KOREAN JOURNAL OF ZOOLOGY
Vol. 20, No. 1, 41—48 (1977)

DNA Repair Synthesis Induced by Bleomycin in HeLa S_3 Cells Pretreated with Base Analogs

Kyung Il Um and Sang Dai Park
(Dept. of Zoology, Seoul National University)

鹽基相似體를 前處理한 HeLa S_3 細胞에 있어 Bleomycin에 의한 DNA 回復合成

嚴 慶 一 · 朴 相 大
(서울大 自然大 動物學科)
(Received February 28, 1977)

적 요

Bleomycin에 의해 유발된 DNA 회복합성은 저농도 처리군에서는 농도의 증가에 따라 증가하며 5μg/m*l* 군에서 조사한 전세포의 15%가 회복합성을 하여 최고율을 보인다. 고농도 처리군에서는 DNA 회복합성율이 감소하며 처리 시간을 연장해도 그율은 변화가 없다.

BUdR이나 IUdR을 전처리한군에서는 DNA 회복합성을 증가 시키는 것으로 판명됐으며 또한 고농도 처리군에서는 정상적인 DNA 합성을 억제한다. 시간 변화에 따른 실험에서는 처리한 bleomycin을 제거한후 24시간까지 DNA 회복합성이 계속됐다.

이들 결과는 bleomycin이 excision repair를 유발하는 효과적인 화학물질이 아니며, bleomycin에 의해 유발되는 DNA의 손상은 DNA 나선 절단뿐만 아니라 다른 형태의 DNA 손상도 유발함을 추측할수 있다.

INTRODUCTION

Bleomycin, a glycopeptide complex isolated from *Streptomyces verticillus* (Umezawa *et al.*, 1966), has widely been used as an antineoplastic antibiotic against lymphoma and squamous carcinoma (Daskal *et al.*, 1976). This drug has been shown to cause an inhibition of DNA synthesis (Müller *et al.*, 1972; Wheatley

et al., 1974), single-strand scissions of DNA (Fujiwara and Kondo, 1973; Iqbal *et al.*, 1976) and double-strand breaks and/or disruption of DNA-lipid complexes (Saito and Andoh, 1973; Byfield *et al.*, 1976). Although mechanism underlying its effects on DNA remains to be elucidated, available information seems to suggest that damages induced in DNA by bleomycin are similar to those by X-rays (Byfield *et al.*, 1976).

Repair processes of such damage may then be expected to be similar to those involved in the repair of X-ray damage, but it has been contradictory. For example, repair of single-strand breaks induced by bleomycin has been reported to occur immediately (Terasima *et al.*, 1970; Byfield *et al.*, 1976), to be inhibited (Miyaki *et al.*, 1971), to have biphasic processes (Clarkson and Humphrey, 1976; Iqbal *et al.*, 1976), or to be inhibited at first and then soon rejoined (Fujiwara and Kondo, 1973). These results indicate that not only the repair processes but also the type of damage induced in DNA by bleomycin have not yet been fully understood. Moreover DNA repair synthesis, which provides information for both DNA damages and their processes, has scarcely been reported in bleomycin-treated cultures.

An incorporation of base analog into DNA has been reported to enhance X-ray or ionizing type chemical, methyl methanesulfonate, induced DNA repair synthesis (Lohman *et al.*, 1972; Sawada and Okada, 1972; Park and Um, 1975; Park, 1976). To date, the effect of base analog on bleomycin-induced DNA repair synthesis has not been attempted to study. As indicated, DNA damages produced by bleomycin have been postulated to be similar to those by ionizing radiation. If so, it is a matter of some considerable interest to determine whether any thymidine analogs would lead to enhancing effect on bleomycin-induced DNA repair synthesis.

The present studies were therefore undertaken to characterize the bleomycin-induced DNA damages and their repair processes in terms of excision repair and to determine the effects of base analogs on bleomycin-induced DNA repair synthesis.

MATERIALS AND METHODS

HeLa S_3 cells, an established cell line derived from human cervical carcinoma, were used throughout this investigation. Monolayer cultures of this cell line were grown using Eagles' minimum essential medium (MEM) (Gibco) supplemented with 10% fetal calf serum and antibiotics (penicillin G, 100 units/m*l*; streptomycin, 100 μg/m*l*; kanamycin, 50 μg/m*l*).

