − | − | − | − | − | − | − | 13 |

* evaluated leucopenia <2000 ($mm^3$) thrombocytopenia <1.0×$10^5$ ($mm^3$)

** ± mild ‡ severe

低下し，胸部写真上淡い膜状陰影が出現した。直ちにカルボコンを中止させ，プレドニンを15mg/日で3週間服用させると肺陰影は消失し呼吸困難はとれた。続発した帯状疱疹によりカルボコンの継続はできなかったが，現在まで再発なく生存中である。当2症例の肺線維症は，その発現の時期，肺陰影像，程度等を異にするが，ともに皮フ，爪の着色，脱毛等の副作用が顕著であり，特に症例8はコース終了ごとにしま状の着色帯を爪につくった。MMC による副作用（表5）は，血液障害が主である。ことに，平均総投与量が37mg と中等量の割に，血小板が10万を割る患者が6例出現した。しかし，このためB-Mコースの続行を中断したのは症例5の1例のみであった。再発時の強化療法として行ったB-M療法は初回時に比べて全体として副作用が増強された。

維持療法として用いたカルボコンは1日投与量が0.5mgと少量のためか，血液障害によって中止する例は1例と少なかったが，2症例に服薬中止をせざるを得ぬ重症の帯状疱疹が現われた。以上の副作用以外に予知せぬものとして，当療法が食道の放射線潰瘍を誘発したと思われる，すでに紹介した症例5の食道の写真（写真 d. e. f. g）を供覧する。当症例の直接死因は再発ではなく，食道潰瘍の心のうへの穿孔による心外膜炎であった。

5)．延命効果

現在まで完全寛解12名のうち4名が死亡した。その原因は，肺線維症1例，再発が2例，食道穿孔による心外膜炎が1例である。部分寛解は2名の内1例が死亡した。その原因はすでに症例11として紹介した。無寛解のうち，腺癌の1例は癌性腹膜炎で死亡した。当療法は開始後，まだ3年を経ず生存率をうんぬんする段階でないが，すでに治療開始後，1年以上経て生存する患者が4名，2年以上の例が1名おり，今後，コントロール例に比べて大幅な生存の延長を期待できそうである。

## 考　按

すでに，内外の諸家の報告より，末期子宮頸癌に対する化学療法の治療成績を検討したが，B-M療法は明らかにこれらの治療成績を凌駕しつつある。

特に寛解導入時に見られた当療法の抗腫瘍効果の特徴は，①，扁平上皮癌に特異的感受性を示す。②，この特異性によって寛解率は，腺癌の1例を除けば93%（14/15）という高いものであり，その内，完全寛解率が80%（12/15）という完璧なものであった。さらに，③，この効果は，転移，浸潤臓器・組織を越えてほぼ平等に及ぶため，④，腫瘍による患者の自覚的，他覚的症状を完全にとり除き，患者を癌の苦痛より解放すると総められる。

この完全寛解効果を解析するため，症例1～9までの肺転移例より腫瘍縮小曲線を求め，各症例の写真のあとに添付した。この腫瘍縮小曲線を比較検討すると，B-M初回コースにおいてMMC投与後，急激に体積の縮小が起った症例3，4，5，6と徐々に縮小した症例1，2，7，8，9に分けることが出来る。

それ以降の縮小は全症例ともほぼ指数関数的に縮小した。90%体積縮小に要したMMCの投与量で求めたこれら腫瘍の縮小率と，初回コースに見られる二種類の体積縮小との間に特別の相関は見られなかった。これらの腫瘍縮小曲線より，治療開始後，腫瘍体積が90%まで縮小するまでに投与

表 6

90% VOLUME REDUCTION DOSE

| Case | BLEOMYCIN (mg) | MITOMYCIN-C (mg) |
|---|---|---|
| 1 | (70) | (50) |
| 2 | 30 | 20 |
| 3 | 120 | 35 |
| 4 | 80 | 20 |
| 5 | 70 | 15 |
| 6 | 70 | 20 |
| 7 | 60 | 13 |
| 8 | 70 | 20 |
| 9 | 70 | 20 |
| average | 67 ± 25 | 20 ± 6 |

されたBLMとMMCの投与総量を求めると表6となる。症例1はMMCの投与間隔が1週間であり，他の症例はすべて2週間である。スケジュールの短縮がそのまま腫瘍の縮小速度と相関しないので，症例1を除いて，BLMとMMCの平均投与量を求めると，それぞれ，(67±25) mgと(20±6) mgであった。特にMMCの投与量でみるかぎり，症例ごとの効果率の差は±30%以下であり，扁平上皮癌の子宮頸癌の完全寛解例は，どの症例でも非常に安定した効果を期待できることがわかった。症例2と5の再発癌の成長曲線を逆延長し，最終コースのMMC投与後の体積減少の絶対値を測定してみると，99%以上となり，1コースのB-M療法で90%以上の体積減少が起ることが判った。自験例の内，MMC単独で最もよい効果を示した症例の腫瘍縮小曲線より，90%体積縮小に要するMMCの量を求めると約200mgとなる。B-M療法では，BLM単独ではほとんど腫瘍縮小を示さないことから，MMCの力価は，BLMの前投与により約10倍に増強されたと計算される。一方，

寛解導入時，当療法のコースの回数を制限するものは，BLMによる肺線維症の発生とMMCによる白血球及び血小板の減少である。しかし，寛解導入時，BLMによる肺線維症は6％（1/16）であり，BLMの平均使用量120mgの割に高い発生率のようであるが，皮フ，爪の変化，脱毛等への注意と肺機能をチエックすることにより十分予知でき，また予防できるものと思われる。また，MMCによる骨髄障害は，総投与を増すにつれて確実に発現する。特に当症例においては血小板減少が多少増強されたように見えるが，これは当症例がすべて，全骨盤に3,000rad以上の照射歴のもつⅡ期，Ⅲ期以上の患者より成ることに由来すると思われる。従ってこの点を考慮に入れるとMMCによる骨髄抑制作用が特に増強されたとは考えられない。以上の副作用を検討した結果，B-M療法により10倍に上昇したMMCの力価は，そのまま10倍の治療効果比をもたらすことになる。とはいえ，寛解導入時，B-M療法のコースの数を確実に規定するものMMCはによる骨髄抑制作用であり，稀には肺線維症の発生や他の予知せぬ副作用であることは変りはない。

症例1は，B-M＋M＋M…のスケジュールで約6カ月間にMMC 70mgを投与し，30カ月以上再発なくほぼ完全に治癒したと思われる例である。

当症例を完全寛解例中の平均的感受性を示す腫瘍と仮定して，あえて治癒に要するMMCの総投与量を求めると，±30％の誤差を考慮に入れて，50～90mgの量と計算される。実際，カルボコンで維持療法を施行した症例はすべて再発なく生存している。症例8はカルボコンの投与量が最も少く，30mgであるが，寛解導入時にすでにMMCは50mg投与されており，計80mgとなる。カルボコンの力価がMMCより高いことを考慮するならすでに治癒量が投与されたものと考えられる。以上の検討より，当スケジュールにおける。B-M療法のより完成された治療計画を示すと，①，B-M2コースを終了した時点（治療開始後1カ月）で，当療法の感受性の有無を判定する。②，感受性のある症例は肺転移，皮フ・リンパ腺転移の縮小曲線を求め90％腫瘍縮小に要するMMCの投与量を算出する。MMCの量が20mg以下の場合は，MMCの投与総量が50～70mgになるように，20mg以上のときは70～90mg以上になるように計画し，寛解導入に当っては前者は2から3コース行い，後者は3から4コース施行し，コース終了後2～3週間あけてカルボコンによる維持療法に入る。この治療計画は副作用を出来るだけ軽減し，治癒を期待しうるものとして，当療法を再検討する目安として提案した。もちろん，原発性肺癌をはじめ他の扁平上皮癌ではさらに工夫を要することはいうまでもない。

当療法が扁平上皮癌に特異的であることはすでに述べたが，自験例において，原発性肺癌，食道癌，においても有効であった。頭頸部，皮フ癌においても恐らく有効であろう。このようなB-M療法の組織特異性は，扁平上皮癌に特異的感受性をもつBLMの作用に帰因すると思われる。現在の所，BLMの組織選択性は，腫瘍，臓器，組織の違い等によるこの薬剤のとり込み率及び不活化酵素含有の差に依存すると説明されている[26]。自験例の子宮体部癌及び他の腺癌に当療法は無効であったが，このような観点からすると，BLMのとり込み率が多く，不活化能力の低い肺とか，皮フ等に発生した腺癌，悪性黒色腫などにもレスポンスを示す可能性は否定できない。また，本来，BLMのみで著効を有するリンパ腺，睾丸腫瘍等はよりよい効果を示すであろう。しかし，すでに調査済みのように，BLM単剤による子宮頸癌のレスポンス率は良くとも20～30％であり，完全寛解を示す例は皆無とされることから，B-M療法でみられた高完全寛解率はBLMとMMCの特異な相互作用によって決まると考えられる。当症例において，初回コースに投与したブレオの投与期間は，最長の症例1が14日間であり，他のすべての症例が7日間で，総投与量で35mg～70mgなので，BLMのみの効果を検討するのは不十分な治療であるが，BLM単剤で明らかな客観的効果のみられた症例は1例もなかった。むしろ，BLM投与中に明らかに成長を続けた腫瘍が若干見られた。しかし，その後に追加したMMCの感受性が著しく高まることから，BLMが腫瘍に何等かの損傷を与えていることは明らかである。理論的には，BLMの潜存的致死効果（亜死）がMMCによって誘発さ

れたか，BLM による何等かの腫瘍損傷効果により MMC の感受性が高まったか，あるいはその双方が組合わさっていることが考えられる。先に検討したように，特に初回コース後に，特異的に腫瘍体積が激減する症例と徐々に減少する症例が見られたが，それ以降減少する指数関数的曲線を逆延長してみると，体積激減例は BLM 単独でも多少のレスポンスが存在していた可能性を推察させる。続く指数関数的体積減少の機序に関して次の4つの機作が考えられる。①，腫瘍支質血管に対する BLM と MMC の特異的障害作用[27]。②，非分裂細胞に対する BLM の特異作用と[28,29,30]$G_1$ 細胞に対する MMC の周期依存性致死作用の相乗作用[21),22)]。③，BLM の頻回投与による腫瘍細胞の $G_2$ block と $G_2$ 期への集積と[14,31,32,33] $G_2$ に特異的致死作用を有する MMC の周期依存性致死作用[21)]の相乗効果。④，BLM による DNA 等への損傷[33)]による MMC の攻撃点の増加等，両薬剤の細胞致死標的レベルでの相乗効果である。現在，このどの可能性についても否定も肯定も出来ない段階であるが，その相互作用を解く鍵が，次の二つの観察事項に存在することを指摘したい。1，90％体積縮小を起すに要する MMC と BLM の相関関係（図12）より，BLM と MMC の投与量の間に逆相関は成立しなかった。2，症例1と2の B-M＋M＋M＋…のスケジュールとそれ以外の症例の B-M＋B-M＋…のスケジュールとの間に腫瘍効果の上で大差はなかった。

以上，種々の面より B-M 療法について考察してきたが，この療法の寛解導入効果は抜群であり，この療法を主軸として局所療法としての手術，放射線療法を巧みに併用し，寛解導入後はさらに免疫療法をとり入れることによって，末期子宮頸癌をはじめ進行扁平上皮癌を治癒させることができる展望が生まれてきた。今後原発性肺癌・食道癌等で多くの臨床的実践をつみ重ねるとともに，この療法の背後に秘められている一般的法則を明らかにし，よりよいスケジュールを決定し，より強力な癌治療の武器に仕上げるとともに，幾多の新しい，強力な併用療法の開発の足がかりとしたいものである。

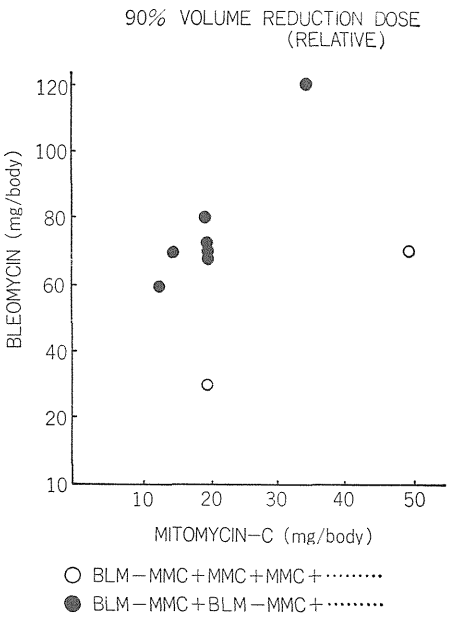



図12

稿を終るに当って，当症例の提供と治療に御協力下さった荒居竜男，森田新六両先生をはじめとする病院部医師，臨床研究部医師，および図表の作成に協力下さった松田和子，古屋正子さんに感謝いたします。

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*Short Communication*

# SYNERGISM OF X-RAYS AND BLEOMYCIN ON EHRLICH ASCITES TUMOUR CELLS

Y. TAKABE*, T. MIYAMOTO*, M. WATANABE* AND T. TERASIMA†

*From the *First Department of Medicine, Chiba University School of Medicine, Inohana, Chiba-si 280 and †Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, Chiba-si 280, Japan*

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COMBINED use of X-rays and bleomycin was initially proposed by Jørgensen (1972). The demonstration of synergism attracted much attention from clinicians as well as basic researchers. The question arose whether the effect can be attributed to a tissue mechanism or to cellular potentiation. In the mean time, several investigations have been carried out to see whether the combination is synergistic in bacterial and mammalian cell systems (Bleehen, Gillies and Twentyman, 1974; Terasima, Takabe and Yasukawa, 1975; Bistrović, Maricić and Kolarić, 1976). Although the experimental results were not consistent among the cell lines used, the synergism was found to a slight or moderate extent.

The potentiation presented here, with Ehrlich ascites tumour cells treated with a combination of X-rays and bleomycin, may involve an interaction of different types of damage and repair induced by both agents.

