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

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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]

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 5×10^7 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 μg/ml) plus T_1 RNase (100 units/ml) and subsequently with pronase (20 μg/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 μg ^{14}C-DNA, 0·003 M ME, 50 μg/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 μg/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 μg 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 μg 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 μg/ml (30 min), which is almost twice as high as the D_0 dose [12 μg/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 μg/ml (30 min). In this resistant part of the survival curve, a BLM dose as high as 100 μg/ml reduces survival to only 15 per cent. Therefore, the toxicity of BLM to HeLa S3 cells seems to be moderate.[13]

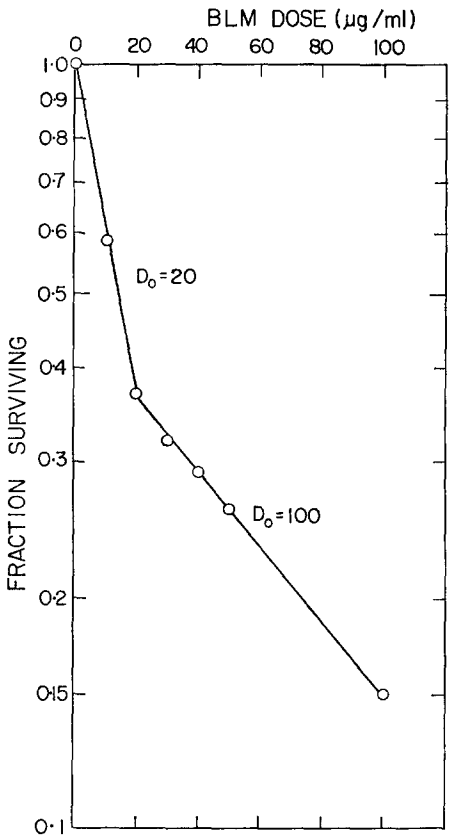

FIG. 1. Effect of BLM (30-min exposure) on survival of asynchronously growing HeLa S3 cells.

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.

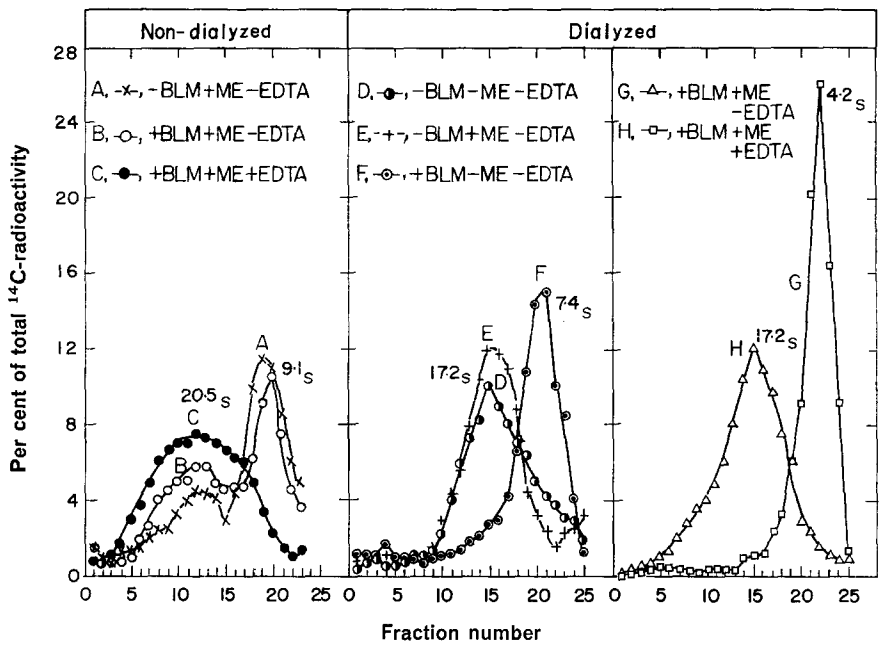

FIG. 2. Effects of BLM, ME and EDTA on the sedimentation profile of extracted HeLa S3 DNA *in vitro*. Purified HeLa ^{14}C-DNA (5 μg; 2300 counts/min) was incubated for 30 min at 37° in 0·0125 M Tris-buffered reaction mixture (0·2 ml, pH 7·6). Variable constituents (50 μg/ml BLM, 0·003 M ME and 0·025 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 μg/ml of BLM breaks a little of the intracellular DNA during a 30-min treatment before the cell lysis.