Bleomycin A_2 (Nihon Kayaku), for clinical use, was dissolved in sterile 0.9% NaCl solution as 1 mg/m*l* stock solution and further diluted to various working

concentration in the serum-free medium. For the induction of DNA repair synthesis, an appropriate number of cells ($1.0\sim5.0\times10^6$ cells/ml) grown on cover slips (9×50 mm, Bellco) in Leighton culture tubes for 48 hours were treated with bleomycin.

For the determination of the effects of base analogs on bleomycin-induced DNA repair synthesis, the cells grown for 24 hours were exposed to 0.2 mM 5-bromodeoxyuridine (BUdR) or 5-iododeoxyuridine (IUdR) (Sigma) for 24 hours, approximately one generation time, prior to the bleomycin treatment.

DNA repair synthesis was carried out in two distinct procedures of unscheduled DNA synthesis, dose response and time dependence. For the dose response experiment, ^{3}H-thymidine (Amersham/Searle) was incorporated into the cultures at a final concentration of 10 μCi/ml (specific activity, 40~60 Ci/mM) for 1 or 1½ hours immediately after treatment with various concentrations of bleomycin for 1 or 3 hours. For the time dependence study, cells were labeled with ^{3}H-thymidine for an hour from 0 to 24 hours after treatment with bleomycin. ^{3}H-thymidine labeling was terminated by washing the cells in cold phosphate buffered saline (PBS) containing unlabeled thymidine. The cells were treated with hypotonic solution, fixed, and then stained. Autoradiograms were prepared using autoradiographic stripping plate (Kodak AR-10), and the DNA repair synthesis was analyzed according to the criteria as described previously (Park and Um, 1975).

RESULTS

The dose responses for the bleomycin-induced DNA repair synthesis (UDS) together with the total labeling index (LI) and the effects of bleomycin on semiconservative DNA synthesis (NDS) are shown in Fig. 1.

In the control, the majority of the labeled cells were found to be semiconservative DNA synthesizing cells and the spontaneous unscheduled DNA synthesis was also detected but the proportion was negligible (0.5%)

The induced DNA repair synthesis by bleomycin was shown to increase in direct proportion up to 5 μg/ml for both 1 and 3 hours treatments and then decreased thereafter. The maximum rate of DNA repair synthesis appeared at this dose represents about 15% of total cells analyzed. The longer treatment of this drug, the more induction of DNA repair synthesis was observed, but at higher doses the rates of it were not affected by the prolongation of drug treatment.

Bleomycin does not seem to inhibit the semiconservative DNA synthesis except at higher dose and for longer treatment, because only 5~7% of heavily labeled cells were reduced at doses above 10 μg/ml for 3 hours comparing to the control. The labeling indices were also shown to increase up to dose of 5 μg/ml, and the

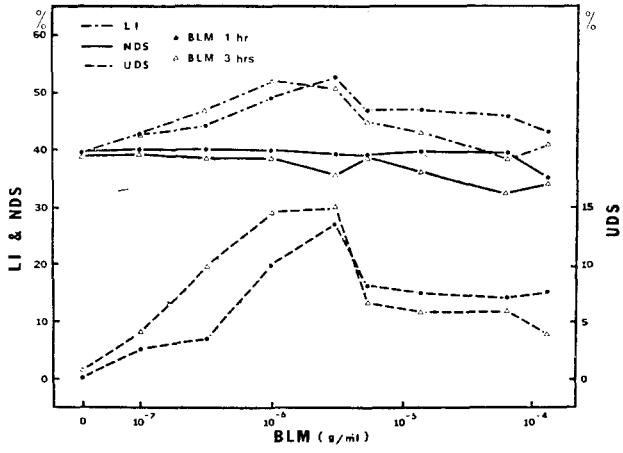

Fig. 1. Dose responses for labeling index, DNA repair synthesis and semiconservative DNA synthesis in bleomycin-treated HeLa S_3 cells.

increased indices were found to be mainly due to increases of lightly labeled cells, but not by decreases of heavily labeled cells.

