INDUCTION OF SINGLE STRAND SCISSION IN BACTERIOPHAGE ϕX174 REPLICATIVE FORM I DNA BY MITOMYCIN C

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The action of mitomycin C on double-stranded replicative form I DNA (RF I DNA; supercoiled, covalently closed, circular duplex DNA) of bacteriophage ϕX174 was investigated using the technique of agarose gel electrophoresis. Mitomycin C reduced with sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$) caused single strand scission in ϕX174 RF I DNA in the presence of Cu^{2+}. Cu^{2+} was essential for this DNA cleavage action, and other transition metal ions such as Fe^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+} and Zn^{2+} were of no effect. This DNA strand scission was inhibited by catalase (EC 1.11.1.6) and various radical scavengers. This DNA strand scission was caused by free oxygen radicals generated during autoxidation of reduced mitomycin C in the presence of Cu^{2+}.

Mitomycin C[1], an anticarcinogenic antibiotic, contains three functionally reactive moieties in the molecule, such as aziridine, aminoquinone and methylurethane. Its cytotoxicity has been supposed to be due to alkylation and crosslinking[2,3] of DNA. Recently, LOWN *et al.*[4] have reported that reduced mitomycin C induces single strand scission in bacteriophage PM2 double-stranded DNA, and that the DNA strand scission is due to oxygen radicals. However, reduced mitomycin C does not directly affect double-stranded replicative form I DNA (RF I DNA; supercoiled, covalently closed, circular duplex DNA) of phage ϕX174[5] or phage ϕA[6], immunologically related to ϕX174. On the other hand, we have found that mitomycin C reduced with sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$) inactivates *in vitro* bacteriophage ϕX174 in the presence of Cu^{2+}[7], and that the inactivation of ϕX174 was caused *via* DNA strand scission of phage single-stranded DNA in the virion by oxygen radicals and mitomycin C semiquinone radical generated during reduction and autoxidation of mitomycin C[7]. A promotive effect of Cu^{2+} is commonly observed in the phage inactivation reaction by oxygen radical-generating agents[8,9,10]. We reexamined the effect of mitomycin C on ϕX174 double-stranded RF I DNA in the presence of Cu^{2+}.

Mitomycin C reduced with sodium hydrosulfite caused single strand scission in ϕX174 RF I DNA in the presence of Cu^{2+}. This DNA strand scission was caused by free oxygen radicals generated during autoxidation of reduced mitomycin C in the presence of Cu^{2+}.

Materials and Methods

Chemicals and enzymes

Mitomycin C was kindly supplied by Kyowa Hakko Co. Ltd., Tokyo, Japan. Superoxide dismutase (EC 1.15.1.1, bovine blood, 2,900 U/mg protein) and catalase (EC 1.11.1.6, bovine liver, 2,500 U/mg protein) were purchased from Sigma Chemical Co. Other chemicals were obtained from Nakarai Chemicals Co.

Preparation of ϕX174 RF 1 DNA

Escherichia coli C_N cells were grown at 37°C to 5×10^8 cells/ml in 2 liters of TPG-CA medium,

which is identical to TPG-2A medium[11] except that 1% of Casamino acids is substituted for the amino acids mixture. The cells were infected with ϕX174 *am*3 at a multiplicity of infection of 5 to 10. After 9 minutes of incubation, chloramphenicol was added to a final concentration of 30 μg/ml[11]. The cells were harvested after incubation for further 3 hours, washed with 50 mM Tris-HCl buffer (pH 8.1), resuspended in 10 ml of ice-cold 50 mM Tris-HCl buffer (pH 8.1) containing 10% (w/v) sucrose, and lysed with lysozyme-EDTA and SDS[12]. Solid NaCl was added to the lysate to a concentration of 1 M, and the solution was kept on ice for 2 hours. Host DNA and proteins precipitated were removed by centrifugation at 8,000$\times g$ for 10 minutes. The supernatant was incubated with 20 μg of RNase A/ml (heated to 90°C for 10 minutes to inactivate DNase contaminated) at 37°C for 30 minutes. Ethidium bromide and CsCl were then added to give a concentration of 300 to 350 μg/ml and 1.58 g/cm^3, respectively. After centrifugation in a RP65TA rotor of a Hitachi 55P ultracentrifuge at 86,000$\times g$ for 40 hours, the DNA bands were visualized with long-wavelength ultraviolet light (365 nm). The lower band which contains exclusively ϕX174 RF 1 DNA was collected by aspiration. After this centrifugation step was repeated, ethidium bromide was removed from the DNA by five extractions with isopropyl alcohol saturated with CsCl. ϕX174 RF I DNA suspension was dialyzed against 50 mM Tris-HCl buffer (pH 8.1).