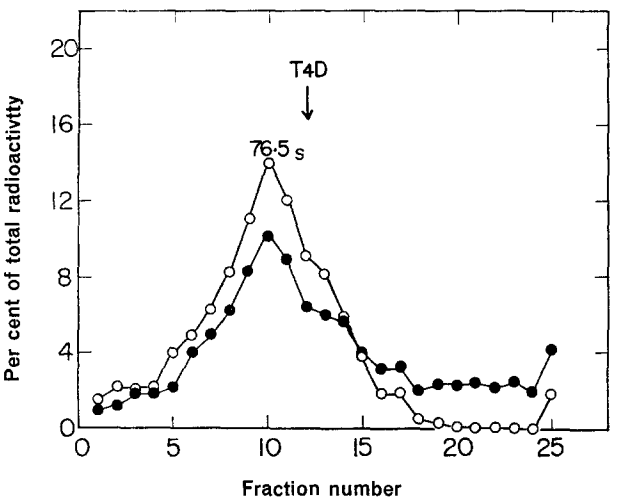

FIG. 3. Effect of EDTA in the lysing solution on the fragmentation of intracellular DNA by BLM. HeLa S3 cells prelabeled with 0·5 μCi/ml of ^{3}H-TdR for 24 hr and chased for 1 hr were treated with 0 (○——○) or 25 μg/ml of BLM (●——●) for 30 min at 37°. The cells were lysed in 0·25% (w/v) SDS–0·015 M EDTA–10% (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

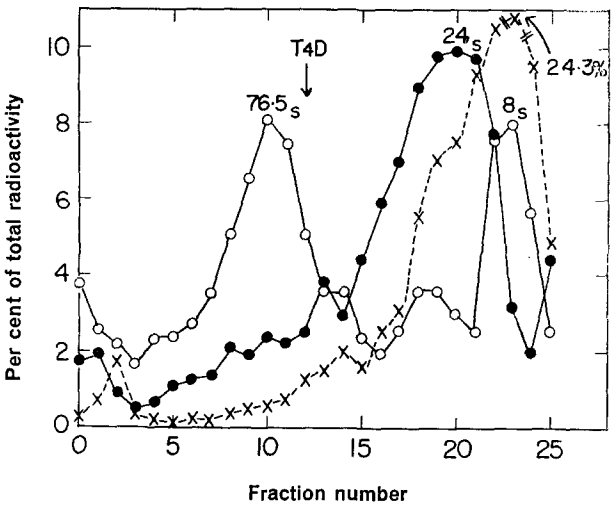

FIG. 4. Effect of cell lysis in the absence of EDTA on the fragmentation of intracellular DNA by BLM. Except that the lysing solution lacks EDTA, all other procedures are the same as described in the legend to Fig. 3. O——O, ×---×, control (no BLM); ●——●, 25 μg/ml BLM-treated.

(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

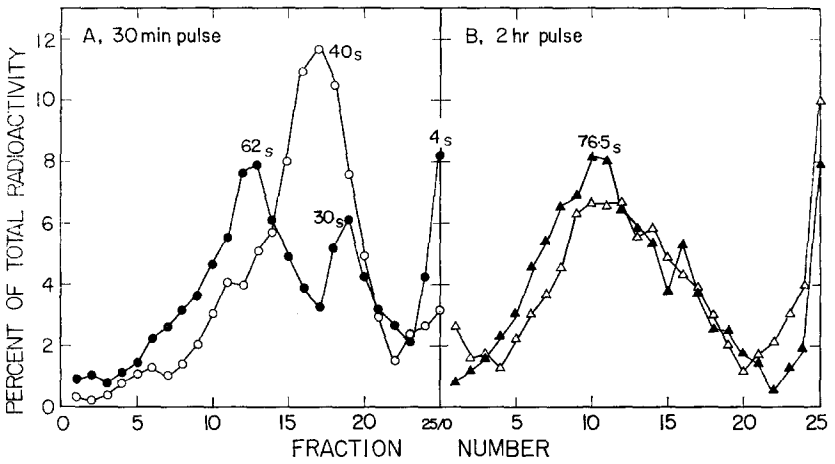

FIG. 5. Effect of BLM on the joining of newly replicating DNA *in vivo*. BLM (25 μg/ml) was added to exponentially growing HeLa S3 cells 30 min before the start of pulse-labeling with 23·4 μCi/ml of ^{3}H-TdR and present continuously during the labeling. The procedures for lysing cells and centrifugation 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 (μg/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 μCi/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 μg/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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