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Peplomycin-induced DNA repair synthesis in permeable mouse ascites sarcoma cells.

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Abstract

DNA repair synthesis induced in permeable mouse ascites sarcoma cells by peplomycin, an antitumor antibiotic, was studied. Mouse ascites sarcoma (SR-C3H/He) cells were permeabilized with a low concentration of Triton X-100 in an isotonic condition. Permeable cells were treated with an appropriate concentration of peplomycin to introduce single-strand breaks in permeable cell DNA. DNA repair synthesis in peplomycin-treated permeable cells was measured by incubating the cells with four deoxynucleoside triphosphates in an appropriate buffer system. The DNA repair synthesis was enhanced by ATP and NaCl at near physiological concentrations. More than 90% of DNA synthesis in the present system depended on the peplomycin-treatment. The repair nature of the DNA synthesis was confirmed by a BrdUMP density shift technique. The repair patches were largely completed and ligated in the presence of ATP. Analyses using selective inhibitors for DNA polymerases showed that both DNA polymerase Beta and aphidicolin-sensitive DNA polymerases (DNA polymerase alpha and/or delta) were involved in the repair DNA synthesis.

KEYWORDS: DNA repair, peplomycin, DNA polymerases, permeable mouse cells

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Peplomycin-Induced DNA Repair Synthesis in Permeable Mouse Ascites Sarcoma Cells

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DNA repair synthesis induced in permeable mouse ascites sarcoma cells by peplomycin, an antitumor antibiotic, was studied. Mouse ascites sarcoma (SR-C3H/He) cells were permeabilized with a low concentration of Triton X-100 in an isotonic condition. Permeable cells were treated with an appropriate concentration of peplomycin to introduce single-strand breaks in permeable cell DNA. DNA repair synthesis in peplomycin-treated permeable cells was measured by incubating the cells with four deoxynucleoside triphosphates in an appropriate buffer system. The DNA repair synthesis was enhanced by ATP and NaCl at near physiological concentrations. More than 90% of DNA synthesis in the present system depended on the peplomycin-treatment. The repair nature of the DNA synthesis was confirmed by a BrdUMP density shift technique. The repair patches were largely completed and ligated in the presence of ATP. Analyses using selective inhibitors for DNA polymerases showed that both DNA polymerase β and aphidicolin-sensitive DNA polymerases (DNA polymerase α and/or δ) were involved in the repair DNA synthesis.

Key words : **DNA repair, peplomycin, DNA polymerases, permeable mouse cells**

It is known that a large number of DNA damages occur daily in cells (1,2). The damaged DNA, in general, is repaired accurately by cellular repair systems, otherwise genetic changes leading to serious results such as cell death, mutation or malignant transformation occur. Although DNA repair is an important function in living cells, the factors involved in the processes are not well known, probably because of the complexity of DNA repair. At least, several tens of types of DNA damage are thought to be induced in cells by chemical, physical and biological stresses, and many cellular factors may be involved in the repair. To make analytical study of DNA repair possible, we have established simplified systems (3,4). Nucleotide-permeable cells, which have been shown to preserve well *in vivo* mechanisms of DNA replication and repair (reviewed in Ref. 5 and 6), were used to assay DNA synthesis using substrates added externally, or to analyze the DNA synthesis using various probes such as inhibitors for DNA polymerases. In addition, by using agents causing one, or few types of DNA damage to induce DNA repair, we could avoid the

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complexity in the priming step of DNA repair.

We have been studying bleomycin-induced DNA repair synthesis in permeable mammalian cells (3,4,7–9), and found a priming factor (an exonuclease) possibly involved in the initiation of repair of bleomycin-induced as well as X-ray-induced single-strand breaks (10,11). Recently, peplomycin, an analog of bleomycin, was synthesized to increase the anticancer activity and to reduce the pulmonary toxicity of bleomycin (12–14). The antitumor effect is ascribed to its DNA damage, but cellular response to the DNA damage is not reported yet. Recently, we studied DNA damage by peplomycin and its repair in a cell-free system to understand the anticancer and DNA repair machanisms (15). In the present paper we report peplomycin-induced DNA repair synthesis in permeable mouse ascites sarcoma cells.