Experiments were carried out with Ehrlich ascites tumour cells grown in 4-week-old male mice ICR/JCL, weighing 20–25 g (CLEA JAPAN Inc., Tokyo). By inoculating $10^6$ tumour cells i.p., the early plateau phase of growth was reached on the 7th day, when all the experiments were initiated.

To assay survival of tumour cells, they were removed from the abdominal cavity and suspended in F10 medium (Ham, 1963) with 10% calf serum. The cells were plated out in triplicate dishes of soft agar after they had been counted with electronic counter (Coulter, Model B) and diluted appropriately with F10 medium. The agar colony assay has been described in detail by Takabe *et al.* (1977). The plating efficiency (PE) for untreated cells was usually 40–90%. To estimate the surviving fraction, a portion of ascites was removed from each animal just before the initial treatment with either agent and the PE of untreated tumour cells was assayed for individual mice. Thus, surviving fraction was expressed as the PE of tumour cells treated with agent(s) divided by the PE of untreated cells from the same animal.

Bleomycin complex (Lot No. F100S41, Nippon Kayaku Co., Ltd, Tokyo) was dissolved in distilled water and diluted in F10 medium at the time of experiments. Approximately 0·3 ml of drug solution was injected through an s.c. route in the back of the mouse. The volume varied slightly with the body weight of individual tumour-bearing mice. All irradiations were given as whole-body doses to unanaesthetized mice by X-ray generator (Sinai, Shimazu Rad. Instr. Co., Ltd, Kyoto) operated at 200 KVp, 20 mA with added filtration (HVL: 1·2 mm Cu). Tumour-bearing mice housed in individual spaces of a round, lucite box were irradiated on a turntable at the dose rate of 80 rad/min.

Mice bearing tumour cells were either

Address for correspondence: T. Terasima, National Institute of Radiological Sciences, Anagawa-4, Chiba-si, Japan 280.



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treated with a single dose of 0·1 mg/kg bleomycin or irradiated with a single dose of 400 rad X-rays. At times indicated in Fig. 1A, ascites fluid was removed repeatedly from the abdominal cavity of the same animal and survival of tumour cells was assayed. Starting from 1 h after administration of bleomycin, the survival increased quickly with time and finally levelled off at 5 h (open circles), whereas the X-ray survival increased rather slowly, and almost attained a plateau at about 7 h (closed circles). The survivals, measured as a ratio of 7-h value to the value of initial determination (surviving fraction ratio, after Evans *et al.*, 1974) were 1·7 and 1·5 respectively. The number of ascites tumour cells was determined following treatment with either single agent. The result showed that cells did not resume their proliferation within the 7-h observation period. Therefore, the observed enhancement of survival represents the repair of damage which was potentially lethal and reparable only when cells were in the abdominal cavity (Belli, Dicus and Nagle, 1970; Little *et al.*, 1973).

Survivals were also determined with time after both agents were given simultaneously, and are shown in Fig. 1A (open triangles). The surviving fraction ratio was about 2·5 during the first 7 h, indicating that repair took place as found after administration of either single agent. The survival level was consistently lower throughout the 7-h period than the level expected when damage produced by each agent was independently repaired (broken line).

A similar experiment was carried out with single doses of 30 mg/kg bleomycin and of 1000 rad X-rays, as shown in Fig. 1B. The surviving fraction ratios given by each agent were much greater than those in the preceding experiment, *i.e.*, 4·1 for bleomycin and 4·2 for X-ray. In this case, the survivals after simultaneous administration of agents (open triangles) were obviously lower than the level expected from independent (or additive) effects of each agent (broken line). This finding indicates that bleomycin potentiated the effect of X-rays, either by interfering with the repair process of, or by

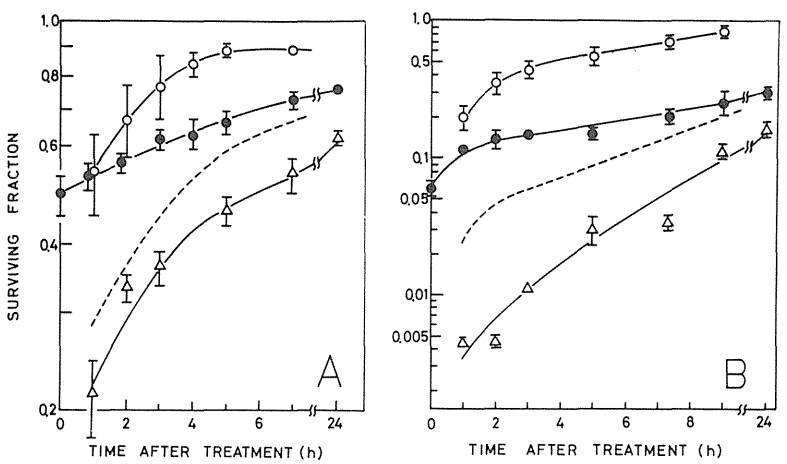


FIG. 1.—Change in survival of Ehrlich ascites tumour cells after separate or simultaneous administration of bleomycin and X-rays. A: open circles—0·1 mg/kg bleomycin alone; closed circles—400 rad X-ray alone; triangles—simultaneous administration. B: open circles—30 mg/kg bleomycin alone; closed circles—1000 rad X-rays alone; triangles—simultaneous administration. Broken line was given by a product of surviving fractions obtained at various times after the single treatment with bleomycin (open circles) and X-rays (closed circles). It represents survival level which would be expected from additive effect of both agents. Survival value with error bar denotes mean ± s.d. of 4 separate determinations.

interacting with, damage induced by X-rays. In the experiment with lower doses as shown in Fig. 1A, the extent of potentiation was 20–25% of survival expected for the additive effect, whereas it was 50–85% in the case of higher doses (Fig. 1B). This may imply that the more the amount of damage, the greater the potentiation.

Fig. 2 shows the result of experiments in which bleomycin was given to mice at various times after X-irradiation. A group of tumour-bearing mice was irradiated with 400 rad at zero time. Then a single dose of bleomycin (0·1 mg/kg) was administered to each mouse at times indicated. A tumour-bearing mouse was used for a single determination. Survival of tumour cells was determined exactly 1 h after injection of bleomycin and was plotted at the time of drug administration (closed circles). Increase of survival after exposure to X-rays alone (open circles) is taken from Fig. 1A. If a single dose of bleomycin (surviving fraction 0·54 in Fig. 1A) exerted only an independent effect, the expected survival level would be given as a product of surviving fraction of both agents, as shown by the broken line. The results showed that the survival determined was clearly lower than the expected value at zero time, then increased with time and reached the level of independent effect after about 3 h.

The next experiments (Fig. 3) were carried out in a similar fashion to the preceding ones, except that bleomycin was injected at various times *before* X-ray exposure. A group of tumour-bearing mice was treated with a single 0·1-mg/kg dose of bleomycin at zero time. From 1 h on, each mouse was irradiated with 400 rad at

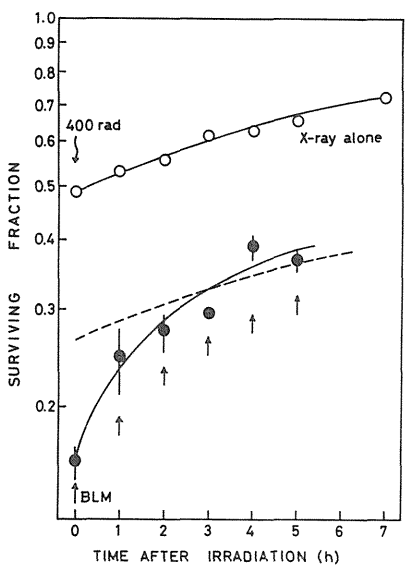


FIG. 2.—Effect of post-irradiation exposure to bleomycin on survival of Ehrlich ascites tumour cells. Straight arrows indicate times when bleomycin (BLM) was introduced. Closed symbols: survival for X-rays followed by BLM. Bar denotes the range of determinations. Open symbols: survival for X-rays alone (from Fig. 1A). Broken line represents the additive effect obtained as a product of surviving fraction at various times after exposure to X-rays alone and surviving fraction assayed 1 h after BLM (*i.e.* 0·54 from Fig. 1A).

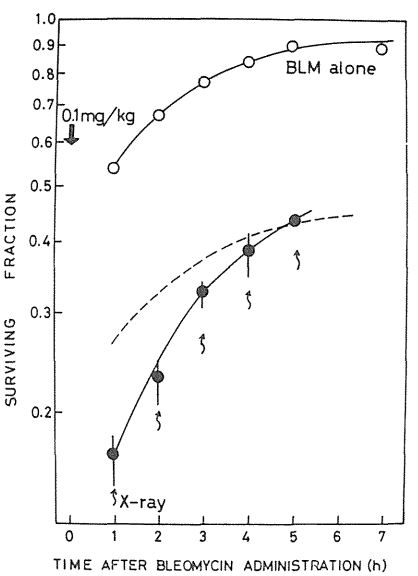


FIG. 3.—Effect of pre-irradiation exposure to bleomycin on survival of Ehrlich ascites tumour cells. Closed symbols: survival for X-ray with BLM pretreatment. Bar denotes the range of determinations. Open symbols: survival for BLM alone (from Fig. 1A). Broken line represents the expected level of additive effect, obtained as a product of surviving fraction at various times after administration of BLM and surviving fraction after 400 rad X-rays (*i.e.* 0·495 from Fig. 1A).

specified times. Immediately afterwards, the ascites fluid was removed and the surviving fraction of tumour cells assayed. Therefore, one animal served for a single determination. Enhancement of survival after administration of bleomycin alone (open circles) is as shown in Fig. 1A. If the effect of each agent were independent, the expected survival would follow the broken line, which is a product of survivals of both agents. Experimental points were obviously lower than the broken line over the first 3 h, and then became close to the level of independent or additive effect. These results revealed that (i) more than additive effect can be obtained only when the interval between two agents was less than 3 h, whatever the order of administration, and (ii) the potentiation is greater if two agents are given at closer intervals.

Recently, the induction and repair of potentially lethal damage was demonstrated in tumour cells treated with bleomycin (Takabe *et al.*, 1974; Barranco, Novak and Humphrey, 1975; Twentyman and Bleehen, 1975). Nevertheless, the antibiotic does not induce sublethal damage and repair, as the simple exponential nature of the survival curve suggested (Terasima *et al.*, 1972; Barranco *et al.*, 1975).

To effect maximal sterilization of tumour cells treated with an agent inducing potentially lethal damage, the repair must be controlled, either by inhibiting enzymatic repair reactions or by fixing potentially lethal damage *per se*. The potentiation found after the simultaneous administration of both agents (Fig. 1) suggests that at least portions of the damage, either potentially lethal or sublethal, induced by the two agents interacted each other and were converted to lethal damage. The limited period of potentiation found in Fig. 2 may suggest that the potentiation involves X-ray-induced sublethal damage, the repair of which normally completes approximately 3 h after X-irradiation. Similarly, the combined effect of bleomycin administered before X-rays may be also related to repair of bleomycin-induced damage (Fig. 3). The fact that both agents induce reparable damage of cellular DNA (Tsuboi and Terasima, 1970; Terasima, Yasukawa and Umezawa, 1970; Fujiwara and Kondo, 1973; Saito and Andoh, 1973) may be a basis for part of the potentiation.

The observed interaction between damages due to X-ray and bleomycin may provide practicable means to control repair. Using transplantable mouse tumour, Jørgensen (1972) demonstrated that simultaneous administration resulted in greater reduction in tumour weight than alternating administration. The result may be understood on the basis of the damage-interaction hypothesis suggested here.

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# Bleomycin: Mammalian Cell Lethality and Cellular Basis of Optimal Schedule [1, 2]

Y. Takabe, [3, 4] T. Miyamoto, [5] M. Watanabe, [3] *and* T. Terasima [5, 6]

**ABSTRACT—Experiments on the dose- and time-dependent changes in the survival of Ehrlich ascites tumor cells exposed to bleomycin were done to determine a useful regimen for the effective inactivation of the tumor cells. The experimental results on time-dependent changes in survival of bleomycin-treated cells indicated that two phenomena were involved in the survival increase observed after single bleomycin treatment: 1) The bleomycin-treated cells were resistant to the second injection when the interval between the two successive doses was within 2 hours (induced resistance), and 2) the survival was increased as a function of time when the interval between treatments was prolonged (repair of potentially lethal damage). The effect of the fractionated treatments was then investigated for the regimen that minimized the induced resistance and repair of potentially lethal damage. The results indicated that administration of a lower dose of bleomycin at shorter intervals was more effective than other fractionation schedules, and, on the basis of the same dose rate, the continuous infusion regimen was more effective than fractionation regimens for the sterilization of Ehrlich ascites tumor cells.—J Natl Cancer Inst 59: 1251-1255, 1977.**

Since the discovery of bleomycin by Umezawa et al. (*1, 2*), the cell biology basis of tumor therapy by this drug has been studied extensively by the use of cultured mammalian cells (*3-7*) and mouse transplantable tumors (*8, 9*). In clinical tumor therapy, this drug is highly effective for the control of squamous cell carcinomas and malignant lymphomas and has a low bone marrow toxicity (*10-13*). The optimal schedule of bleomycin therapy, however, has not yet been well established.

The dose-survival relationship between bleomycin and cultured mammalian cells was characterized by the upward concavity of survival curves (*3-5*); cells were more sensitive at lower concentrations of the drug than at higher concentrations. In time-survival experiments, bleomycin induced the resistance of cells as the time of incubation elapsed (*5*). The induced resistance disappeared within 4 hours after removal of the antibiotic, as demonstrated by a two-dose fractionation experiment (*5*). We showed that fractionated treatment given at adequate intervals sterilized cultured mammalian cells more effectively than did continuous treatment (*6*).

Recently, the repair of PLD induced by bleomycin has been demonstrated in in vitro and in vivo systems (*14-16*). We also reported that the repair of PLD occurs in Ehrlich ascites tumor cells after the sc administration of bleomycin to tumor-bearing mice (*17*). These results stimulated us to seek the most effective schedule of bleomycin treatment. In the present study, bleomycin was tested with Ehrlich ascites tumor cells, grown in mice, for its antitumor activity, resistance induction, and therapeutic effectiveness dependent on the treatment schedule.