Table 1 shows the effects of thymidine analogs on labeling index and labeling patterns of bleomycin-treated cells, and the sensitization effects of these base analogs on bleomycin-induced DNA repair synthesis is depicted in Fig. 2.

The single treatment with either of these analogs does not seem to induce DNA repair synthesis. In the combined treatment with BUdR or IUdR and bleomycin, the labeling indices and lightly labeled cells (UDS) were shown to

Table 1. Effects of thymidine analogs on Bleomycin-induced DNA repair synthesis.

Treatments*		Labeling index (%)	Labeling pattern(%±S.E.)	
BUdR or IUdR 0.2 mM	BLM (μg/ml)		Heavily labeled (NDS)	Lightly labeled (UDS)
	—	38.9	38.1±2.1	0.8±0.3
BUdR	—	42.9	40.8±2.1	2.1±0.6
BUdR	0.1	48.4	39.3±2.1	9.1±1.2
BUdR	0.5	57.9	38.3±2.1	19.6±1.7
BUdR	1.0	66.6	29.8±2.0	36.8±2.1
BUdR	5.0	59.6	25.3±1.9	34.3±2.1
IUdR	—	40.8	38.6±2.1	2.2±0.6
IUdR	0.1	50.9	37.7±2.1	13.2±1.5
IUdR	0.5	52.3	28.7±2.0	23.6±1.8
IUdR	1.0	52.4	25.0±1.9	27.4±1.9
IUdR	5.0	45.5	23.3±1.8	22.4±1.8

* BUdR or IUdR for 24 hours, BLM for 3 hours and ^{3}H-TdR for 1.5 hours.

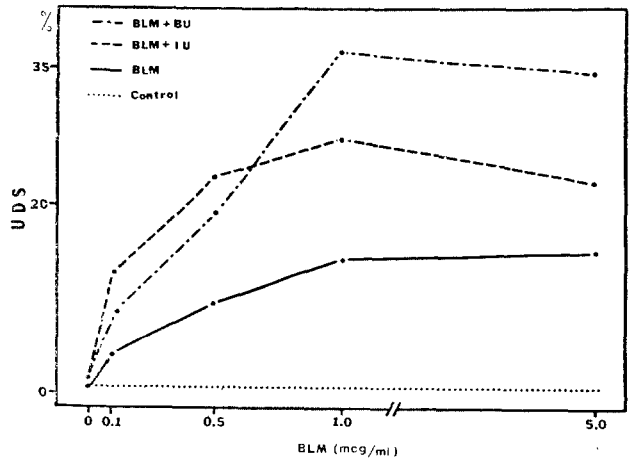

Fig. 2. Effects of base analogs on bleomycin induced DNA repair synthesis.

increase in all dose ranges as compared with the corresponding bleomycin treated group (Fig. 1). At higher dose ranges, however, the percentages of heavily labeled cells (NDS) were found to be reduced and these tendencies were more marked in IUdR pretreated group. These results strongly indicate that these base analogs are to enhance DNA repair synthesis and also to interfere with semiconservative DNA synthesis at higher doses (Fig. 2 and Table 2)

Table 2. Comparison of Bleomycin-induced DNA repair synthesis in HeLa S_3 cells with or without pretreatment with thymidine analogs.

Treatments	Average UDS within the dose ranges (%±S.E.)	Average NDS within the dose ranges (%±S.E.)
Control	0.8±0.3	38.1±2.1
BLM	10.9±1.3	37.2±2.1
BUdR + BLM	24.9±1.9	33.1±2.1
IUdR + BLM	21.6±1.8	28.7±2.0

The overall results for dose response study suggest that bleomycin seems not to be an effective chemical in inducing excision repair and the rate of it is dosedependent within certain level, and that the thymidine analogs used are both potent sensitizers enhancing bleomycin-induced DNA repair synthesis.

Fig. 3 represents the time dependence of DNA repair synthesis induced by three different doses of bleomycin. As shown in the figure, bleomycin-induced excision repair occurs for as long as 24 hours after removal of bleomycin. At lower dose, the maximum percentage of DNA repair synthesis appeared at 12 hours but this tendency was not found in other two dose groups. These data may suggest that the damage induced in DNA by bleomycin might include not only single strand breaks but also other types of damages.