Materials and Methods

The reagents used were obtained from the following sources: [^{3}H]dTTP from Amersham Japan Corp., Tokyo, Japan, ribonucleotides (NTPs) and deoxyribonucleotides (dNTPs) from Seikagaku Kogyou Co. Ltd., Tokyo, Japan, 2′, 3′-dideoxythymidine 5′-triphosphate (ddTTP) and 5-bromodeoxyuridine 5′-triphosphate (BrdUTP) from Pharmacia LKB Biotechnology, Uppsala, Sweden, *E. coli* exonuclease III from Takara Shuzo Co. Ltd., Kyoto, Japan, and N-ethylmaleimide from Sigma Chemical Co., St. Louis, MO, USA. Peplomycin (PEP) and aphidicolin were kindly provided by Nippon Kayaku Co., Tokyo, Japan and Dr. A.H. Todd of Imperial Chemical Industries, Macclesfield, Cheshire, UK, respectively. Mouse ascites sarcoma (SR-C3H/He) cells were obtained and have been maintained as reported previousely (16). The sarcoma cells were collected from ascites fluid of a mouse 5–7 days after ascites sarcoma cell inoculation of 0.05 ml/mouse ($0.5–1 \times 10^7$ cells).

The following procedures were carried out at 0–4 °C unless otherwise indicated.

Preparation of permeable cells. SR-C3H/He cells were suspended at $2–5 \times 10^6$ cells/ml in Triton-buffer B (0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, 0.0175 % Triton X-100, pH 8.0), and kept at 0 °C for 10 min. After centrifugation the precipitated cells were resuspended in Triton-buffer B and centrifuged again at $1,500 \times g$ for 5 min. The cells were washed twice with Triton-buffer B by repeated centrifugation and resuspension. Permeable cells thus obtained were suspended in Triton-buffer B at 1×10^7 cells/ml and distributed into assay tubes.

Preparation of PEP-pretreated permeable cells. Permeable cells suspended in Triton-buffer B at 5×10^6 cells/ml were incubated with 1 mM ATP and 110 μM PEP at 0 °C for 60 min. The cells were washed twice with 2-mercaptoethanol-free Triton-buffer B, suspended in the same buffer at 1×10^7 cells/ml, and distributed into assay tubes.

Assay of DNA synthetic activity. DNA repair synthesis in permeable cells was measured in a mixture (final volume, 0.6 ml; the standard assay mixture) consisting of 2×10^6 cells pretreated with 110 μM PEP, 0.0117 % Triton X-100, 0.167 M sucrose, 0.67 mM EDTA, 40 mM Tris-HCl, 5 mM $MgCl_2$, 80 mM NaCl, 2.5 mM ATP, 50 μM dATP, 10 μM dCTP, 50 μM dGTP and 2.5 μM [^{3}H]dTTP (0.5 Ci/mmol), pH 8.0, essentially as described previously (8,17). To assay replicative DNA synthesis, permeable cells were not pretreated with PEP and incubated with the standard assay mixture. Incubation was performed at 37 °C for 30 min unless otherwise indicated. Acid-insoluble radioactivity incorporated into permeable cells was measured by a disk method (16).

Isopyknic centrifugation of DNA labeled with BrdUMP and [^{3}H]dTMP in permeable cells. Density label of DNA newly synthesized in permeable cells and isopyknic centrifugation of the labeled DNA were performed basically as described previously (8). Briefly, DNA synthesis in permeable cells pretreated with or without PEP was performed as described above except that 0.083 μM [^{3}H]dTTP (50 Ci/mmol) and 8.3 μM BrdUTP replaced 2.5 μM [^{3}H]dTTP. DNA was extracted from 1.6×10^7 BrdUMP-[^{3}H]dTMP-labeled cells. Alkaline CsCl density gradient centrifugation of the labeled DNA was performed at 20 °C at 37,000 rpm for 48 h. Fifteen drop fractions were obtained from the bottom of the tube, and 100 μl of each fraction was spotted onto a glassfiber disc (Whatman; GF/C) and the radioactivity was measured. The absorbance of the remaining samples was measured at 260 nm after addition of 0.6 ml distilled water per tube.