## MATERIALS AND METHODS

*Experimental animals and cells.*—The experiments were performed with 4-week-old male ICR/JCL inbred mice weighing 20-25 g (CLEA Japan Inc., Tokyo, Japan). They were kept under standard conditions, 5 per cage, with pellet food and water ad libitum. Ehrlich ascites tumor cells were maintained by weekly ip transplantation into mice. After the ip inoculation of $10^6$ tumor cells, the cells multiplied exponentially until day 4, with a mean generation time of 12 hours. The experiments were all done on day 7 after cell inoculation, when tumor cells were in the early plateau phase.

*Test agent.*—The test agent was bleomycin complex (copper-free; Nippon Kayaku Co., Tokyo, Japan). The agent was dissolved in distilled water and stored at −20° C. Immediately before use, the solution was thawed and diluted with F10 medium (*18*). In all experiments except those with continuous infusion treatment, 0.01 ml drug solution per g body weight was administered sc in the backs of mice.

*Continuous sc infusion.*—The mice with 7-day tumors were anesthetized by ip administration of 10% Nembutal. The catheter (intravenous catheter for cut down; external diameter, 1 mm; Atom Co., Tokyo, Japan) was inserted into the subcutis of the back through the root of the tail. Bleomycin, diluted with F10 medium, was then infused through the catheter for 6 hours by a motor-driven infusion pump at the rate of 0.035 ml/hour.

*In vitro survival assay.*—The ascites tumor cells were aspirated and suspended in F10 medium supplemented with 10% calf serum. The number of cells was measured by Coulter electronic counter. The surviving fraction was assayed by the soft agar technique (*19*). The cultures consisted of two layers of agar medium (Noble Agar; Difco Laboratories, Detroit, Mich.) that were prepared in plastic 60×15-mm petri dishes. In the top layer, an appropriate number of cells was suspended in 3 ml soft agar medium containing 80% F10 medium, 20% calf serum, and 0.3% agar. The bottom layer was composed of 90% F10 medium, 10% calf serum, and 0.5% agar. This mixture was usually prepared in 6-ml amounts and incubated in a $CO_2$ chamber

ABBREVIATIONS USED: PLD = potentially lethal damage; PE = plating efficiency.

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[2] Supported in part by a grant from the Ministry of Education, Japan.

[3] First Department of Medicine, Chiba University School of Medicine, Inohana, Chiba, Japan.

[4] *Address reprint requests to* Dr. Yosinobu Takabe, Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa 4-9-1, Chiba-shi, Chiba, Japan.

[5] Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa-4, Chiba, Japan.

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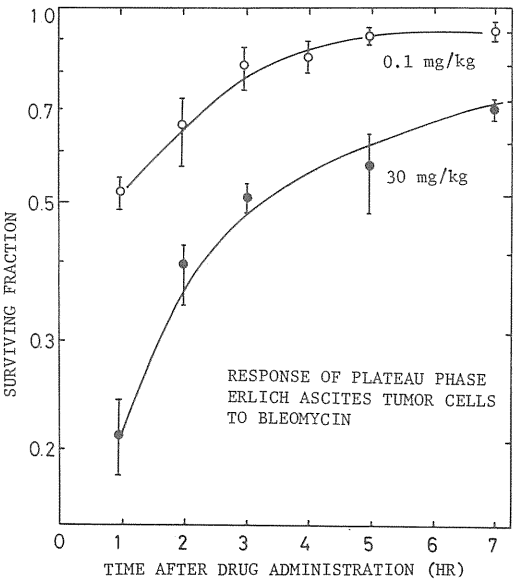
at 37° C 1 day before use. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were always added to the F10 medium. The cultures were incubated for 10 days in a $CO_2$ chamber. The number of colonies containing more than 100 cells was counted by an inverted microscope. PE for untreated cells ranged from 40 to 90% under these assay conditions. The surviving fraction of drug-treated tumor cells was expressed as the ratio of PE (drug-treated cells) to PE (non-treated cells).

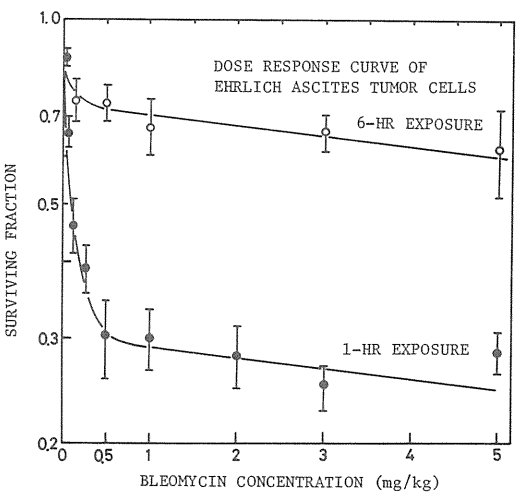
## RESULTS

Text-figure 1 shows the dose-survival response of Ehrlich ascites tumor cells after a single administration of bleomycin. The lower curve determined from cells obtained 1 hour after treatment showed a rapid initial and a subsequent gradual fall. The upper curve represents the surviving fraction of ascites tumor cells removed 6 hours after bleomycin injection. The general shape was essentially similar to the 1-hour survival curve. However, the survival values obtained by 6-hour exposure were much higher than those obtained by 1-hour exposure.

The change in survivals was examined as a function of time after bleomycin administration (text-fig. 2). Several repeated experiments with two different doses consistently showed an increase of the surviving fraction. The increase was rapid during the first 3 hours and finally appeared to level off. The enhancement ratio of the surviving fraction between 1 hour and 7 hours was 1.5 for 0.1 mg/kg and 3.5 for 30 mg/kg. This ratio appears related to the amount of



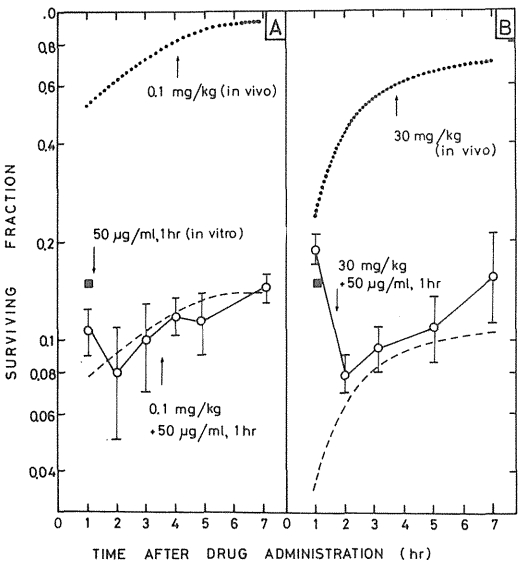

TEXT-FIGURE 2.—Change in surviving fractions of Ehrlich ascites tumor cells after exposure to bleomycin. Ehrlich ascites tumor cells were treated with single doses of 0.1 (*open symbols*) and 30 (*solid symbols*) mg bleomycin/kg. Ascitic fluid was aspirated at indicated times, and surviving fraction of tumor cells was determined. A series of determinations over a 7-hour period was done with the same animals. Limit shown is the standard deviation of the mean obtained from several repeated expts.



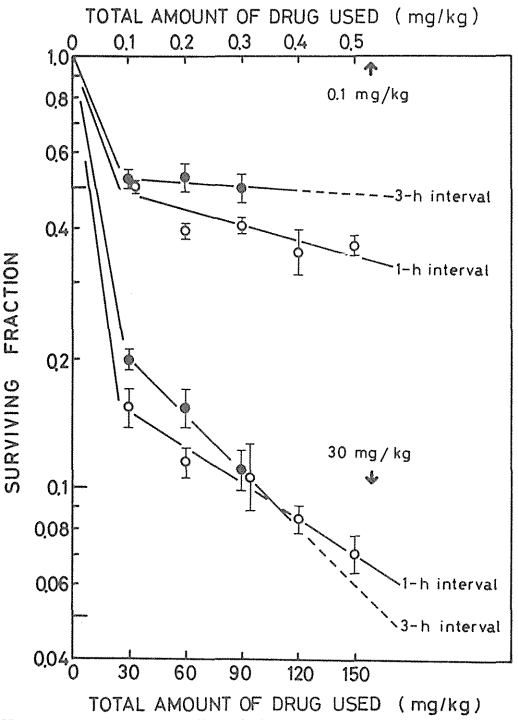

TEXT-FIGURE 1.—Dose-response curve of Ehrlich ascites tumor cells exposed to bleomycin in vivo. Mice with 7-day Ehrlich ascites tumor received single doses of bleomycin at different concentrations. We determined surviving fraction 1 hr (*solid symbols*) and 6 hr (*open symbols*) after sc injection of the drug by removing ascitic fluid and assaying colony survival as described in "Materials and Methods." Limit shown is the standard deviation of the mean obtained from 3 repeated expts.

initial damage. This enhancement of the surviving fraction cannot be explained on the basis of either selective loss of dead cells from the abdominal cavities of mice or preferential proliferation of viable cells during the post-treatment interval, because no significant change was found in total cell number and number of dye-stained cells until 7 hours after administration of doses of 0.1 and 30 mg bleomycin/kg (data not shown). In addition, the mean generation time of the ascites tumor cells on day 7 after inoculation was approximately 50 hours. A possible increase in cell number due to post-treatment proliferation would be approximately one-seventh of the tumor cell population during the 7-hour period even if the growth was not delayed. Therefore, the observed enhancement of survival with time indicated that the bleomycin-induced damage could be repaired in vivo.

In earlier reports (*5*), we described that cell resistance to bleomycin was reversibly induced when cultured L5 cells were incubated with the drug. To see whether a similar kind of resistance was induced in in vivo tumor cells, we did two-dose (in vivo-in vitro) fractionation experiments with Ehrlich ascites tumor cells (*see* legend to text-fig. 3). The lower curve of text-figure 3A represents the two-dose survivals. The broken line indicates the two-dose survival level expected on the assumption that the resistance of cells to bleomycin was not induced by the first administration of the drug. The two-dose survival level at 1 hour after injection




TEXT-FIGURE 3.—In vivo-in vitro dose fractionation expt. The single dose of 0.1 (A) and 30 (B) mg bleomycin/kg was injected into 6 tumor-bearing mice at 0 hour. At the indicated times after injection, the ascitic fluid was aspirated from each mouse and diluted. Cells ($10^6$) were then incubated with growth medium containing 50 μg bleomycin/ml for 1 hour (test dose) in a $CO_2$ chamber at 37° C. When the treatment was terminated, the cells were removed from the chamber and rinsed three times with F10 medium; a known number of cells was plated to triplicate soft agar dishes for survival assay. *Dotted line* is the time-survival curve obtained from single shot of 0.1 (A) and 30 (B) mg bleomycin/kg (redrawn from text-fig. 2). *Solid square* represents surviving fraction of 50 μg bleomycin/ml, 1-hour treatment of tumor cells aspirated from untreated control mouse. *Broken line* is obtained from the product of the surviving fractions shown by *dotted line* and survival shown by *solid square* (0.15). Limit shown is standard deviation of the mean obtained from 3 repeated expts.

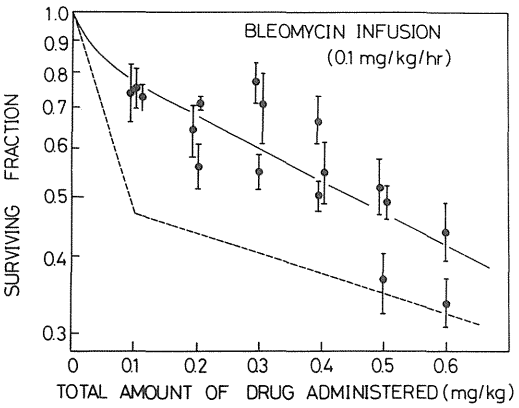



TEXT-FIGURE 4.—Tumor cell survival curve after fractionated treatment according to different schedules. Upper two curves show surviving fraction of Ehrlich ascites tumor cells after treatment with 0.1 mg bleomycin/kg dose fractions given at 1-hour (*open symbols*) and 3-hour (*solid symbols*) intervals. Lower two curves show results of similar expt except that 30 mg bleomycin/kg dose fractions were used. Survivals were determined by the removal of ascitic fluid for colony assay 1 hour after every administration of bleomycin. The values were plotted as a function of total amount of bleomycin used.

was higher than expected, whereas survival at the other points from 2 to 7 hours were slightly lower than expected. The results indicated that the resistance was induced 1 hour after injection of 0.1 mg bleomycin/kg but disappeared within the next 1 hour. The survival increase from 2 hours onward appeared due to the repair of PLD. Similar experiments were repeated with a single dose of 30 mg bleomycin/kg. Resistance greater than that with 0.1 mg bleomycin/kg was induced 1 hour after injection but disappeared within 2 hours (text-fig. 3B).

Text-figure 4 shows the results of the fractionated treatments with bleomycin on Ehrlich ascites tumor cells. The two groups of tumor-bearing mice were treated repeatedly with 0.1 or 30 mg bleomycin/kg. The interval was 1 and 3 hours for each group. The survival curves were biphasic for all four regimens. In the upper two curves with 0.1 mg bleomycin/kg, the cell inactivation rate of the terminal portion by the 1-hour interval was greater than that by the 3-hour interval. In the lower two curves obtained from 30 mg bleomycin/kg fractions, the results were contrary to those with the low dose, i.e., the cell inactivation rate was greater for the 3-hour interval than for the 1-hour interval.

From the results shown in text-figure 4, we expected that the continuous infusion of bleomycin might be most efficient for the inactivation of ascites tumor cells when a dose rate equivalent to 0.1 mg/kg fractions at a 1-hour interval was used. To confirm this, bleomycin was continuously infused to a mouse with a 7-day tumor at the dose rate of 0.1 mg/kg/hour during 6 hours (text-fig. 5). The survival curve, drawn by the method of least-square regression, showed a slight upward concavity, and the extrapolation of the terminal component to zero dose was 0.87. The inactivation rate by fractionated treatments (text-fig. 4) and continuous infusion (text-fig. 5) is given in table 1. The result shows that the terminal slope obtained from continuous infusion was greater by a factor of 1.5 than that of the fractionated treatment at the comparable dose rate.




