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## Abstract

To investigate factors involved in excision repair DNA synthesis, a soluble extract was prepared from permeable mouse sarcoma (SR-C3H/He) cells by homogenization and ultracentrifugation. DNA synthesis measured by using native calf thymus DNA as the template-primer and the extract as the polymerase source showed low activity. The DNA synthesis was enhanced more than ten-fold by the addition of an appropriate concentration of bleomycin, a radiomimetic DNA-damaging drug. Using selective inhibitors of DNA polymerases, it was shown that the DNA polymerase involved in the bleomycin-induced DNA synthesis was DNA polymerase beta. In addition to DNA polymerase beta, an exonuclease which converts bleomycin-damaged DNA into suitable template-primers for repair DNA synthesis appeared to be present in the permeable cell extract.

**KEYWORDS:** DNA repair, DNA polymerase ?, exonuclease, bleomycin, permeable mouse sarcoma cells

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# Bleomycin-induced DNA Synthesis in a Cell-free System Using a Permeable Mouse Sarcoma Cell Extract

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To investigate factors involved in excision repair DNA synthesis, a soluble extract was prepared from permeable mouse sarcoma (SR-C3H/He) cells by homogenization and ultracentrifugation. DNA synthesis measured by using native calf thymus DNA as the template-primer and the extract as the polymerase source showed low activity. The DNA synthesis was enhanced more than ten-fold by the addition of an appropriate concentration of bleomycin, a radiomimetic DNA-damaging drug. Using selective inhibitors of DNA polymerases, it was shown that the DNA polymerase involved in the bleomycin-induced DNA synthesis was DNA polymerase $\beta$. In addition to DNA polymerase $\beta$, an exonuclease which converts bleomycin-damaged DNA into suitable template-primers for repair DNA synthesis appeared to be present in the permeable cell extract.

*Key words :* DNA repair, DNA polymerase $\beta$, exonuclease, bleomycin, permeable mouse sarcoma cells

Bleomycin, a glycopeptide antibiotic with antineoplastic activity (1-3), causes DNA damage and induces unscheduled DNA synthesis (UDS) in mammalian cells both *in vivo* and *in vitro* (4-7). The major cleavage product induced by bleomycin is shown to have 3′-phosphoglycolate termini (8, 9), the same termini observed in some ionizing radiation-induced strand breaks (10). In the repair of lesions having 3′-phosphoglycolate termini by DNA polymerase, the damaged sites are thought to be recognized and modified to short gaps with 3′-hydroxyl termini by an exonuclease(s) (unidentified in mammalian cells) as observed in *E. coli* by Niwa and Moses (11). The fact that bleomycin-treated permeable mammalian cells show highly active repair DNA synthesis indicates that the recognition and modification (activation for reparative DNA synthesis) of bleomycin-damaged sites can occur actively in permeable cells (12). The purpose of the present experiment was to establish a cell-free system for studying bleomycin-induced excision repair DNA synthesis by dissolving the permeable mammalian cells.

## Materials and Methods

Mouse ascites sarcoma (SR-C3H/He) cells were obtained and maintained as described previously (13). SR-C3H/He cells were permeabilized by treatment with buffer A containing 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0 (13), and stored at −20°C until use. The stored permeable cells were suspended in buffer B (0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercapto-

ethanol, pH8.0) supplemented with Triton X-100 at 0.0175% (Triton-buffer B)(12). The suspension was centrifuged at 1,000×g for 15 min, and the precipitated cells were suspended in Triton-buffer B at $5\times10^7$ cells per ml. The suspension was homogenized 80 strokes in a Dounce homogenizer. The homogenate was centrifuged at 80,000×g for 60 min, and the supernatant was stored at −20°C and used as the cell extract in the following experiments.

DNA synthesis in a cell-free system was assayed in a mixture (0.3 ml in final volume, the standard assay mixture) consisting of 0.05 ml of the extract, 0.10 ml of a substrate mixture for DNA synthesis (100 mM Tris-HCl, 7.5 mM $MgCl_2$, 240 mM NaCl, 150 μM dATP, 30 μM dCTP, 150 μM dGTP and 7.5 μM [$^3$H]dTTP at 1 Ci/mmol, pH8.0), and 0.15 ml of Triton-buffer B containing 30 μg of sheared calf thymus DNA (Type I, Sigma Chemical Co., St. Louis), 1 μg bleomycin $A_2$ (Nippon Kayaku Co., Tokyo) and 20 μM Fe(II) (ferrous ammonium sulfate, Katayama Chemicals, Osaka). DNA polymerase activity was assayed in a mixture (0.3 ml in final volume, the polymerase assay mixture) consisting of 0.10 ml of the substrate mixture for DNA synthesis, 0.05 ml of the extract and 0.15 ml of Triton-buffer B containing 30 μg of activated DNA(14). Duplicate samples were incubated at 37°C for 30 min. The reaction was stopped by chilling in an ice-water bath, and 200 μg bovine serum albumin was added to each assay tube as a carrier. The radioactivity incorporated into acid-insoluble materials was measured by a disc method(13).