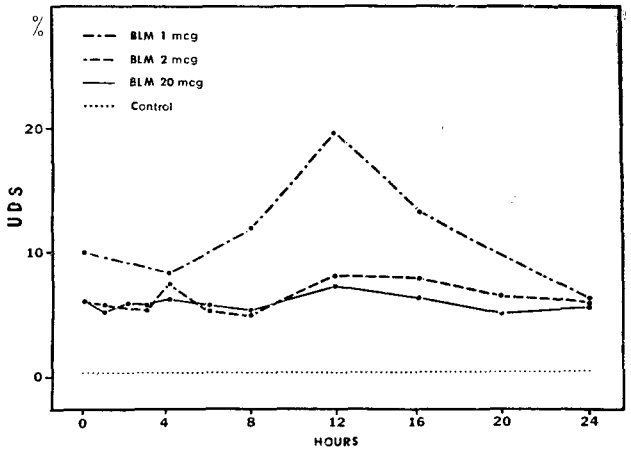

Fig. 3. Time dependence of DNA repair synthesis induced by bleomycin.

DISCUSSION

Ever since bleomycin was reported to have an antineoplastic activity by Umezawa *et al.* (1966), a number of investigators have attempted to elucidate the action mechanism of this drug using a variety of normal and cancer cells both *in vivo* and *in vitro*. Accumulated data indicate that one of main damages caused by this antibiotic seems to be single strand breaks of DNA (Iqbal *et al.*, 1976; Clarkson and Humphrey, 1976). This type of damage is characteristically produced by ionizing radiation, and the repair of it is characterized by an insertion of few nucleotides per break (Park, 1975). Accordingly the rate of excision repair is usually lower as compared with that of base damage produced by UV-light.

Fujiwara and Kondo (1973) were first able to detect bleomycin-induced unscheduled DNA synthesis following treatment with 25~100 μg/m*l* for 30 minutes. They failed to find the dose-dependent increase of DNA repair synthesis within the dose range. Byfield *et al.* (1976) subsequently reported repair replication after 250 μg/m*l* treatment. Both studies consistently showed a small amount of DNA repair synthesis stimulated by this drug. The present results for dose response experiment are generally consistent with those data.

Because the DNA repair synthesis is involved in the processes of ligation and polymerization after excision of the damaged strand, the present results do not support the hypothesis that bleomycin inhibits the repair of single strand breaks by DNA ligase (Miyaki *et al.*, 1971). In this respect, these results are good accord with other published data.

Byfield *et al.* (1976) suggested that damages induced by X-rays and bleomycin are quite similar both in their induction and repair. However, the results pres-

ented here concerning the time dependence study showed that bleomycin-induced excision repair occurrred as long as for 24 hours. This might strongly indicate that single strand breaks seem not to be sole damage caused by bleomycin. Evidences for this speculation have also been accumulated. Clarkson and Evans (1972) already suggested that the fast reaction processes of DNA repair synthesis might be involved in the rejoining of single strand breaks, and that the slow processes may be associated with damages other strand breaks. Iqbal *et al.* (1976) reported that bleomycin cause extensive breakage in some DNA strands, while leaving others relatively intact. Clarkson and Humphrey (1976) showed that although majority of strand rejoining is completed within two hours, a small percentage of mitotic DNA is in the form of unrepaired short strands. In addition, Byfield *et al.* (1976) demonstrated bleomycin caused disruption of DNA-lipid complexes.

So far no one has yet attempted to study effects of the combined treatment with base analog and bleomycin. The present results clearly show that both BUdR and IUdR were found to have a sensitization effect on bleomycin-induced DNA repair synthesis. These results confirmed the previous finding which showed that thymidine analogs enhanced methyl methanesulfonate stimulated-DNA repair synthesis (Park and Um, 1975; Park, 1976). However, the mechanism involved in how the substituted base with BU or IU interact with DNA resulting in the sensitization effect of this drug to enhance DNA repair synthesis is unknown.

A more detailed experiment with dose protraction and varying time of treatment combined with these base analogs may be expected to provide more useful information on the action mechanism of bleomycin.