TEXT-FIGURE 5.—Tumor cell survival curve after infusion of bleomycin. Ehrlich ascites tumor cells were treated with bleomycin infused at the dose rate of 0.1 mg/kg/hr. Surviving fraction was assayed every hour from the beginning of infusion. Each value is the mean survival ± SD. *Solid line* represents the regression line based on data of three separate expts. *Broken line* shows the survival curve by treatment with 0.1 mg bleomycin/kg at 1-hour intervals (redrawn from text-fig. 4).

TABLE 1.—*Inactivation rate of Ehrlich ascites tumor cells by different regimens*

| Treatment regimens | Inactivation constant unit dose$^{-1}$ |
|---|---|
| 0.1 mg bleomycin/kg | |
| 1-hr interval | 0.862 |
| 3-hr interval | 0.363 |
| 30 mg bleomycin/kg | |
| 1-hr interval | 0.006 |
| 3-hr interval | 0.013 |
| 0.1 mg/kg/hr | |
| (continuous infusion) | 1.270 |

## DISCUSSION

Repair of PLD in bleomycin-treated cells has been shown by several investigators (*14-17*). These experimental results strongly suggest that the repair of PLD by bleomycin also develops in human tumor cells and affects the therapeutic results. Therefore, the rational treatment schedule should be based on cell repair kinetics to improve therapeutic results.

Firstly, the results shown in text-figure 1 indicated that the dose-response curve of Ehrlich ascites tumor cells was biphasic, with the inflection point at the region of 0.25-0.5 mg bleomycin/kg. The D37[7] doses of initial and final slopes were 0.15 and 23 mg/kg, respectively. This indicates that in fractional use of the drug, doses below the inflection point are far more efficient to inactivate Ehrlich ascites tumor cells than are doses beyond the inflection point. Secondly, we must discuss the particular time-survival relationship. There may be two factors, i.e., resistance induction and PLD repair, both of which affect the time-survival relationship of treated Ehrlich ascites tumor cells. Text-figure 3 indicates that 1) the induction of resistance was demonstrated 1 hour after injection of bleomycin and then disappeared within 2 hours, and 2) the extent of resistance induced after a dose of 0.1 mg bleomycin/kg was distinctly smaller than that induced after 30 mg bleomycin/kg. If induction and disappearance of the resistance alone were taken into account, the most effective schedule of bleomycin administration in this tumor system would be a fractionated treatment at 2-hour intervals. However, the time-survival curve of Ehrlich ascites tumor cells was also characterized by PLD repair (text-figs. 1, 2). The amount of repair becomes greater as the time of interval between fractions increases. This finally results in the higher rate of survival of tumor cells. The ultimate consequence of the two contradictory effects was examined by fractionated treatments of Ehrlich ascites tumor cells with 0.1 and 30 mg bleomycin/kg (doses below and above the inflection point) at 1- and 3-hour intervals (text-fig. 4). To compare the effectiveness of the four fractionation regimens, we determined the inactivation constant of terminal slopes by assuming that these slopes are exponential (table 1). From the data, it is clear that the greatest cell inactivation rate is obtained with 0.1 mg bleomycin/kg given at 1-hour intervals. The low inactivation constant obtained with the same dose at 3-hour intervals may be attributed to the PLD repair developed during the interval.

The results with 0.1 mg bleomycin/kg dose fractions suggest that the drug administration at the shortest interval can minimize PLD repair which normally develops between fractions. This was demonstrated by the continuous infusion experiment. The inactivation constant determined from the terminal slope of the survival curve was 1.270, which was 1.5 times greater than the value obtained from administration of drug at 1-hour intervals (*see* table 1). This finding implies that infusion is more efficient than fractionation in sterilizing Ehrlich ascites tumor cells, if the dose rate (mg/kg/hr) is comparable.

A question arises whether a treatment schedule so optimal in this particular tumor system can be applied to other experimental tumors or human cancers. Recently, experimental results have been obtained with transplantable mammary carcinoma of C3H mice, demonstrating that tumor growth was significantly more affected by continuous infusion than by fractionated treatment with the same total dose (Urano M: Personal communication). Cvitkovic et al. (*20*) reported the therapeutic results of treatment of squamous cancer of the cervix and of head and neck areas with continuous iv infusion of bleomycin. They obtained the high response rate of 53% in a small series of 17 patients. Furthermore, Samuels et al. (*21*) reported the high rate of complete response in patients with stage III testicular neoplasia to infusion of bleomycin with the combined use of vinblastine. The present studies strongly suggest that the PLD repair is minimized by infusion and that similar kinetics may underlie several successful clinical trials.

Finally, in view of the lethal side effects of bleomycin, a possibility cannot be disregarded that continuous infusion of the drug at a low concentration may reduce the total amount consumed and, thereby, the occurrence of lung fibrosis (*10-13, 22*).

[7] Concentration needed to yield 37% survival in the exponential region of the survival curve.

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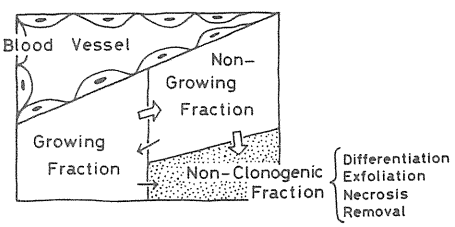
# CELL CYCLE DEPENDENT RESPONSE AND COMBINED TREATMENT OF TUMOR

T. TERASIMA, H. OHARA, T. MIYAMOTO*, M. WATANABE,
Y. TAKABE and I. WATANABE

Division of Physiology & Pathology, and Hospital*,
National Institute of Radiological Sciences,
Chiba, Japan

Tumor growth is affected in a number of ways, *e.g.*, endocrine function, immune capacity and general nutritional state of host as well as direct and indirect action of exogenous agents administered. To understand the response of tumor to various agents, we would usually envisage a simple scheme of tumor structure shown in Fig. 1. It represents that growing fraction of tumor is nourished through well developed blood vessel and as it grows, a part of the fraction shifts toward non-growing fraction the cells of which are, in turn, subjected either to differentiation, exfoliation or to necrosis as indicated by thick arrows. When exogenous agents are administered, each fraction in a tumor responds differently as shown by thin arrows. Blood vessel may also be affected directly by the treatment or indirectly through parenchymal response. In general, a response of tumor to a given treatment can be ascribed to three major factors, *i.e.*, tumor cell sensitivity, tumor cell kinetics and stromal reaction.

Fig. 1. Schematic illustration of tumor structure




Making use of cultured mammalian cells, a sensitivity of tumor cells was studied extensively by radiobiologists, and it was well known that a sensitivity depends on cell cycle stages, growth phases, extent of oxygenation, tissue origin and even extent of differentiation, of tumor cells. In a recent decade, more and more information has been obtained regarding mechanisms which involve in a sensitivity of tumor cells. Capacity of cells to repair sublethal or potentially lethal damages given by various exogenous agents essentially determines a sensitivity of cells. As we have demonstrated with bleomycin, the cell-drug interaction greatly modifies cell's sensitivity[1]. All these processes would be worth considering, if a rational basis should be obtained for an enhancement of therapeutic efficacy which may be produced either by combined use of, or even by scheduled administration of, two or more agents. In this communication, however, we would like to confine the subject area to the cell cycle dependence of sensitivity.

## I. Cell Cycle Dependence of Radiation Response

It has long been assumed that a target for mammalian cell killing by radiation appears to be DNA molecule and its associated structures. Many, either strong or suggestive, evidences in favor of the theory have been occasionally reviewed[2,3]. Assuming the genome theory it would be expected that mammalian cells change their sensitivity to radiation as they progress through the cell cycle. This was first demonstrated by TERASIMA & TOLMACH[4,5] in 1961 by virtue of synchrony technique of cultured mammalian cells developed by themselves[6]. Fig. 2 schematically illustrates the change in colony survival obtained after a single dose of X-rays given at each age of the HeLa cell cycle. The single dose survival pattern revealed that the G1-S transition point and the G2-M period were found more sensitive

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than the remainder of the cell cycle whereas the early G1 and the late S periods were relatively resistant, mainly reflecting the change in a slope (or Do value: mean lethal dose) of the dose-survival curve. The basis of the change cannot be attributed only to the amount of DNA as expected initially, and will be discussed elsewhere.

Since then, various mammalian cell lines were tested for cell cycle dependence of radiosensitivity and found to be essentially identical in the pattern of response, although a resistant peak of the early G1 was not discernible for some strains of Chinese hamster cells probably because the early G1 property was phenotypically defective in such cells as characterized by their extraordinary short G1 period (designated as G1′)[7].

Another remarkable action of radiation on cells, as demonstrated by the synchrony technique, concerns the progression (or traverse) of cells through the cell cycle. This was also cell cycle dependent, as shown in Fig. 3 where the response of HeLa cells to 300 rad X-rays was schematically given. If G2 cells are irradiated the next mitosis delays remarkably (G2 arrest). If a radiation is given to S cells, the S period prolongs by a slowing-down of DNA synthesis. This is due to damage in the machinery of replication which can be ultimately repaired[8]. G1 cells are relatively insensitive to radiation. Irradiation of G1 cells does not produce any appreciable prolongation of cell cycle periods except for G2. In this connection, it may be important to note that the G1 period of normal cells tends to be prolonged by irradiation. It may suggest a possible difference in proliferation machinery between normal and tumor cells[9]. Damage given at M period does not become apparent until the cells reach the S and G2 period. As a whole, a damage responsible for G2 prolongation is induced commonly in cells whatever the period they were. It seems that such particular damage is maintained as a form of chromosome aberration as revealed by premature chromosome condensation technique[10]. Above findings in relation to cell cycle dependence may be of great importance in understanding the kinetics of tumor cell population affected by exogenous agents as well as radiation.

Fig. 2. Change in X-ray survival during mammalian cell cycle
M: mitosis, G1: pre DNA-synthetic period, G1′: pre DNA-synthetic period lacking early G1 portion, S: DNA-synthetic period, G2: post DNA-synthetic period.

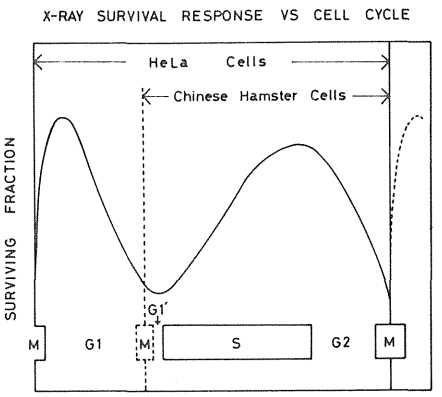
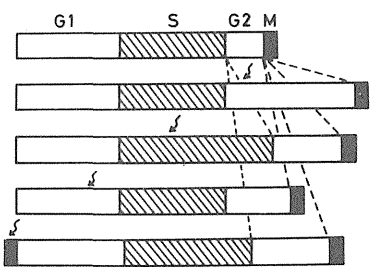



Fig. 3. Radiation-induced progression delay of HeLa S3 cells resolved into cell cycle periods




## II. Data for Cell Cycle Response to Antitumor Agents

Since the demonstration of cell cycle dependent radiation response by TERASIMA & TOLMACH and other researchers, similar sort of information has accumulated for various antitumor agents including cytotoxic drugs and anti-metabolites. Most studies have concerned effect of agents on cell progression, especially on DNA synthesis period. With regard to survival response pattern during the cell cycle,

relatively small number of reports have been available since the middle of 1960s, except for a few extensive investigations[11,12,13].

In an attempt to find a general pattern of cellular response in relation to the cell cycle, we have selected data which clearly indicate a cell survival response over the entire cell cycle, and tried to conform our and others' data to constant cell cycle parameters in rather schematical way. It was also expected that the attempt may permit us to compare action mechanism of various agents. Among data was apparent inconsistence in various respects, *e.g.*, dosage of agents, cell strains used and method of synchronizing cells. For the first approximation, a normalization of DNA cycle parameters was made on a basis of adequate assumption and, in some cases, arbitrarily. However, survival data were not able to normalize to a given level since a dose-survival relationship was not generally known.

Fig. 4. Survival response during the cell cycle of various cultured mammalian cells to single doses of bleomycin and mitomycin

Bleomycin: ○ L5 mouse fibroblast cells (20 μg/ml, 1 hour)[14], △ T1 human lymphatic leukemia cells (50 μg/ml, 1 hour)[15], □ CHO Chinese hamster cells (100 μg/ml, 0.5 hour)[16], ● HeLa S3 human cervical carcinoma cells (20 μg/ml, 1 hour)[17], ▲ P3HR-1 Burkitt lymphoma cells (20 μg/ml, 1 hour)[18], Mitomycin: • HeLa S3 cells (0.5 μg/ml, 1 hour)[19].

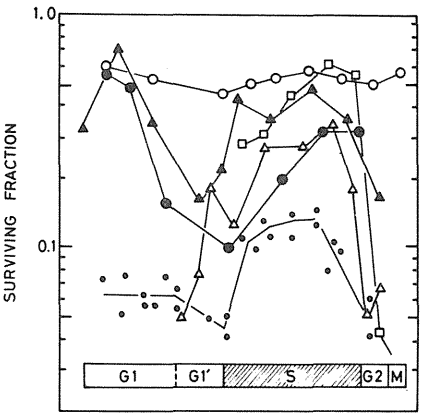



Fig. 4 shows the bleomycin survival during the cell cycle of several cultured mammalian cells[14~18]. Survival level is different among cell lines, depending upon their sensitivity and the dosage given. A consistent finding was that the early G1 and the S period appeared to be less sensitive than the remainder of the cell cycle, although human lymphoma T1 cells did not show a G1 resistance since the G1 period was extremely short (G1′) as compared to those of other cell lines, presumably lacking its early portion. From the figure we can readily see that various mammalian cells respond in essentially similar way to bleomycin over their cell cycle, just as found in X-ray response.