Exonuclease III sensitivity of DNA synthesized in PEP-pretreated permeable cells. To measure the completion and ligation of repair patches, exonuclease III sensitivity of newly synthesized DNA was assayed

basically as described previously (3). Briefly, mouse ascites sarcoma (SR-C3H/He) cells were permeabilized, pretreated with PEP, and incubated for DNA synthesis *in vitro* as described above. DNA was extracted from 1.6×10^7 ^{3}H-labeled cells and dissolved in 0.7 ml of distilled water. The reaction mixture (pH 7.8, 0.5 ml in final volume) for the exonuclease digestion contained 60 μl of the labeled DNA, 50 μg of unlabeled salmon testis DNA, 10 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$ and 50 unit exonuclease III. The mixture was incubated at 37 °C for various lengths of time. The radioactivity in acid-soluble supernatant and acid-insoluble pellets were measured by a liquid scintillation counter.

Assays were replicated, and the variation in the results for the duplicate test was for the most part less than 10 %.

Results

DNA repair synthesis in PEP-pretreated permeable cells. DNA synthetic activity measured in the present condition in permeable SR-C3H/He cells depended on the PEP-pretreatment. The activity increased as the dose of PEP was increased, between 0.055 μM–22 μM, and reached almost plateau beyond 22 μM of PEP (Fig. 1). The time course of [^{3}H]dTMP incorporation in the 110 μM PEP-pretreated permeable cells showed that DNA synthesis occurred rapidly for 10 min, and proceeded thereafter at a diminished rate (Fig. 2). The DNA synthesis was enhanced about 1.5-fold by the addition of 2.5 mM ATP without significant change in the pattern of the time course. The DNA synthesis was proportional to cell number to 3×10^6 (data not shown). The PEP-induced DNA synthesis depended on salt concentration in the incubation mixture (Fig. 3). Maximum DNA synthesis occurred with 80 mM NaCl in the presence of 2.5 mM ATP or 100 mM in the absence of ATP. The stimulation of DNA synthesis by salt was more remarkable when DNA synthesis was measured without ATP than with ATP (Fig. 3).

DNA polymerases possibly involved in PEP-induced DNA synthesis. Aphidicolin, a specific inhibitor for DNA polymerases α and δ partially inhibited PEP-induced DNA synthesis measured in the presence of 2.5 mM ATP, while ATP-independent DNA synthesis

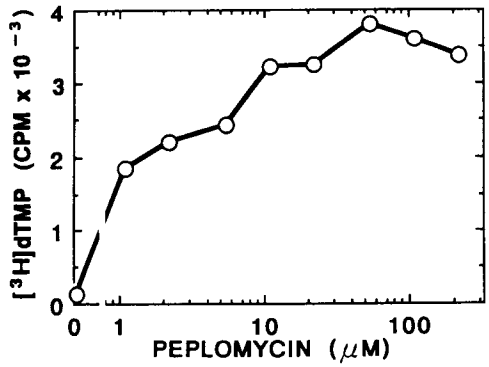

Fig. 1 Effects of varying PEP concentrations on repair synthesis in permeable SR-C3H/He cells. Permeable cells were pretreated with PEP at the concentration indicated, and 1 mM ATP. The cells were incubated at 37 °C for 30 min with the standard assay mixture described in Materials and Methods. The data are expressed as cpm of [^{3}H]dTMP incorporated per 2×10^6 cells per 30 min.

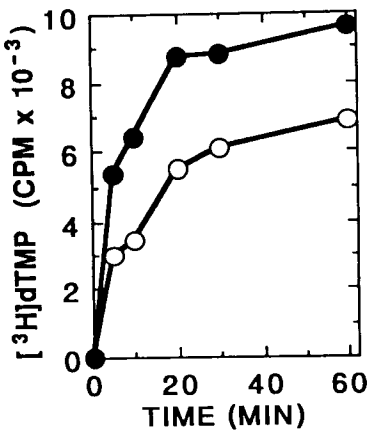

Fig. 2 Time course of [^{3}H]dTMP incorporation into PEP-treated permeable SR-C3H/He cells. Permeable cells were treated with 0.11 mM PEP, and 1 mM ATP. DNA synthesis in the cells was assayed in the standard assay mixture (●), or in the standard assay mixture without ATP (○), as described in Materials and Methods. The incubation was conducted at 37 °C for the indicated intervals. The results are expressed as cpm of [^{3}H]dTMP incorporated per 2×10^6 cells.