Nuclease activity in the extract was determined by measuring acid-soluble radioactivity released from $^3$H-labeled DNA. SR-C3H/He cell DNA was labeled with [$^3$H]deoxythymidine ([$^3$H]dThd, 50 Ci/mmol, 0.2 μCi/ml) for 48 h. The $^3$H-labeled DNA was extracted and purified by a previously described method using sodium lauryl sulfate, proteinase K, ribonuclease A and a chloroform-isoamylalcohol (24 : 10) solution (12). The nuclease assay mixture (0.3 ml/tube) contained the same components as that for the assay of bleomycin-induced DNA synthesis in the cell-free system, except that cold dTTP replaced [$^3$H]dTTP and $^3$H-labeled DNA (2.5 μg, about 6,000 cpm) was added to the mixture. The mixture was incubated at 37°C for 30 min. The reaction was stopped by chilling in an ice-water bath. Two-tenths ml of ice-cold distilled water and 0.5 ml of an ice-cold solution containing 1 N perchloric acid and 1.4 M NaCl were added to the mixture. The samples were centrifuged at 10,000×g for 20 min. Radioactivity of the acid-soluble supernatant and of the acid-insoluble residue was measured as described previously(12).

## Results and Discussion

*Effects of varying the concentrations of bleomycin on DNA synthesis in the cell-free system.* DNA synthetic activity measured by using sheared calf thymus DNA and the permeable cell extract was low, because the DNA had few priming sites for DNA synthesis (Fig. 1). The DNA synthesis increased dose-dependently with the addition of bleomycin in the concentration range of 0.025 and 0.5 μg per 0.3 ml of the reaction mixture, and more than ten times the DNA synthesis of the bleomycin-free control was attained at the bleomycin concentrations of 0.25-2.5 μg/0.3 ml. The DNA synthesis decreased when the concentration of bleomycin was over 1 μg/0.3

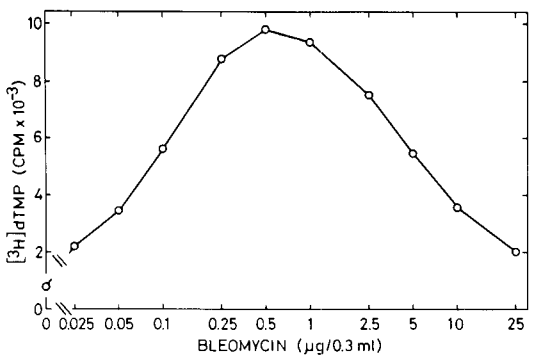


**Fig. 1** Effects of varying concentrations of bleomycin on DNA synthesis in the cell-free system using a permeable cell extract. The permeable cell extract was prepared from SR-C3H/He cells, and DNA synthesis was measured in the standard assay mixture as described in Materials and Methods except that the concentration of bleomycin was varied as indicated. The data are expressed as cpm of [$^3$H]dTMP incorporated in the acid-insoluble fraction per tube/30 min.

ml. No DNA synthesis was observed by the omission of either the template DNA or the cell extract from the standard assay mixture.

*Effects of varying concentrations of Fe(II) on bleomycin-induced DNA synthesis.* The bleomycin-induced DNA synthesis was markedly reduced by the omission of Fe(II), increased by the addition of increasing concentrations of Fe(II), and reached a plateau at the concentration of 20 $\mu$M of Fe(II), the concentration used in the standard assay mixture (Fig. 2). Bleomycin-independent DNA synthesis was not stimulated by Fe(II). The stimulation of bleomycin-induced DNA synthesis by Fe(II) was thought to be due to the stimulative effect of Fe(II) on DNA damage by bleomycin, as reported previously (7). Ample evidence indicates that DNA damage by bleomycin depends on Fe(II) and oxygen (8, 15).

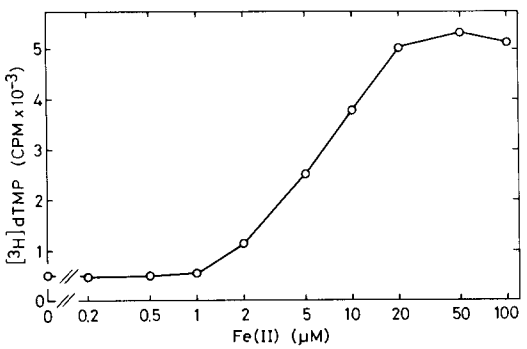


Fig. 2 Effects of varying concentrations of Fe(II) on bleomycin-induced DNA synthesis. DNA synthesis was measured in the standard assay mixture as described in Materials and Methods except that the concentration of Fe (II) was varied as indicated. The data are expressed as described in the legend to Fig. 1.

*Characterization of the DNA polymerase involved in the DNA synthesis.* The DNA synthesis measured in the standard assay mixture was largely (91%) inhibited by 20 $\mu$M 2′, 3′-dideoxythymidine 5′-triphosphate (ddTTP, a selective inhibitor of DNA polymerases $\beta$ and $\gamma$), but was totally resistant to 30 $\mu$M aphidicolin (a specific inhibitor of DNA polymerase $\alpha$) and highly resistant (95%) to 1 mM N-ethylmaleimide (a selective inhibitor for DNA polymerases $\alpha$ and $\gamma$). These results indicate that the DNA polymerase contained in the extract and involved in the bleomycin-induced DNA synthesis in the present cell-free system was DNA polymerase $\beta$.