Mitomycin response given in the same figure revealed a particular single peak pattern where G2-M-G1 consecutive periods were more sensitive than S period[19].

The data for survival response of two Chinese hamster cell lines and of HeLa cells to several other antitumor agents are put together in Fig. 5[11,12,20,21]. As indicated typically with some mustards, all the drugs showed sensitive troughs at G1-S transition and G2-M period, and the patterns were practically consistent with those of bleomycin and X-rays. Although G1 survival peak was not always remarkable with Chinese hamster cells, response of HeLa cells to actinomycin and nogalamycin (data not shown) could supplement it[11]. It has also been reported that relatively high concentration of inhibitors for protein synthesis appears to give the similar pattern to those in this figure[11].

Contrary to preceding agents, there are a group of agents showing entirely different pattern of response[11,12,22,23,24]. As shown in Fig. 6, DNA synthesis inhibitors like hydroxyurea affected S cells exclusively. Cytosine arabinoside, 5-fluorodeoxyuridine, adriamycin and others belonged to this group. Therefore, the response pattern represents a single sensitive trough in S period and a relatively resistant period for the rest of the cell cycle. Interesting was that a few mitotic inhibitors, such as colchicine and vincristin, exerted the similar effect.

As roughly depicted in Fig. 7, several antitumor agents examined appear to fall into two types, *i.e.*,

Fig. 5. Survival response during the cell cycle of mammalian cells to various antitumor agents (I)
BCNU: ● Don Chinese hamster cells (2 μg/ml, 1 hour)[12]
Neocarzinostatin: ■ Don cells (1 μg/ml, 1 hour)[12]
Actinomycin D:
▲ Don cells (0.2 μg/ml, 1 hour)[12]
▲---V79 Chinese hamster cells (1.4 μg/ml, 0.5 hour)[20]
Nogalamycin:
○ Don cells (2 μg/ml, 1 hour)[12]
○---HeLa S3 cells (1 μg/ml, 3 hours)[11]
Nitrogen mustard: □ HeLa S3 cells (0.5 μg/ml, 0.5 hour)[11]
Uracil mustard: ◇ HeLa S3 cells (7.5 μg/ml, 0.5 hour)[11]
Sulfur mustard: △ V79 cells (0.18 μg/ml)[21]

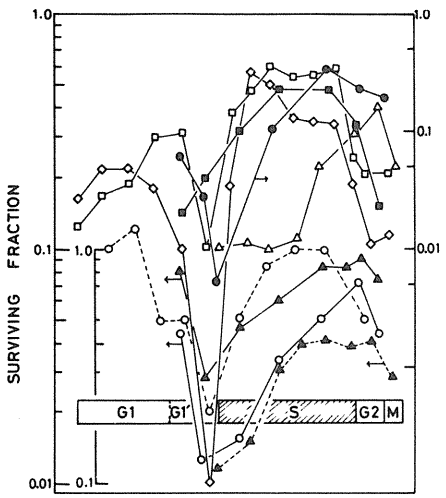
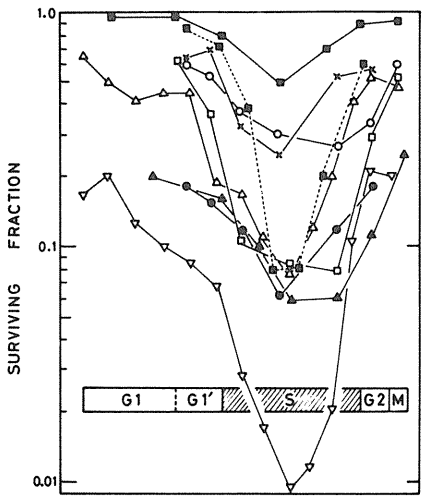



Fig. 6. Survival response during the cell cycle of mammalian cells to various antitumor agents (II)
Hydroxyurea:
■ HeLa S3 cells (10 mM, 4 hours)[22]
■---V79 cells (1 mM, 1.2 hours)[23]
5-FUdR: × Don cells (20 μg/ml, 1 hour)[12]
5-Azacytidine: ● Don cells (100 μg/ml, 2 hours)[12]
Adriamycin: ▲ HeLa S3 cells (0.3 μg/ml, 1 hour)[24]
Cytosine arabinoside: ○ Don cells (100 μg/ml, 2 hours)[12]
Camptothecin: □ Don cells (2 μg/ml, 1 hour)[12]
Colchicine: △ HeLa S3 cells (0.5 μg/ml, 3 hours)[11]
Vincristine: ▽ HeLa S3 cells (0.1 μg/ml, 3 hours)[11]




X-ray type (designated as R) and hydroxyurea type (designated as H), in terms of response pattern through the cell cycle. Only exception appears to be mitomycin as shown by dotted line. Notable findings are that (i) the agents "R" are usually known to be radiomimetic, alkylating or DNA-attacking agents, whereas "H" are grouped as DNA synthesis inhibitors and mitotic poisons, (ii) the patterns are grossly reciprocal; therefore, their effects may be complementary. Some of other agents studied as yet may be classified into either group and some others, which were not shown, may be rather dissimilar. The complete grouping of drugs awaits further detailed investigation.

Not only a lethal effect of agents but an effect on cell progression appear to be clearly different between above two categories of agents. Among R agents mitomycin and bleomycin have been known to manifest cell cycle dependent progression delay which were qualitatively similar to those induced by X-rays, as summarized in Fig. 8[19,25,26]. In addition to the effect on G1 and S period, BCNU is also known to induce considerable G2 prolongation[27]. However, the relative response of each cell cycle period may be quantitatively different from one agent to another.

Hydroxyurea and other H agents normally prevent the entry of G1 cells into S period as long as the agents are present in the medium. Exceptional may be camptothecin which does not prevent the entry[28]. It has also been known that hydroxyurea affected G2 progression[29] and adriamycin produced the

Fig. 7. Pattern of cell cycle dependent sensitivity of mammalian cells to antitumor agents
Dotted line represents the mitomycin survival.

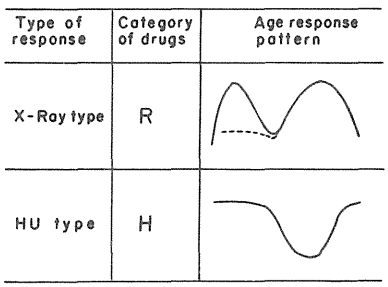
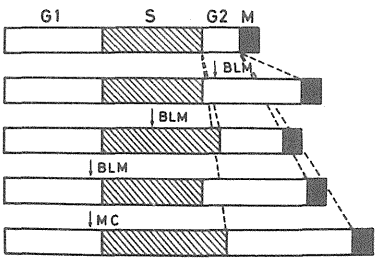



Fig. 8. Drug-induced progression delay resolved into cell cycle periods
BLM: bleomycin, MC: mitomycin




progression delay at all stages of the cell cycle, especially in late S and G2 period[30,31]. In general, it can be noted that R agents affect cell progression even after a pulse treatment, while the effect of H agents on DNA synthesis or DNA-synthesizing cells seems likely to be reversible with some exceptions.

### III. Designing of Tumor Therapy Based on Cell Cycle Dependent Response

As mentioned in foregoing sections, antitumor agents sterilize, or delay progression of, tumor cells at specific stage(s) more effectively than those in the rest of cell cycle. Accordingly, a combined use of two or more agents would enhance the tumor cell sterilization, if combination and timing of administration were adequately fixed.

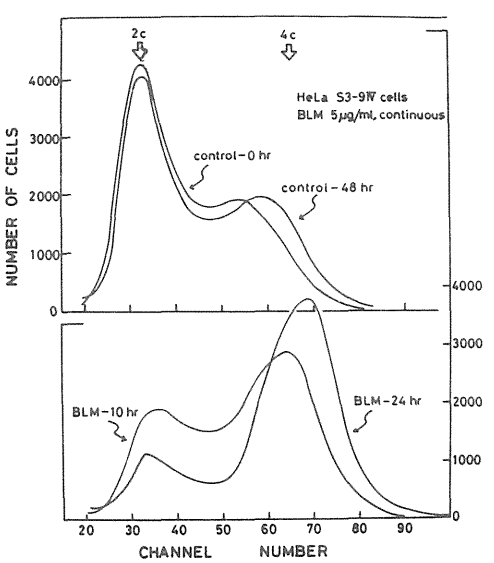
Possible basic schemes are listed in Table 1, where dot, bar and broken line denote simultaneous use of two agents, timed administration of agents and interval between unit treatment, respectively.

Scheme Ia may be useful only when R potentiates effect of R'. In that case, an interaction between damages given by each agent or a repair inhibition by either agent would be involved[32,33,34]. In scheme Ib the synchrony induced by the first agent may be taken as advantage. For instance, bleomycin causes cells to arrest in G2 period[25,35]. Fig. 9 illustrates the direct demonstration of G2 accumulation

Table 1. Possible combination of agents for tumor cell sterilization

| | |
|---|---|
| Ia | R·R' ---- R·R' ---- |
| Ib | R-R' ---- R-R' ---- |
| IIa | R·H ---- R·H ---- |
| IIb | R-H ---- R-H ---- |
| III | H-H ---- H-H ---- |

Fig. 9. Distribution of cellular DNA contents in untreated and bleomycin-treated HeLa S3 cells
Cells grown 40 hours after trypsinization were treated with 5 μg/ml of bleomycin for 10 and 24 hours. Cells were stained with propionium iodide solution and the DNA content was determined in aliquots of $10^5$ cells with a flow cytofluorometry. As compared with untreated control shown in the upper panel, the profile of treated cell populations in the lower panel indicates a remarkable accumulation of G2 cells.



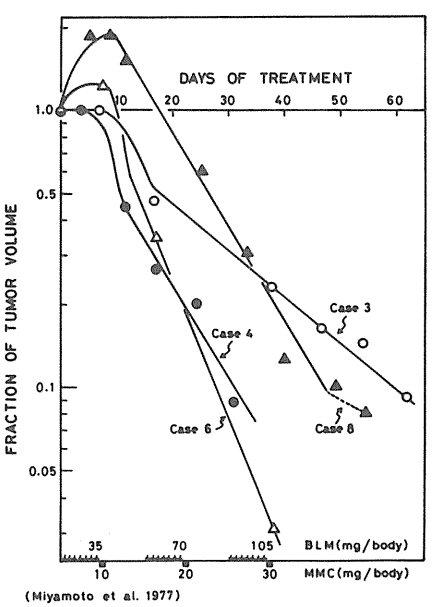
developed in bleomycin-treated HeLa S3 cells. It would then become possible to sterilize the G2 population by the second agent. Based on the reciprocal pattern shown in Fig. 7, one would expect that the scheme IIa exerts a complementary effect. A verification by well-designed experiments is awaited. The scheme IIb also seems promising. Experiment carried out by one of the authors (I.W.) demonstrated that single 1 krad dose of X-rays induced cultured mouse leukemic cell population a remarkable retention (almost 90 %) in the S period after 4 hours. The resultant surviving S population may be effectively managed by H agents. Scheme III was suggested previously by BHUYAN *et al.*[28]. In either scheme the interval between unit treatments constitutes an essential problem for the present therapeutic design. The solution must be left to further investigation of tumor cell kinetics.

## IV. New Clinical Trial: Bleomycin-Mitomycin Combination

Since 1974 one of us (T.M.) has initiated the combination chemotherapy of lung metastasis of cervical carcinoma[36]. The remarkable results so far obtained will be briefly touched on in the final section.

Primary tumors of squamous cell type at stage II or III had been treated by radiation in NIRS. No local recurrence was recognized when the chemotherapy was given. The unit treatment was 5 mg bleomycin per day infused i.v. for consecutive 5 to 7 days followed by single i.v. administration of 10 mg mitomycin (as shown by Ib in Table 1). Usually 4 to 5 courses of the unit treatment given at a week interval were carried out as the induction therapy and followed by the additional treatment for maintenance. Out of 15 patients treated until the end of 1976, 12 (80 %) showed complete remission and only 1 did not respond. In most cases, metastatic tumors in lung and other organs (bone and liver) disappeared after 2 to 3 courses. Fig. 10 indicates a change in tumor volume as measured on chest X-ray films which were taken at intervals after initiation of the treatment. Response in terms of tumor volume to the treatment appeared to lag for approximately a week and, then, the volume began to decrease at an exponential fashion. The volume reduction rate ranged from 3 % to 11 % per day. Average 90 % volume reduction dose determined from response of 9 cases was 67 mg bleomycin and 20 mg mitomycin, being equivalent to the dosage of two courses of the unit treatment. It would be concluded that bleomycin pretreatment evidently potentiated the effect of mitomycin by a factor of about 10, since the treatment of lung cancer with mitomycin alone required approximately 200 mg for the 90 % volume reduction.

Fig. 10. Change of volume of metastatic lung tumors during the course of B-M therapy




The reason for the remarkable potentiation may not be answered immediately. However, our assumption based on cell cycle dependent response data is that the consecutive bleomycin treatment arrests most tumor cells in the G2 period, allowing a portion of them to move into the early next cycle and makes surviving tumor cell population more susceptible to the action of

mitomycin as a G2-G1 attacking agent. Also, synergistic effect on non-proliferative fraction of tumor as well as on stromal component may not be disregarded.

## V. Summary

Attempt was made to summarize data indicating cell cycle dependence of survival response to various antitumor agents. Normalized patterns of survival response permitted us to group agents into two major categories. It was known that effect of agents on cell progression was not only related to respective periods of the cell cycle but also rather specific for each category of agents. The extent of progression inhibition was not compared on a quantitative basis. On the basis of above findings a rational combination and a scheduled administration of agents for effective sterilization of tumor cells were considered.