Reaction of mitomycin C with ϕX174 RF I DNA

The reaction mixture (20 μl) contains 0.93 μg (47 μg/ml) ϕX174 RF I DNA, 1 mM mitomycin C, 0.1 mM sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$) and 0.01 mM $CuCl_2$ in 50 mM Tris-HCl buffer (pH 8.1), unless otherwise noted. Reaction was carried out for 3 hours at 37°C, and stopped by addition of 5 μl of 0.1 M EDTA solution containing 50% (W/V) sucrose and 0.1% bromophenol blue. The sample, a final volume of 25 μl, was directly analysed by agarose gel electrophoresis.

Agarose gel electrophoresis

Electrophoretic analysis was performed as described by JOHNSON and GROSSMAN[13]. Agarose slab gels (1.4%) were run at 3.3 V/cm for 4 hours in 40 mM Tris-acetate buffer (pH 8.1) containing 5 mM sodium acetate and 1 mM EDTA. The gels were run in the presence of 1 μg/ml ethidium bromide, and the stained bands were visualized using an ultraviolet lamp (Chromato-Vue, Transilluminator model C-62) and photographed.

Results

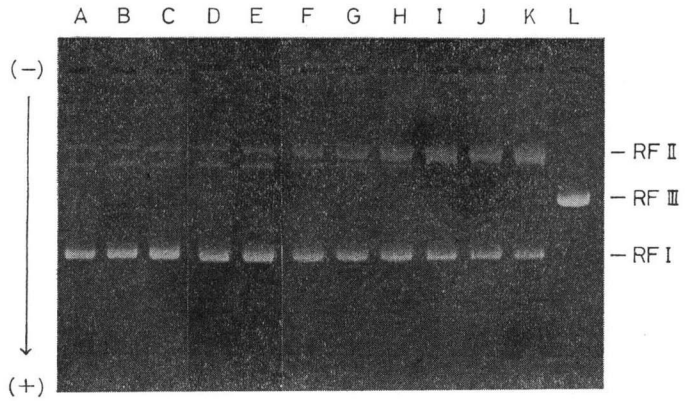
Interaction of Mitomycin C with ϕX174 RF I DNA

ϕX174 RF I DNA was not affected by mitomycin C or mitomycin C reduced with sodium hydrosulfite (Fig. 1 B, C). As shown in Fig. 1 F~K, in the presence of 0.01 mM Cu^{2+}, mitomycin C reduced with sodium hydrosulfite caused DNA strand scission in RF I DNA, and the RF I DNA was converted to RF II DNA (nicked, open circular duplex DNA). The conversion of RF I DNA to RF II DNA was proportional to the concentration of mitomycin C. When the produced RF II DNA was heat-denatured and analyzed using agarose electrophoresis, smaller DNA fragments, other than circular and length of linear single-stranded DNA, were observed (unpublished data). Even when RF I DNA was treated with 5 mM mitomycin C in the presence of sodium hydrosulfite and Cu^{2+} (Fig. 1 K), RF III DNA (linear duplex DNA), which is generated by double strand scission in RF I DNA, was not observed. Nonreduced mitomycin C (Fig. 1 D) or sodium hydrosulfite (Fig. 1 E) did not convert RF I DNA to RF II DNA even in the presence of Cu^{2+}. Another reducing agent, sodium borohydride did not serve as a substitute for sodium hydrosulfite, and other transition metal ions such as Fe^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+} and Zn^{2+} were of no effect (data not shown).

These results indicate that mitomycin C reduced with sodium hydrosulfite causes one or more single strand scissions, but not double strand scission, in ϕX174 RF I DNA, and that Cu^{2+} is essential for this DNA cleavage action.

Fig. 1. Induction of single strand scission in ϕX174 RF I DNA by mitomycin C in the presence of sodium hydrosulfite and Cu^{2+}.

A : Drug-free control
B : 1 mM mitomycin C
C : 1 mM mitomycin C, 0.1 mM $Na_2S_2O_4$
D : 1 mM mitomycin C, 0.01 mM $CuCl_2$
E : 0.1 mM $Na_2S_2O_4$, 0.01 mM $CuCl_2$
F : E+0.01 mM mitomycin C
G : E+0.05 mM mitomycin C
H : E+0.1 mM mitomycin C
I : E+0.5 mM mitomycin C
J : E+1.0 mM mitomycin C
K : E+5.0 mM mitomycin C
L : Pst I-digested ϕX174 RF I DNA (indicates the position of linear duplex DNA, RF III.)