*Effect of bleomycin on DNA polymerase activity.* To study the reason why DNA synthesis in the present system decreased by increasing the concentration of bleomycin to over 1 $\mu$g/0.3 ml (as shown in Fig. 1), the effects of varying the concentration of bleomycin on DNA synthesis were measured using activated DNA and the cell extract. DNA synthetic activity was high in the activated DNA-extract system without bleomycin and was not stimulated by bleomycin, because activated DNA had enough priming sites for DNA synthesis. This DNA synthesis was also inhibited by bleomycin at concentrations higher than 1 $\mu$g/0.3 ml (Fig. 3), similarly to the result shown in Fig. 1. DiCioccio and Srivastava (16) showed that bleomycin inhibited DNA polymerases $\alpha$ and $\beta$ by interacting with the DNA template. Considering these

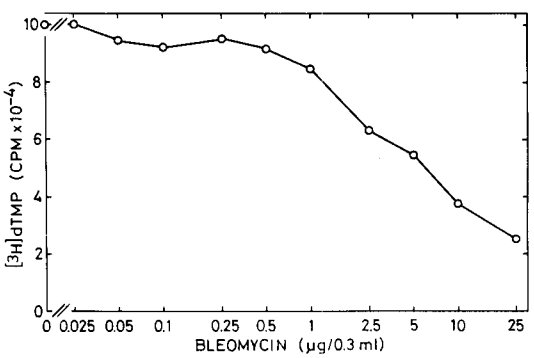


Fig. 3 Effects of varying concentrations of bleomycin on DNA polymerase activity in the permeable cell extract. Polymerase activity was assayed in the polymerase assay mixture as described in Materials and Methods except that bleomycin was added to the assay mixture at the concentration indicated. The data are expressed as described in the legend to Fig. 1.

findings, the decrease in activity observed at high concentrations of bleomycin in the present experiment (Fig. 1) was thought to be due to the inhibition of DNA polymerase $\beta$ by bleomycin. DNA synthesis was thought to reach a maximum, as shown in Fig. 1, when the increase in priming sites due to DNA damage by bleomycin reached a balance with the inhibition of DNA polymerase $\beta$.

*Existence in the cell extract of an exonuclease acting on bleomycin-damaged DNA.* Niwa and Moses (11) showed that bleomycin-damaged $\phi$X174 RFI DNA did not serve as a template-primer for *E. coli* DNA polymerase I, and that purified exonuclease III from *E. coli* and extracts from wild-type *E. coli* strains were able to convert the bleomycin-treated DNA to a suitable template-primer by a modification of 3′-termini. Other studies (8, 9) have shown glycolic acid esterified to the 3′-phosphate termini (3′-phosphoglycolate termini) at bleomycin damaged sites of DNA. *E. coli* exonuclease III can hydrolyze DNA with bleomycin-induced 3′-phosphoglycolate termini and form to gapped DNA with 3′-hydroxyl termini, which is a good template-primer for repair DNA polymerase (10, 11).

Considering that DNA polymerase $\beta$ has no nuclease activity (17) and that highly active bleomycin-dependent DNA synthesis was observed in the present cell-free system, an exonuclease, such as *E. coli* exonuclease III, involved in the removal of the phosphoglycolate termini from bleomycin-damaged DNA and conversion of the DNA into gapped DNA with 3′-hydroxyl termini was thought to be present in the permeable cell extract. Bleomycin and Fe(II) dependent $^3$H-release from $^3$H-labeled double-stranded DNA was markedly enhanced by addition of the permeable cell extract, but not by addition of the extract pretreated at 60℃ for 10 min (Table 1). This result indicated that an exonuclease(s) acting on bleomycin-damaged DNA was present in the extract.

**Table 1** Detection of an exonuclease(s) dependent on bleomycin-induced DNA damage[a]

| Reaction conditions | % of $^3$H solubilized[b] (Mean ± S. D.) |
|---|---|
| Complete | 4.13 ± 1.55 |
| −bleomycin | 0.8 ± 0.02 |
| −extract | 0.38 ± 0.08 |
| −Fe(II) | 0.86 ± 0.01 |
| −extract +heat-treated extract[c] | 1.01 ± 0.27 |

*a*: Omissions (−) from or additions (+) to the complete reaction mixture (described in Materials and Methods) are shown in the table.

*b*: The percentage of solubilized radioactivity to the radioactivity (mean: 6,100 cpm) of $^3$H-labeled DNA used for the assay. The data are presented as the mean ± S. D. of two independent experiments.

*c*: The permeable cell extract was preincubated at 60°C for 10 min. The heat-treated extract was used in place of the non-treated extract.

We are now trying to purify the exonuclease involved in bleomycin-induced repair DNA synthesis from permeable cell extracts. This study is thought to be important for the clarification of the repair mechanism of DNA damage caused by ionizing radiation, because some strand breaks by ionizing radiation contain 3′-phosphoglycolate termini (10).

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