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# EFFECTIVENESS OF A SEQUENTIAL COMBINATION OF BLEOMYCIN AND MITOMYCIN-C ON AN ADVANCED CERVICAL CANCER

TADAAKI MIYAMOTO, MD,* YOSHINOBU TAKABE, MD,† MICHINORI WATANABE, MD,† AND TOYOZO TERASIMA, MD‡

Fifteen patients with squamous type of metastatic cervical cancer were treated with a sequential combination of bleomycin (BLM) and mitomycin-C(MMC) as follows: 5 mg of BLM daily for 7 days followed by a single injection of 10 mg of MMC. After 1 week of rest, this course was repeated two to five times depending on the responses or adverse effects. Fourteen out of 15 patients (93%) responded, with a complete remission in 12(80%) and a partial remission in two (13%). During the courses one patient died of lung fibrosis. Four of 12 complete responders had reccurrence after 4.5 months; and three of them died of relapse with a median survival of 7 months, but the fourth one is living. After the treatment one patient who received a booster shot of MMC and five patients with maintenance therapy of Carboquon, all are alive without relapse at an average of over 17 months. These results offer a hopeful prospect for the control of metastatic solid cancers of the squamous cell type.
*Cancer* 41:403–414, 1978.

MITOMYCIN-C(MMC) AND BLEOMYCIN (BLM) were discovered by Hata *et al.* in 1950[11] and Umezawa *et al.* in 1966,[27] respectively. After extensive studies of these antibiotics over many years, they have come to assume an important position in cancer chemotherapy. BLM is effective for lymphomas, testicular cancer and various types of squamous carcinoma. The remarkable effect of this drug on squamous cancer, together with the low bone marrow toxicity, makes it an unique agent. After the initial observation of Ichikawa *et al.*,[12] there have been a number of reports demonstrating the moderate sensitivity of all types of squamous cancer to BLM. However, this drug, if used singly, was more effective for well-differentiated types, and there has been an attempt to enhance its effect by combining it with other agents such as x-ray or other drugs.[1,7]

Following Ichikawa's first proposal of the protocol for BLM therapy, this drug has generally been given in a dose of 15 mg twice a week. The rationale of this protocol remains questionable, however. During the last several years we have investigated a principle for BLM therapy based on cell inactivation kinetics.[22] Our conclusion has been that fractionated treatment with BLM at the lowest practicable dose inactivates cells most efficiently.[21] Based on this, we usually gave BLM at a dose of below 5 mg every day.

In Japan MMC is now considered as one of the most reliable anti-cancer agents. This drug has chemotherapeutic effects against various kinds of solid tumor such as cancers of the gastrointestinal tract, ovary, breast and lung. The long-term clinical trials with MMC have disclosed that the drug should be administered in a larger dose at a longer interval rather than at a smaller dose at a shorter interval. This empirical schedule agrees with the experimental one sug-

From the *: National Institute of Radiological Sciences, Anagawa-4, Chiba; and †: Chiba University School of Medicine, Inohana, Chiba, Japan.
* Hospital (N.I.R.S.).
† First Department of Medicine.
‡ Division of Physiology and Pathology.
Address for reprints: Takaaki Miyamoto, MD, Hospital, National Institute of Radiological Sciences, Anagawa-4, Chiba-shi, 280 Japan.

Bleomycin: Nippon Kayaku Co. Ltd. Tokyo, Japan.
Mitomycin-C: Kyowa Habbo Co. Ltd, Tokyo, Japan.
Carboquon (Carbazilquinone): 2,5-bis (1-azinidinyl)-3-(2-carbamoyloxy-1-methoxyethel)-6-methyl-beuzoquinone (NSC-134679). A new synthesized anti-tumor agent, developed by Sankyo Co. Ltd. Tokyo, Japan, which contains azinidinyl, carbomoxyloxy, and quinonyl groups; also present in Mitomycin-C.

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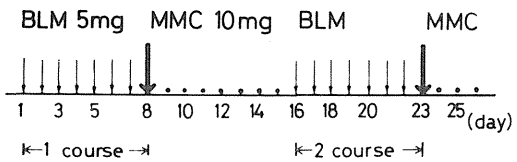


FIG. 1. An illustrated protocol of bleomycin and Mitomycin-C (B-M) therapy.

gested by the dose response curve of MMC with a shoulder,[18,19] which implied that the drug killed the cells more efficiently as the dose exceeded the shoulder region of the curves. Considering the hematopoietic damage and recovery, we usually adopt a dose of 6 to 16 mg for MMC depending upon the patient's weight.

As discussed later, the effect of BLM and MMC in single and systemic use on metastatic cervical cancer was about 20 to 30% in response rate without complete remission. In contrast, a combination chemotherapy using BLM and MMC in a sequential manner resulted in a complete remission rate of 80% without increasing adverse effects of any significance in patients with squamous cancer. This unique combination, for which the first hint was provided by one of the authors (T.M.), was based on the *(in vitro)* information of the specific effect of MMC on the cells at G2 synchronized by successive treatment with BLM. The clinical results obtained with our protocol using this combination will be presented.

## MATERIALS AND METHODS

Since July 1974, 16 patients with uterine carcinoma were treated with BLM and MMC given in a sequential manner at the Hospital of the National Institute of Radiological Sciences, Chiba. Fifteen of them had cervical cancer of the non-keratinizing squamous type. Only one had endometrial cancer with adenocarcinoma. All except one (case 2) were in stage II or III at the time of radiotherapy but had distant metastases in the lungs and elsewhere at the time of chemotherapy. Case 2 was thought to be in stage IV because the lungs were involved when the primary lesion was found.

### Drug Regimens

The protocol for therapy (abbreviated as B-M therapy) is illustrated in Fig. 1. BLM in a dose of 5 mg dissolved in 500 ml of 5% glucose was given intravenously every day for one week followed by the injection of MMC at a dose of 10 mg dissolved in a 20 ml of 20% glucose on day 8. The course was usually repeated five times with

TABLE 1. Patient and Initial Treatment

| Patient | Age | Sites of metastases | Initial treatment: Regimen | No. of B-M courses | Dose (mg): BLM | Dose (mg): MMC | Response | Length of CR (mos) | Relapse |
|---|---|---|---|---|---|---|---|---|---|
| 1. Y.I. | 64 | Lung | (B-M) + M + M +.. | 1 | 70 | 50 | CR | > 30 | − |
| 2. A.N. | 69 | Lung | (B-M) + M + M +.. | 1 | 30 | 26 | CR | 4 | + |
| 3. S.O. | 43 | Lung | (B-M) + (B-M) +.. | 5 | 175 | 50 | CR | 5 | + |
| 4. Ki.K | 39 | Lungs | (B-M) + (B-M) +.. | 5 | 175 | 50 | CR | > 18 | − |
| 5. A.K. | 55 | Lung, mediastinum abdominal node | (B-M) + (B-M) | 2 | 70 | 20 | CR | 4 | + |
| 6. N.S. | 53 | Lungs, (local relapse) | (B-M) + (B-M) +.. | 4 | 140 | 40 | CR | | − |
| 7. Ka.K. | 53 | Lungs, bones | (B-M) + (B-M) +.. | 5 | 140 | 40 | CR | 2 | + |
| 8. Y.M. | 49 | Lungs, liver, bones | (B-M) + (B-M) +.. | 5 | 175 | 50 | CR | > 11 | − |
| 9. I.U. | 43 | Lungs, inguinal node | (B-M) + (B-M) +.. | 3 | 105 | 40 | CR | > 5 | − |
| 10. S.Y. | 73 | Pelvic and abdominal nodes, bone | (B-M) + (B-M) (Radiotherapy) | 2 | 70 | 20 | > 12 | | − |
| 11. M.I. | 51 | Liver, bone, pelvic node | (B-M) + (B-M) +.. | 3 | 105 | 30 | PR | | + |
| 12. K.Y. | 41 | Pelvic and abdominal nodes, sacral bone | (B-M) + (B-M) +.. (Radiotherapy) | 4 | 105 | 40 | CR | > 5 | − |
| 13. H.K. | 43 | Neck node, lungs | (B-M) + (B-M) | 2 | 70 | 20 | NR | | |
| 14. T.N. | 67 | Bone, liver, lungs, skin, neck node | (B-M) + (B-M) +.. | 4 | 130 | 40 | PR | | + |
| 15. T.B. | 43 | Bone, skin, neck and inguinal nodes | (B-M) + (B-M) +.. | 4 | 140 | 40 | CR | > 3 | − |
| 16. F.S. | 53 | Bone, lung, liver | (B-M) + (B-M) | 2 | 70 | 20 | NR | | |

CR: Complete Remission, PR: Partial Remission, NR: No Remission, BLM, B: bleomycin, MMC, M: mitomycin-C, (B-M): one course of B-M therapy.

TABLE 2. Retreatment, Maintenance and Survival

| Patient | Retreatment: Chemotherapy: Regimen | Dose (mg): BLM | Dose (mg): MMC | Radiotherapy (rad) | Maintenance: Duration (month) | Maintenance: Total dose of CQ (mg) | Survival (mos) | Cause of death |
|---|---|---|---|---|---|---|---|---|
| 1 | M + M | | 20 | | | | > 30 | |
| 2 | (B-M) + M + M | 17.5 | 18 | | | | 13 | Relapse |
| 3 | (B-M) | 35 | 10 | 4000 | 4.5 | 67.5 | > 20 | |
| 4 | | | | | 6.0 | 90 | > 18 | |
| 5 | | | | | | | 4 | Esophageal perforation |
| 6 | | | | | | | 3 | Lung fibrosis |
| 7 | (B-M) | 35 | 10 | 4000 | 4.0 | 32 | 7 | Relapse |
| 8 | | | | | 2.0 | 30 | > 11 | |
| 9 | | | | | | | > 5 | |
| 10 | | | | | 5.0 | 75 | > 12 | |
| 11 | | | | | 0.5 | 8.5 | 4 | Necrosis of liver meta., relapse |
| 12 | | | | | 3.0 | 45 | > 5 | |
| 13 | | | | | | FT-207* CQ† | > 5 | |
| 14 | | | | | | | > 4 | |
| 15 | | | | | | | > 3 | |
| 16 | | | | | | FT-207* CQ† | 5 | Progression |

* Ftoraful: $N_1$-(2'furanidyl)-5-fluorouracil † CQ: Carboquon (Carbazilguinone)

intervals of 1 week's rest period. When retreatment for relapse was necessary, another course was given followed by single dose of MMC and/or irradiation. In case 1 MMC in a dose of 10 mg was injected twice for consolidation 2 and 3 months after complete remission had been achieved. In order to prevent recurrence, maintenance therapy with a daily oral dose of 0.5 mg of Carboquon (CQ) was given. This medication starting 2 or 3 weeks after complete remission was planned to last for 6 months.

### Evaluation of Responses

Complete remission (CR) was defined as the disappearance of all clinical evidence of tumor. Some tumors that received B-M therapy were apt to change into scarlike on roentogenogram. Therefore, complete remission for the patients with these tumors was carefully identified by confirming no tumor regrowth of over 3 months. Partial remission (PR) was defined as a 50% or greater decrease in tumor volume. Other responses were classified as no remission (NR).

## RESULTS

The overall clinical data in this study are summarized in Tables 1 and 2.

### Response to Initial Treatment

Of the 15 patients with cervical cancer, 14 (93%) had remission, CR in 12 patients (80%) and PR in two (13%). One patient with endometrial cancer did not respond. Table 3 shows the signs and symptoms of the responders before therapy and the number of B-M courses to achieve complete remission. All the signs and symptoms disappeared within two courses on the average, and in three courses at the longest. Case 12 in Table 3 is described because she is a representative patient.

She was a 40-year-old housewife. In September 1975, she first had genital bleeding which was found to be from a cervical cancer stage III. She immediately received radiotherapy in our hospital with good response. In March 1976, however, bilateral leg pain and numbness developed and she could not walk. On readmission, she had urinary and fecal incontinence. Bone roentogenogram showed destruction of the 5th lumbar spine and sacral bone, and scintigram (Fig. 2, upper left) also exhibited a lack of uptake of $^{99m}$Tc pyrophosphate in the sacrum. Soon after hospitalization, she was given 4,000 rads to the involved bones with no improvement in the complaints. Thereafter, she received B-M therapy. Pains were relieved completely after the

TABLE 3. Signs and Symptoms and Their Response

| Case No. (with complete remission) | Symptoms and signs before treatment | No. of B-M courses before disappearance of symptoms and signs |
|---|---|---|
| 1 | Cough | 1 |
| 2 | Cough | 1 |
| 3 | Bloody sputum, cough | 2 |
| 4 | Mild dyspnea | 2 |
| 5 | Cough, dysphagia, lumbago | 2 |
| 6 | Cough, dyspnea | 2 |
| 7 | Bloody sputum, cough, bone pain | 3 |
| 8 | Cough | 1 |
| 9 | Cough | 1 |
| 10 | Lumbago | 2 |
| 12 | Leg pain, lumbago paralysis of legs incontinence | 3 |
| 14 | Lumbago, chest pain | 2 |
| 15 | Leg and thigh pain | 3 |
| Average | | 2 |

first course. After the second course there was no more incontinence. After the third course, she started walking. The bone scintigram at the end of the fourth course showed an uptake of the isotope by the involved bone equal to that by the surrounding bones (Fig. 2, upper right), but re-ossification did not occur (Fig. 2, lower left). In December, three months after discharge, re-ossification appeared in the involved sacral bone (Fig. 2, lower right).

In each patient with lung metastasis (cases 1 to 9) in which complete remission was obtained, drastic tumor regression occurred in chest roentogenograms (Figs. 3 to 11). In addition to lung metastasis, cases 7 and 8 had bone and liver metastases, respectively. Case 7 complained of severe bone pain caused by metastasis to the left scapula and the third proximal phalanx, which also produced swelling (Fig. 9, lower upper). After three courses the bone pain was relieved with disappearance of swelling; there was no more dyspnea or hemoptysis. After the fourth course, the finger became shorter, and the roentogenogram demonstrated shortening of the involved bone because of tumor regression. Case 8 had liver metastases (Fig. 10, lower left). After the third course, there was no more space occupying lesion (Fig. 10, lower right). In case 10, the flank mass with lumbosacral pain disappeared after the second course of B-M therapy. In case 15 the multiple metastases in the skin and lymphnodes of the neck and groins disappeared after the third course, together with the relief of the pain caused by a tumor involving the third lumbar vertebra.