was highly resistant to the inhibitor (Fig. 3). The aphidicolin-sensitivity of DNA synthesis measured in the presence of 2.5 mM ATP was gradually decreased by increasing NaCl concentration over 120 mM, and reached zero at 200 mM NaCl (Fig. 3B). The results suggest that DNA polymerase β is involved in the DNA synthesis measured in the absence of ATP, while both DNA polymerase β and aphidicolin-sensitive DNA polymerase (α and/or δ) are involved in the DNA synthesis measured in the presence of 2.5 mM ATP. In the presence of the optimal concentrations of NaCl (80 mM) and ATP (2.5 mM), DNA polymerases involved in PEP-induced DNA synthesis were studied further by using selective inhibitors of DNA polymerases (Table 1). The DNA synthesis was partially inhibited by 20 μM ddTTP (a selective inhibitor of DNA polymerases β and γ), 30 μM aphidicolin and 1 mM N-ethylmaleimide (a selective inhibitor of DNA polymerases α, γ and δ). The DNA synthesis was strongly inhibited by the combination of ddTTP and aphidicolin, or ddTTP and N-ethylmaleimide, but not by the combination of aphidicolin and N-ethylmaleimide. These results indicate that both DNA polymerase β and aphidicolin-sensitive DNA polymerases (α and/or δ) are involved in the DNA synthesis occurred in PEP-pretreated permeable SR-C3H/He cells at nearly physiological ionic strength and ATP concentration.

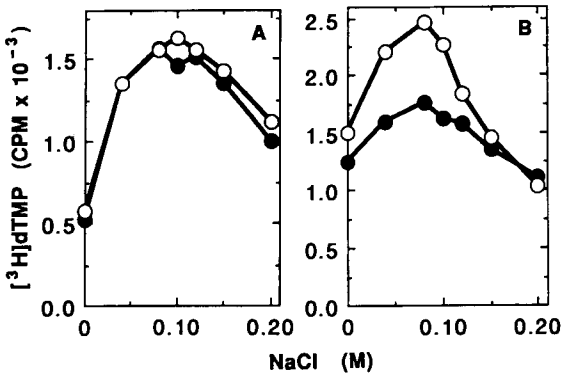

Fig. 3 Effects of varying NaCl concentrations on PEP-induced repair synthesis in permeable SR-C3H/He cells. PEP-pretreated permeable cells were incubated at 37 °C for 30 min in the standard assay mixture for DNA synthesis except that NaCl concentration was varied as indicated, and that 30 μM aphidicolin was added (●) or not added to the reaction mixture (○). A, measured in the absence of ATP; B, measured in the presence of 2.5 mM ATP. The data are expressed as cpm of [³H]dTMP incorporated per 2×10^6 cells per 30 min.

The nature of DNA synthesized in PEP-pretreated permeable cells. The repair nature of DNA synthesis in PEP-pretreated permeable

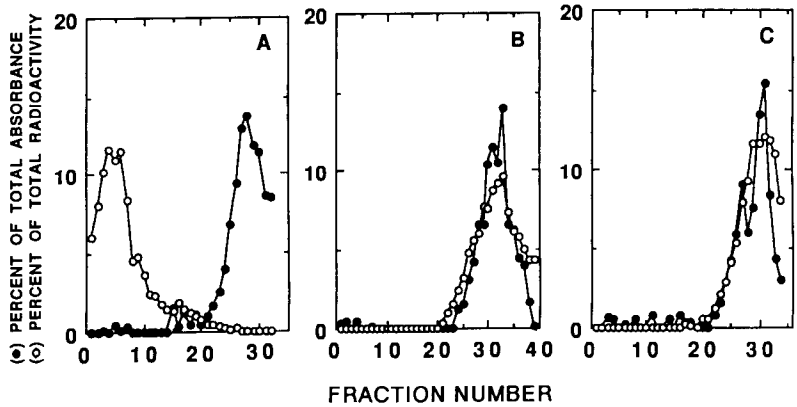

Fig. 4 Isopyknic centrifugation of DNA labeled with BrdUMP and [³H]dTMP in permeable SR-C3H/He cells. Sedimentation is from right to left. ●, % of total absorbance; ○, % of total radioactivity. A, replicative DNA synthesis in permeable cells; B, DNA synthesis measured without ATP in PEP-pretreated permeable cells; C, DNA synthesis measured with 2.5 mM ATP in PEP-pretreated permeable cells.

cells was confirmed by equilibrium sedimentation analysis in alkaline CsCl of BrdUMP and [^{3}H] dTMP double-labeled DNA. The DNA synthesized in PEP-pretreated permeable cells either in the absence of ATP or in the presence of ATP sedimented with bulk DNA at a normal density (Fig. 4B, C), whereas the DNA synthesized in non-PEP-pretreated permeable cells sedimented at a higher density than the normal density bulk DNA (Fig. 4A). The results indicate that the DNA synthesis in PEP-pretreated permeable cells, either in the absence of ATP or in the presence of ATP is not replicative but unscheduled (reparative DNA synthesis).