Effect of Enzymes and Radical Scavengers on the RF I DNA Strand Scission by Mitomycin C in the Presence of Sodium Hydrosulfite and Cu^{2+}

It is suggested that free oxygen radicals and mitomycin C semiquinone radical generated during reduction and autoxidation of mitomycin C are involved in the ϕX174 single-stranded DNA strand scission and subsequent phage inactivation[7]. Therefore, enzymes and several radical scavengers were tested for their abilities to inhibit the RF I DNA strand scission by mitomycin C in the presence of sodium hydrosulfite and Cu^{2+}. As shown in Fig. 2 D, catalase (10 μg/ml), which removes hydrogen peroxide, completely inhibited the DNA strand scission. The same concentration of the heat-inactivated enzyme did not inhibit the DNA strand scission (Fig. 2 E). Tiron[14], a scavenger for superoxide anion, completely inhibited the DNA strand scission (Fig. 3 C, D), but superoxide dismutase did not inhibit the DNA strand scission (Fig. 2 F, G). This is probably because the enzyme

Fig. 2. Effect of catalase and superoxide dismutase on the induction of single strand scission in ϕX174 RF I DNA.

A : Drug-free control
B : Complete (1.0 mM mitomycin C, 0.1 mM $Na_2S_2O_4$, 0.01 mM $CuCl_2$)
C : +1 μg/ml catalase
D : +10 μg/ml catalase
E : +10 μg/ml catalase inactivated by heating for 10 minutes at 100°C
F : +10 μg/ml SOD
G : +25 μg/ml SOD
H : +25 μg/ml SOD inactivated by heating for 10 minutes at 100°C

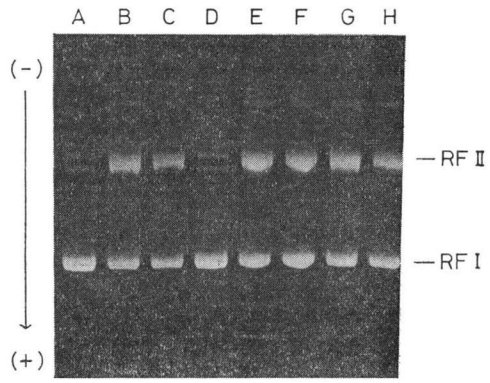
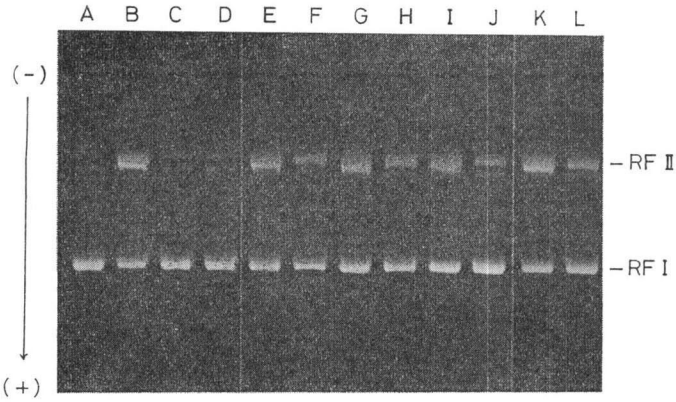


Fig. 3. Effect of radical scavengers on the induction of single strand scission in ϕX174 RF I DNA.

A : Drug-free control
B : Complete (1.0 mM mitomycin C, 0.1 mM $Na_2S_2O_4$, 0.01 mM $CuCl_2$)
C : +0.4 mM Tiron
D : +4.0 mM Tiron
E : +10 mM sodium benzoate
F : +100 mM sodium benzoate
G : +10 mM sodium formate
H : +100 mM sodium formate
I : +0.5 mM KI
J : +5.0 mM KI
K : +1.0 mM DABCO
L : +10 mM DABCO



dismutates superoxide anion to oxygen and hydrogen peroxide which produces hydroxyl radical. Sodium benzoate (100 mM), sodium formate (100 mM) and potassium iodide (5 mM), scavengers for hydroxyl radical[15], inhibited the DNA strand scission (Fig. 3 F, H, J). The DNA strand scission was inhibited also by 1,4-diazabicyclo[2, 2, 2]octane (DABCO) which scavenges singlet oxygen[16] (Fig. 3 L).