SUMMARY

Dose response of DNA repair synthesis induced by bleomycin was dose-dependent in lower doses, and maximum rate of it at 5 μg/m*l* represents about 15% of total cells analyzed. At higher doses DNA repair synthesis was reduced and the rate of it remained unchanged even prolonged treatment.

Pretreatment with BUdR or IUdR was found to enhance DNA repair synthesis and also to interfere with semiconservative DNA synthesis at higher doses. Time dependence study showed that DNA repair synthesis occurrred as long as for 24 hours after removal of bleomycin.

These results seem to suggest that bleomycin is not to be an effective chemical in inducing excision repair and that damages induced in DNA by this drug might include not only strand breaks but other types of DNA damage.

REFERENCES

Byfield, J.E., Y.C. Lee, L. Tu, and F. Kulhanian, 1976. Molecular interactions of the combined effects of bleomycin and x-rays on mammalian cell survival. *Cancer Res.* **36** : 1138—1143.

Clarkson, J.M., and H.J. Evans, 1972. Unscheduled DNA synthesis in human leucocytes after exposure to UV light, γ-rays and chemical mutagens. *Mutation Res.* **14** : 413—430.

Clarkson, J.M., and R.M. Humphrey, 1976. The significance of DNA damage in the cell cycle sensitivity of chinese hamster ovary cells to bleomycin. *Cancer Res.* **36** : 2345—2349.

Daskal, Y., F. Gyorkey, P. Gyorkey, and H. Busch, 1976. Ultrastructural study of pulmonary bleomycin toxicity. *Cancer Res.* **36** : 1267—1272.

Fujiwara, Y., and T. Kondo, 1973. Strand-scission of HeLa cell deoxyribonucleic acid by bleomycin in vitro and in vivo. *Biochem. Pharmac.* **22** : 323—333.

Iqbal, Z.M., K.W. Kohn, R.A.G. Ewig, and A.J. Fornace, Jr., 1976. Single-strand scission and repair of DNA in mammalian cells by bleomycin. *Cancer Res.* **36** : 3834—3838.

Lohman, P.H.M., D. Bootsma, and A.H. Hey, 1972. The influence of 5-bromodexyuridine on the induction of breaks in the deoxyribonucleic acid of cultivated human cells by X-irradiation and ultraviolet light. *Radiation Res.* **52** : 627—641.

Miyaki, M., T. Ono, and H. Umezawa, 1971. Inhibition of ligase reaction by bleomycin. *J. Antibiotics* **24** : 587—591.

Müller, W.E.G., Z-i. Yamazaki, H-J. Breter, and R.K. Zahn, 1972. Action of bleomycin on DNA and RNA. *Eur. J. Biochem.* **31** : 518—525.

Park, S.D., 1975. Gamma-ray induced DNA repair synthesis in relation to chromosome exchanges in mammalian cells in vitro. *Korean J. Zool.* **18** : 41—49.

Park, S.D., and K.I. Um, 1975. Sensitization effects of thymidine analogs on methyl methanesulfonate induced DNA repair synthesis. *Korean J. Zool.* **18** : 131—140.

Pārk, S.D., 1976. Methyl methanesulfonate stimulated ^{3}H-thymidine uptake in synchronized HeLa S_3 suspension cells pretreated with thymidine analogs. *Korean J. Zool.* **19** : 113—122.

Saito, M., and T. Andoh, 1973, Breakage of a DNA-protein complex induced by bleomycin and their repair in cultured mouse fibroblasts. *Cancer Res.* **33** : 1696—1700.

Sawada, S., and S. Okada, 1972. Effects of BUdR-labeling on radiation-induced DNA breakage and subsequent rejoining in cultured mammalian cells. *Int. J. Biol.* **21** : 599—602.

Terasima, T., M. Yasukawa, and H. Umezawa, 1970. Breaks and rejoining of DNA in cultured mammalian cells treated with bleomycin. *Gann* **61** : 513—516.

Umezawa, H., K. Maeda, T. Takeuchi, and Y. Okami, 1966. New antibiotics, bleomycin A and B. *J.Antibiotics* **19** : 200—209.

Wheatley, D.N., G.C. Mueller, and K. Kajiwara, 1974. Antimitotic activity of bleomycin: time of action in the manmalian cell cycle. *Br. J. Cancer*, **29** : **117—131**