Table 1 Effects of various combinations of inhibitors on repair DNA synthesis in presence of 2.5 mM ATP in PEP-pretreated permeable SR-C3H/He cells

Inhibitor[a]			Activity (%)[b]
ddTTP	Aphidicolin	N-ethylmaleimide	
−	−	−	100
+	−	−	54
−	+	−	82
−	−	+	26
+	+	−	23
+	−	+	6
−	+	+	26

a: Where added (+), the concentrations of ddTTP, aphidicolin and N-ethylmaleimide were 20 μM, 30 μM and 1 mM, respectively. *b*: Assay was performed in duplicate. Variation in the results for duplicate tests was mostly less than 10 %.

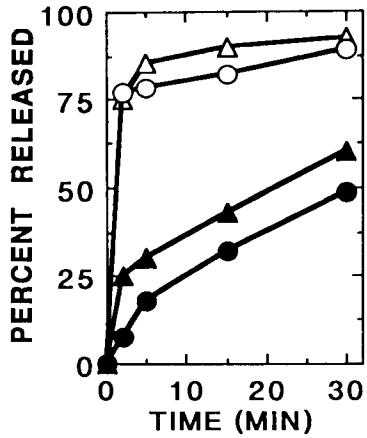

Fig. 5 Exonuclease III sensitivity of DNA synthesized in PEP-pretreated permeable cells. PEP-pretreated permeable cells were incubated at 37 °C for 10 min with 2.5 mM ATP (▲) or without ATP (△), or at 37 °C for 10 min without ATP and then chased for 50 min by the addition of 0.25 mM cold dTTP (○), or by the addition of 0.25 mM cold dTTP and 2.5 mM ATP (●). DNA isolated from the [^{3}H]dTMP-labeled cells was incubated at 37 °C for the indicated intervals with exonuclease III, and the % of ^{3}H-nucleotides released from DNA was determined.

Demonstration of repair patch ligation in the present system. Exonuclease III sensitivity of DNA repaired in PEP-pretreated permeable cells was measured to determine whether or not the completion of repair patches and ligation occurred in the present systems. DNA synthesized in the presence of ATP in PEP-pretreated permeable cells was resistant to exonuclease III digestion, although DNA synthesized in the absence of ATP was highly sensitive to the enzyme (Fig. 5). DNA synthesized in the absence of ATP also became resistant to exonuclease III when the DNA synthesis was chased by the addition of cold dTTP and 2.5 mM ATP, but not by the addition of cold dTTP alone (Fig. 5). The results suggest that in the presence of an appropriate concentration of ATP, the DNA repair in PEP-pretreated permeable SR-C3H/He cells proceeds to completion and ligation of repair patches.

Discussion

Peplomycin (PEP), an analog of bleomycin, has been developed to increase the antitumor activity and to reduce the pulmonary toxicity of bleomycin mixture (12–14). Clinical studies and practical use of the drug are increasing (13,18). However, these basic studies are extremely limited (19, 20). The structural difference between PEP and bleomycin resides in the amine terminal which is known to be involved in DNA binding (21). Effects of the structural difference on cellular DNA damage by these drugs, and repair of PEP-damaged DNA by cellular repair systems have scarcely been studied. PEP is expected to provide more clearcut experimental results than

those of the bleomycin mixture because of the simplicity of the former preparation.

In the present paper, repair of PEP-damaged DNA was studied in permeable mouse sarcoma cells. It was shown that the DNA damage caused by PEP was repaired clearly in appropriate conditions in permeable cells. Appropriate concentrations of salt and ATP were required in the DNA repair. DNA polymerases involved in the repair DNA synthesis were also studied.

The conditions required for repair of PEP-damaged DNA in permeable cells were quite similar to those required for repair of bleomycin-damaged DNA reported previously (3,4,9). DNA polymerases involved in both these of DNA repair seemed to be identical (4,6,9). These results suggested that PEP caused essentially the same DNA damage in permeable cells as bleomycin did.

The present experiments confirmed again the usefulness of the permeable cell system for studying DNA repair synthesis in mammalian cells.

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