Cases 11 and 14 had PR. Case 14, a 68-year-old female, had a relapse of cervical cancer occurring in the skin, liver, lungs and bones and lymphnodes. A skin metastasis grew as large as an adult hand in size. After the third course of treatment, this lesion completely disappeared with relief of the pain, but lung and lymphnode tumors remained, although they were reduced by over 50% in volume. The bone and liver scintigrams still showed some changes and the response was evaluated as PR.

Only one out of 15 patients (case 13) with squamous cell type of cervical cancer failed to respond to B-M therapy. Two courses brought about only cessation of tumor growth. In case 16

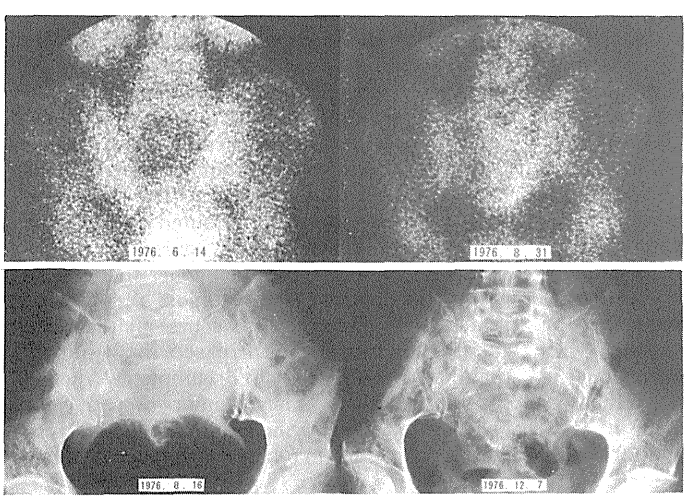


FIG. 2. A 44-year-old woman with sacral bone metastasis of cervical cancer (case 12). Upper photographs are the scintigrams of the pelvis. Before treatment, a round area of negative uptake of isotope is evident in the sacral bone (left). After four courses, the defect disappeared (right). Re-ossification had not as yet occurred, as shown in the bone roentogenogram (left, below), but it did occur three months after the fourth course (right, below).

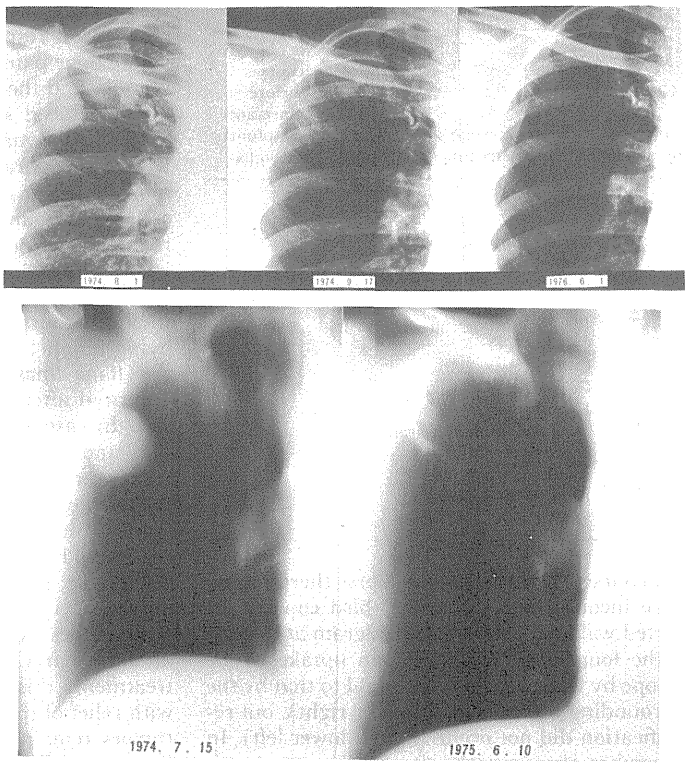


FIG. 3. A 64-year-old woman with lung metastases of cervical cancer (case 1). Upper photographs show the simple chest roentogenograms before treatment (left), after fifth dose of MMC (middle), and one-and-a-half years after the final administration of MMC (right). Lower photographs show the tomogram of the lung before treatment (left), and one-and-a-half years after treatment (right).

with endometrial cancer, no regression of metastatic tumors occurred.

### Relapse and Re-treatment

There were four patients with relapse after 4.5 months on the average following the achievement of CR. In case 2, lung tumors re-appeared 4 months later with inguinal pain caused by ischial bone metastasis. Upon re-admission, one course of B-M therapy in a reduced dose of 2.5 mg of BLM and 6 mg of MMC was given, followed by intermittent administration of 6 mg of MMC at intervals of 2 weeks. The lung tumors regressed remarkably at the second treatment as they did at the first treatment with disappearance of bone pain. Further treatment could not be instituted because of a severe drug eruption caused by BLM. Nine months after the re-treatment, she died of general weakness with ascites. In case 3, tumor re-appeared on the chest roentogenogram 5 months after CR. After re-admission, she received one course of B-M therapy followed by irradiation of 4,000 rads, and had remission again.

Case 5, a 55-year-old women, had successive recurrence in Virchow's lymphnode, mediastinum and esophagus (in this order) 3 years after receiving radiotherapy to the primary. These metastatic lesions were controlled successfully by irradiation. But finally metastases appeared in the lungs with complaints of severe lumbago and dysphagia. Two courses of the B-M therapy relieved these complaints with complete disappearance of lung tumors (Fig. 7, upper, left to right). Further therapy, however, was impossible because of thrombocytopenia. Relapse occurred 4 months later. The relapse in these three patients was attributed mainly to an inadequate number of B-M courses for initial treatment or to the lack of further therapy. In case 7, in contrast, three tumors re-appeared immediately after the fifth course at the same sites in the lungs where there were many tumors that had once disappeared completely during the fourth course. These tumors were clearly resistant to therapy. She eventually died of general weakness due to relapse, despite other treatments.

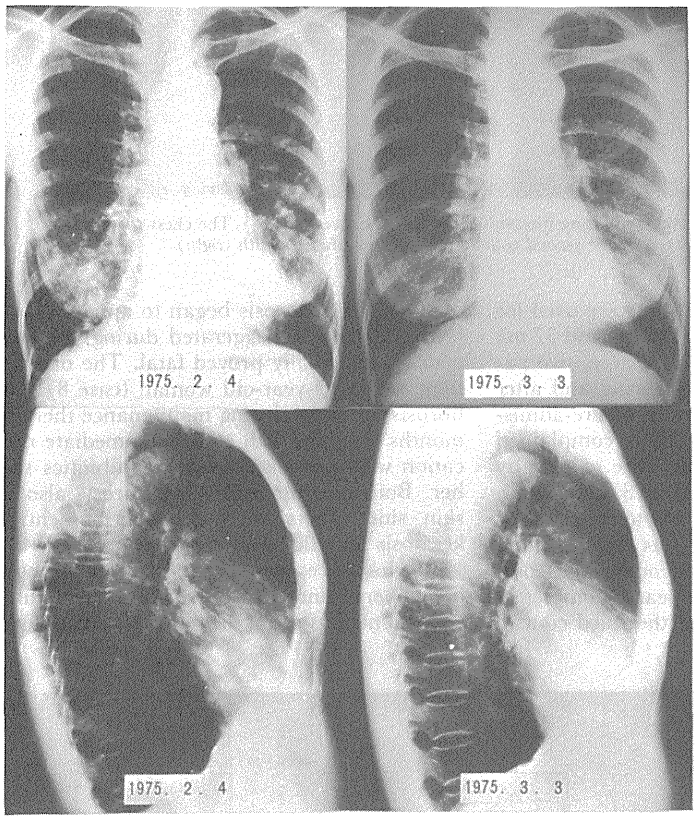


FIG. 4. A 69-year-old woman with multiple lung metastases of cervical cancer (case 2). Left upper and lower chest films are before treatment and right upper and lower chest films are after the second shot of MMC.

## Maintenance Therapy

Seven patients had received or are receiving maintenance therapy. Only one was able to continue the medication of CQ for 6 months without trouble. The others had to give up the scheduled medication due to such complications as lung fibrosis, severe herpes zoster, acute cholecystitis and severe anemia. None of the patients had relapse after or during maintenance therapy, however.

## Toxicity

The effect of the therapy on the nutritional state of the patients receiving more than four courses was evaluated by the changes of body weight, serum total protein and cholesterol. As shown in Fig. 12, no consistent changes were noted.

Other adverse effects are listed in Table 4 which indicates that they are infrequent and mild. This seems to be due mainly to the low

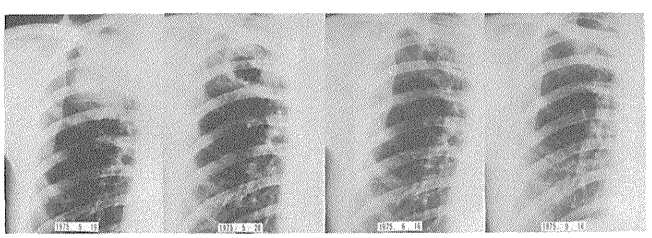

FIG. 5. A 43-year-old woman with lung metastasis of cervical cancer (case 3). From left to right, the chest roentogenograms before treatment, after the first course, after third and after fifth.

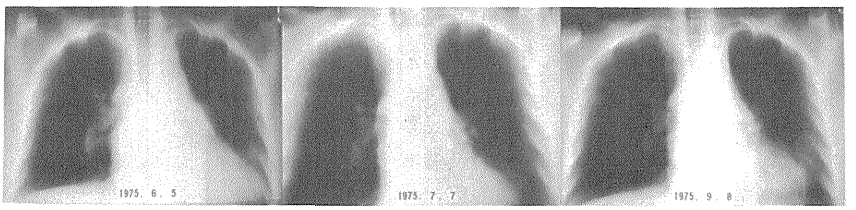


FIG. 6. A 39-year-old woman with lung metastases of cervical cancer (case 4). The chest roentgenograms before treatment (left), after the second course (middle) and after the fifth (right).

doses of BLM and MMC which were used for initial treatment in a total of 120 mg and 37 mg on the average, respectively. However, two patients developed lung fibrosis during and after induction treatment, respectively. On re-admission, case 6, a 55-year-old woman, complained of cough and dyspnea which were caused by lymphangitis carcinomatosa (Fig. 8, upper left). Combined with the radiotherapy to the relapsed primary lesion, B-M therapy was given for lung metastases. After the second course, the complaints and lung tumors disappeared completely (Fig. 8, upper right). During the third course, however, lung fibrosis began to appear (Fig. 8, lower left), was exaggerated during the fourth course, and finally proved fatal. The other patient was a 49-year-old woman (case 8). Lung fibrosis occurred during maintenance therapy 2 months after the fifth course. Immediate medication with prednisolone and antibiotics saved her. Both patients with lung fibrosis also had skin side effects such as hyperpigmentaion, keratosis and alopecia as shown in Table 4.

Myelosuppression is also noted. Thrombocytopenia under $1.0 \times 10^5/mm^3$ occurring in six patients in spite of the relatively small dose of

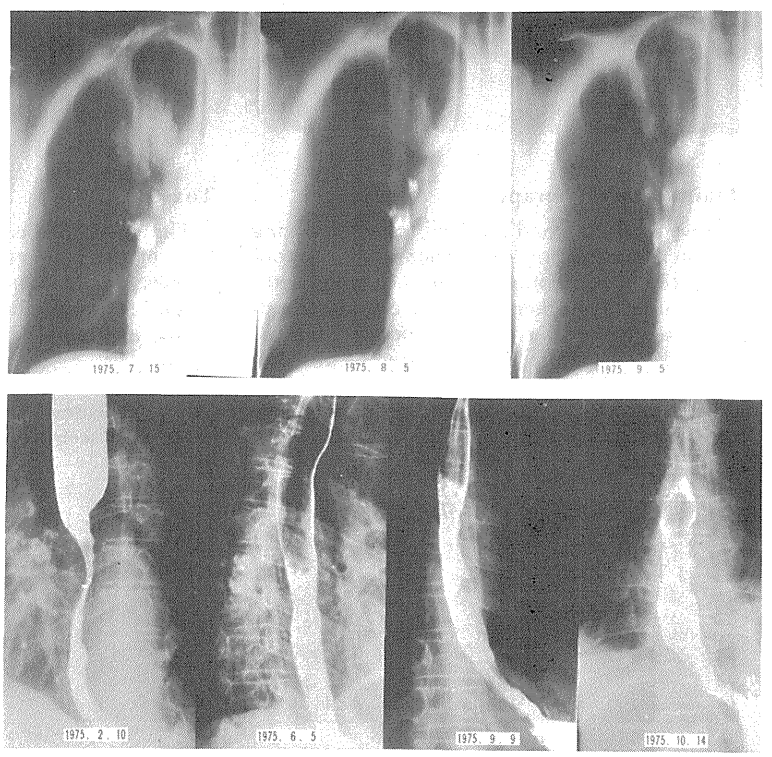


FIG. 7. A 55-year-
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5). Upper photograp
are the tomograms
fore treatment (left),
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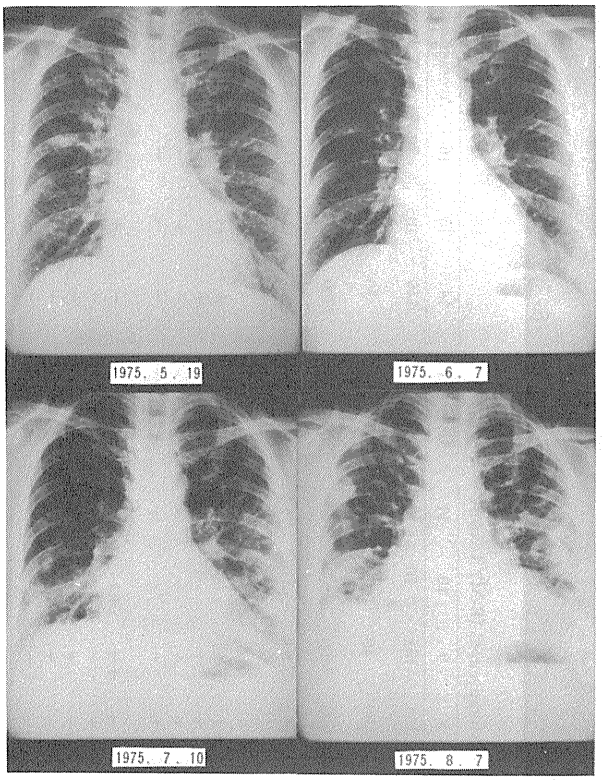


FIG. 8. A 53-year-old woman with lymphoangitis carcinomatosa in lungs of cervical cancer (case 6). Upper photographs show the chest films before treatment (left) and after the second course (right). Lower photographs show one after the third course (left) and after the fourth course (right).