These results suggest that all of the above mentioned oxygen radicals participate in the RF I DNA strand scission.

Discussion

The present results reveal that mitomycin C reduced with sodium hydrosulfite causes single strand scission in ϕX174 double-stranded RF I DNA. Cu^{2+} is essential for this RF I DNA cleavage in the presence of mitomycin C reduced with sodium hydrosulfite. Sodium borohydride can not serve as a substitute for sodium hydrosulfite, and other transition metal ions than Cu^{2+} were of no effect. These characteristics of ϕX174 RF I DNA cleavage very closely resemble those of the ϕX174 phage inactivation and the ϕX174 single-stranded DNA cleavage produced by action of mitomycin C[7].

The RF I DNA strand scission by mitomycin C in the presence of sodium hydrosulfite and Cu^{2+} was inhibited by catalase and various radical scavengers. Oxygen radicals such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen are, therefore, suggested to be involved in the ϕX174 RF I DNA strand scission. A possible radical-generating mechanism is as follows;

(1) Mitomycin C $\xrightarrow{Na_2S_2O_4,\ Cu^{2+}}$ Mitomycin $C^{\cdot-}$, Mitomycin CH_2

(2) Mitomycin $C^{\cdot-}+O_2 \longrightarrow$ Mitomycin $C+O_2^{\cdot-}$

(3) Mitomycin $CH_2+O_2 \longrightarrow$ Mitomycin $C^{\cdot-}+HO_2^{\cdot}+H^+$

(4) Mitomycin $C^{\cdot-}+H_2O_2 \longrightarrow$ Mitomycin $C+OH^-+OH^{\cdot}$

(5) $HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot-}$ (6) $2HO_2^{\cdot} \rightleftharpoons H_2O_2+{}^1O_2$

(7) $O_2^{\cdot-}+H_2O_2 \rightarrow HO^{\cdot}+HO^-+{}^1O_2$ (8) $H_2O_2+H_2O_2 \rightarrow 2H_2O+{}^1O_2$

(9) $O_2^{\cdot-}+Cu^{2+} \rightarrow {}^1O_2+Cu^+$ (10) $H_2O_2+Cu^+ \rightarrow OH^{\cdot}+OH^-+Cu^{2+}$

(11) ϕX174 RF I DNA $+ \begin{Bmatrix} O_2^{\cdot-},\ H_2O_2,\ OH^{\cdot},\ {}^1O_2 \\ \text{Mitomycin } C^{\cdot-} \end{Bmatrix} \rightarrow$ Single strand scission

Mitomycin C is slowly, non-enzymatically reduced by sodium hydrosulfite to form semiquinone or hydroquinone. On the subsequent rapid autoxidation of the reduced form of quinone, oxygen radicals are produced. Hydroxyl radical is supposed as a responsible species for DNA strand scission by many oxygen radical-generating agents[4,8,9]. The effect of scavengers for hydroxyl radical on the present RF I DNA strand scission suggests that hydroxyl radical is mainly responsible for the ϕX174 RF I DNA strand scission by mitomycin C in the presence of sodium hydrosulfite and Cu^{2+}. Singlet oxygen may also be involved in the RF I DNA strand scission, because DABCO partly inhibited the conversion of RF I DNA to RF II DNA. LOWN *et al.*[4] suggested that strand scission of phage PM2 covalently closed circular (ccc) DNA by reduced mitomycin C is induced by hydroxyl radical. The ϕX174 RF I DNA cleavage action of mitomycin C is, however, different from the PM2 ccc DNA cleavage action of mitomycin C in the requirement of Cu^{2+} and in the availability of sodium borohydride as a reducing agent. TOMASZ *et al.*[17] reported that the addition of a large excess of non-reduced mitomycin C during reduction of mitomycin C increases the concentration of semiquinone radical, and that mitomycin C semiquinone radical combines with DNA in a noncovalent manner. Since ϕX174 RF I DNA strand scission was increased when a large excess of mitomycin C was added against a concentration of sodium hydrosulfite (Fig. 1 I~K), mitomycin C semiquinone radical is considered to play an important role in the ϕX174 RF I DNA strand scission. Mitomycin C semiquinone radical has been detected by the electron paramagnetic resonance method and shown to have a lifetime of several seconds[18]. Oxygen radicals may be generated extremely near DNA at the point where mitomycin C semiquinone radical is combined, and these oxygen radicals may attack DNA effectively.

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