MMC, is particularly noteworthy. In general, an adverse effect was intensified by re-treatment compared to the initial treatment.

As an unexpected adverse effect, a radiation ulcer was induced by B-M therapy. In case 5, the esophagus involved with tumor (already mentioned) had been irradiated and cured radiographically (Fig. 7, lower, second from left). One month after B-M therapy of the newly formed lung tumors, the radiographic defect of the esophagus suddenly re-appeared (Fig. 2, lower, third from left) and enlarged gradually (Fig. 7, lower, right). She finally died of pericarditis following esophageal perforation, as revealed by autopsy.

## Survival

Of four patients with CR who died from lung fibrosis, two relapsed and one had pericarditis. One of two patients with PR died of general weakness and tumor necrosis in the liver. One of the two non-responders succumbed to carci-

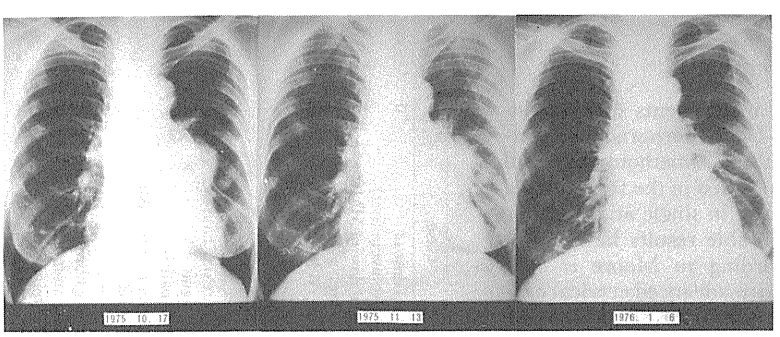


FIG. 9A. A 53-year-old woman with lung and bone metastases of cervical cancer (case 7). Upper photographs show the chest films before treatment (left), after the second course (middle) and after the fourth (right).

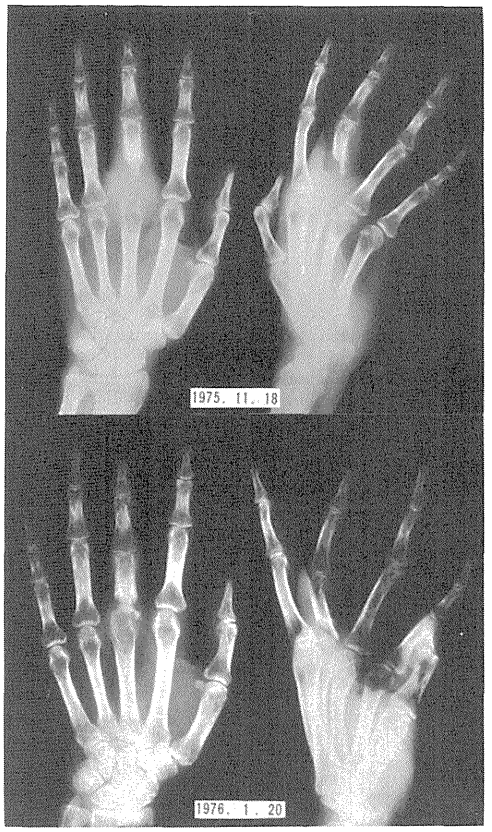


FIG. 9B. Lower photographs are bone roentogenogram of the left hand after the second course (upper) and after the fifth course (lower).

nomatous peritonitis. The mean survival for six patients already dead was 6.6 months while the other nine patients are still alive after 3 to 30 months. Although the period of study was not sufficient for the assessment of survival, we are confident that the prognosis will prove to be better than previous reports.

## DISCUSSION

A number of anti-cancer agents such as vincristine, actinomycin-D, 5-fluorouracil, 6-Mercaptopurine, chlorambucil, methotrexate and Adriamycin have been tested in the treatment of advanced cervical cancer in single and systemic use. However, no favorable results have as yet been reported.[6] According to Moore *et al.*,[16] three of five patients with advanced cervical cancer had partial response to MMC. In spite of the

TABLE 4. Bleomycin-Mitomycin-C (B-M) Toxicity

| Case | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | Average dose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total dose of bleomycin (mg) | 70 | 30 | 175 | 175 | 70 | 140 | 175 | 175 | 105 | 70 | 105 | 140 | 70 | 140 | 105 | 70 | 120 |
| Total dose of MMC (mg) | 50 | 26 | 50 | 50 | 20 | 40 | 50 | 50 | 40 | 20 | 30 | 40 | 20 | 30 | 20 | 20 | 37 |
| Min. WBC ($mm^3$) | 1000 | 1700 | 3300 | 2600 | 1200 | 3200 | 2200 | 1900 | 5400 | 2500 | 7500 | 3300 | 3600 | 1900 average (3100) | 5700 | 4100 | Incidence (%) |
| Min. platelet ($\times 10^4/mm^3$) | 6.5 | 6.5 | 13.5 | 8.0 | 3.0 | 10.5 | 9.5 | 11.0 | 5.5 | 10.0 | 22.5 | 17.5 | 20.5 | 19.5 average (16.0) | 34.0 | 41.0 | 33* 40* |
| Fever 38°C | + | – | – | – | – | – | + | – | – | – | – | – | – | – | – | – | 13 |
| Anorexia, nausea | + | – | + | – | – | + | – | ++ | + | – | – | – | – | + | – | – | 36 |
| Fatigue | + | – | + | + | – | + | – | + | + | – | – | – | – | + | – | – | 43 |
| Stomatitis | – | – | – | – | – | – | – | + | – | – | – | – | – | – | – | – | 6 |
| Pigmentation (skin and nail) | – | – | – | + | – | + | – | + | – | – | – | – | – | – | – | – | 20 |
| Alopecia | – | – | – | – | – | – | ++ | – | ++ | ++ | – | – | – | – | – | – | 20 |
| Lung fibrosis | – | – | – | – | – | ++ | – | ++ | – | – | – | – | – | – | – | – | 13 |
| Hepatotoxicity | – | + | – | – | ++ | – | – | – | + | – | – | – | – | – | – | – | 18 |
| Proteinuria | – | – | – | – | – | – | – | – | + | – | – | – | – | – | – | – | 6 |
| Drug eruption | + | ++ | – | – | + | – | – | – | – | – | – | – | – | – | – | – | 18 |

+ mild, ++ severe.
* Percentage of leucopenia ($< 2000/mm^3$), thrombocytopenia ($< 1.0 \times 10^5/mm^3$).

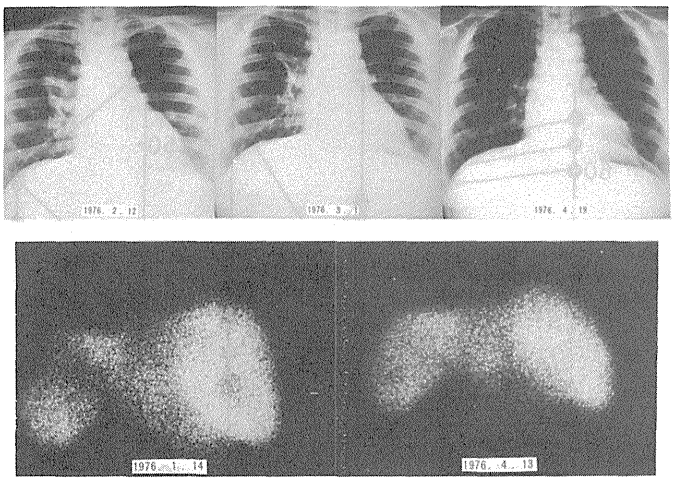

FIG. 10. A 49-year-old woman with lung and liver metastases of cervical cancer (case 8). Upper photographs show the chest films before treatment (left), after the second course (middle) and after the fifth (right). Lower photographs show the liver scans with $^{99m}$Tc phytate before treatment (left) and after the fifth course (right).

expectation based on the potential anti-tumor effect of BLM on squamous cancer, several reports described an overall response rate of about 20 to 30% with no complete remission when BLM was used singly.

As a next step, combinations of these drugs have been designed. Recently, methotrexate and Adriamycin have been tested in connection with BLM. Methotrexate alone brought about approximately the same response rate as BLM, but no cross resistance was demonstrated to the drug.[9] In single use of Adriamycin, three of five patients responded including one with complete remission, but there was no enhancement in response rate when combined with BLM.[1] Conroy *et al.*,[7] however, reported that by a combination treatment with BLM and methotrexate, 12 out of 20(60%) had a greater than 50% shrinkage of measurable tumor masses, lasting a median remission period of 7.5 months. Ota *et al.*[17] also reported that 43 patients with metastatic lung tumors from the cervix had a response rate of 35% including 16% with tumor shrinkage in diameter of over 50% with multi-agent chemotherapy which included MMC, Cytoxan, thiotepa, Toyomycin, and others; the response lasted 4.0 months.

In contrast to these reports, our results obtained by B-M therapy have been remarkable in all clinical aspects. Above all the remission effects are characterized by the high response rate (93%) and high complete response rate (80%) which were obtained irrespective of the tissues involved with metastatic cancer. The rapid disappearance of signs and symptoms (Table 3) and tumor regression observed in the patients with CR make it possible to determine if a tumor was responsive to the therapy or not before significant side effects would appear on unresponsive patients. The patients not receiving further therapy after the achievement of CR had relapse. The medication of CQ for maintenance, however, seems to prevent the relapse successfully and contribute to the improvement of sur-

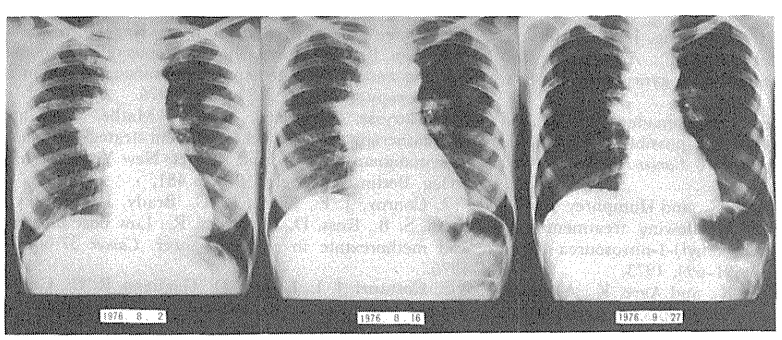

FIG. 11. A 43-year-old woman with lung metastases of cervical cancer (case 9). The chest roentogenograms before treatment (left), after the second course (middle) and after the fourth (right). A remaining coin lesion in the left upper lung in the film (right) had no thickness in a lateral film.

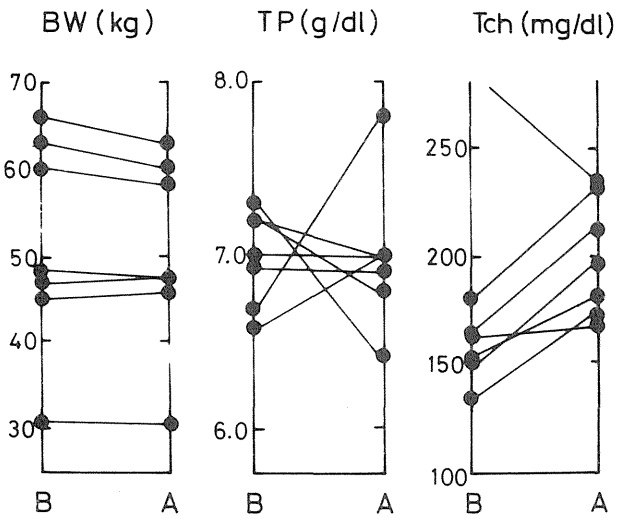


FIG. 12. Changes in body weight (BW), serum total protein (TP) and cholesterol (Tch) before (B) and after (A) the treatment in patients receiving more than four courses.

vival, though the problem of the dose and time of CQ treatment remains to be solve.

The dose-limiting toxicity of the therapy was lung fibrosis for BLM and myelosuppression for MMC, respectively. The frequency of lung fibrosis throughout the treatment was 12%(2/16). From the small number of patients it is difficult to conclude that the above value was significantly higher than that in single use.[4,29] Myelosuppression appeared consistently as the dose of MMC increased. Frequent thrombocytopenia with an average dose of 37 mg of MMC seemed to be due to the past irradiation of over 3,000 rads to the entire pelvis of these patients.

Our recent clinical trials for cancers of the lung, head and neck, and esophagus are revealing that these drastic effects seem to be specific for squamous cell carcinoma, which has the capability to take up BLM efficiently without inactivating the drug.[26]

As mentioned in the introduction, BLM alone gave a response rate of 20 to 30%, and, in fact, in the present regimen none of the patients showed regression of tumor before the administration of MMC. The enhanced effect of MMC after treatment with BLM clearly suggested that there was certain potential damage induced by BLM in the tumor. The possible explanations for the enhanced response are that: (1) MMC may exert preferential killing effect on cells[18] which were blocked at G2 by successive treatment of proliferating tumor cells with BLM,[2,8,24,28] or (2) tumor cells may be precipitated by an unknown combined action of the two drugs on cellular DNA, which is broken by BLM[23] and cross-linked by MMC,[20] respectively. In addition, a preferential lethal effect of BLM[3,25] and MMC[13-15] on non-proliferating cells may not be dismissed. Studies on the cell biological basis of the present modality are now under way.

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