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INHIBITION OF DEOXYRIBONUCLEIC ACID POLYMERASES α AND β AND TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE FROM CALF THYMUS BY MITOMYCIN C–DEOXYRIBONUCLEIC ACID*

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Abstract—Mitomycin C, cross-linked with DNA, strongly inhibited DNA polymerase α and β (EC 2.7.7.7) and terminal deoxynucelotidyl transferase (EC 2.7.7.31) from calf thymus, while the free form of mitomycin C produced no inhibition. The reaction of DNA polymerase β was slightly more sensitive than that of DNA polymerase α (K_i: 6.9 μg/ml for DNA polymerase α and 5.7 μg/ml for DNA polymerase β). The mode of inhibition by the mitomycin C–DNA complex was competitive with respect to template-primers, i.e. activated DNA.

The extent of inhibition by the mitomycin C–DNA complex depended on the amount of mitomycin C bound to DNA. These results suggest that DNA polymerizing enzymes may have strong affinities to DNA sites that have conformational changes induced by cross-linking with mitomycin C.

Mitomycin C is a potent anti-cancer agent used widely for the treatment of human malignancies such as leukemias and sarcomas. It is well established that mitomycin C requires reductive activation to become an ultimate reactant, that is, a bifunctional alkylating reagent of biological macromolecules, including DNA [1, 2]. It has been reported that mitomycin C, as a result of cross-linking with DNA strands, inhibits DNA synthesis by interfering with the separation of double-stranded DNA [3–5]. This antibiotic has not been reported to inhibit the polymerization of DNA *in vitro* which is catalyzed by DNA polymerases. Here we report that mitomycin C–DNA complexes strongly inhibited the DNA polymerases α and β and the terminal deoxynucleotidyl transferase from calf thymus, whereas the free form of mitomycin C showed no inhibition. The mode of inhibition was studied extensively.

MATERIALS AND METHODS

Chemicals. [Me-^{3}H]Deoxythymidine-5′-triphosphate ([^{3}H]dTTP) and the other labeled compounds were purchased from the New England Nuclear Corp., Boston, MA, U.S.A. Mitomycin C was purchased from Kyowa Hakko Inc., Tokyo, Japan. Unlabeled deoxynucleoside-5′-triphosphate (dNTP) was obtained from Boehringer, Mannheim, GFR, and P-L Biochemicals, Inc., Milwaukee, WI, U.S.A. Activated calf thymus DNA was prepared as described [6].

Enzymes. DNA polymerase α was purified from the soluble fraction of calf thymus as described previously [7]. The DEAE-cellulose fraction of the DNA polymerase α was further purified on a hydroxylapatite column. The specific activity of the DNA polymerase α fraction (HA 2, Ref. 7) was 3000–4000 nmoles/mg protein under the assay condition described below. It sedimented through a sucrose gradient at about 6.5 S.

DNA polymerase β was purified from the soluble fraction of calf thymus by serial column chromatography as described previously [8]. The specific activity of the DNA polymerase β used in this study was approximately 20,000 nmoles/mg protein, with poly (rA)·$(dT)_{10}$ template-primer under the assay condition described previously [6]. Terminal deoxynucleotidyl transferase (TdT) from calf thymus was purified by the modified method of Bollum [9]. The specific activity of TdT used in this study was 29,900 units/mg protein under the assay condition described below. This TdT preparation was completely free from contamination by DNA polymerase α or β. Initiator DNA for the assay of TdT was prepared by heating activated calf thymus DNA at 90° for 10 min and cooling quickly in ice (heat-activated DNA).

Assay of DNA polymerase α. The standard reaction mixture (62.5 μl) contained 40 mM potassium phosphate (pH 7.2), 8 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM each of dATP, dGTP, dCTP and [^{3}H]dTTP (40,000 cpm/nmole), 10 μg of activated DNA and 0.1 μg of DNA-polymerase of calf thymus.

Assay of DNA polymerase β. The reaction mixture (62.5 μl) contained 40 mM Tris–HCl (pH 9.0), 80 mM $NaCl_2$, 0.1 mM each of dATP, dGTP, dCTP and [^{3}H]dTTP (40,000 cpm/nmole), 10 μg of activated DNA and 0.1 μg of DNA polymerase β from calf thymus.

Assay of TdT. The reaction mixture (62.5 μl) contained 50 mM Tris–HCl (pH 8.3), 100 mM

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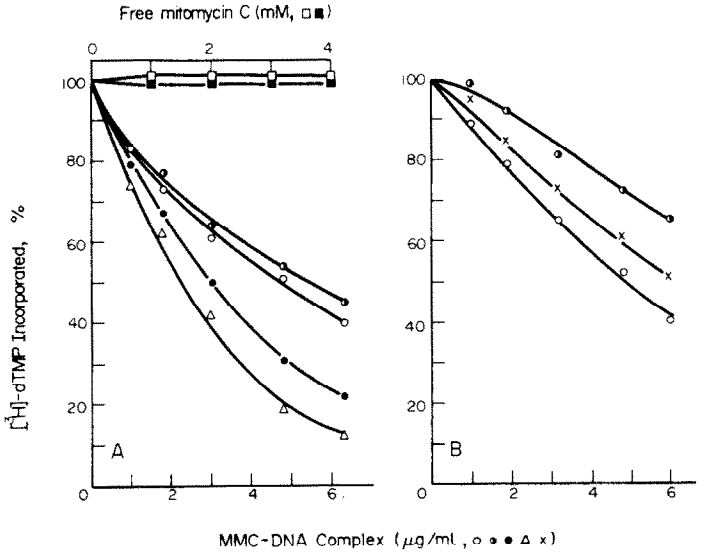

Fig. 1. Panel A: Inhibition of DNA polymerases α and β and TdT from calf thymus by mitomycin C-DNA complex. DNA polymerases α and β and TdT were assayed under conditions described in Materials and Methods. Mitomycin C–DNA complex with a binding ratio of 3, prepared from activated calf thymus DNA, was added to the reaction mixtures of DNA ploymerase α (○) and β (●) and TdT (△) at the concentrations indicated on the bottom abscissa. Mitomycin C–DNA complex prepared from native calf thymus DNA was also tested for inhibition of the reaction of DNA polymerase α (◑). Free mitomycin C was added to the reaction mixture of DNA polymerase α (□) and β (■) at the concentrations indicated on the top abscissa. Panel B: Inhibition of DNA polymerase α by mitomycin C–DNA complex with binding ratios of 3 (○), 7 (X), and 21 (◑).

KCl, 0.3 mM $MnCl_2$, 0.1 mM [^{3}H]dGTP (40,000 cpm/nmole), 10 μg of heated activated DNA, 2 mM dithiothreitol and 0.1 μg of TdT from calf thymus [10]. After incubation at 37° for 30 min, acid-insoluble radioactivity was measured as described previously [6]. One unit of enzyme activity was defined as the amount which catalyzed the incorporation of 1 nmole of deoxynucleotide into the acid-insoluble fraction in 1 hr.

In vitro *formation of mitomycin C–DNA complex*. Activation of mitomycin C was performed as described by Tomasz *et al.* [11]. Briefly, activated or native calf thymus DNA was mixed in 0.017 M sodium phosphate buffer at pH 7.5 (1.6 μmoles DNA/ml) and deaerated by bubbling N_2 gas through the solution for 20 min. DNA was then reacted with 0.1 M $Na_2S_2O_4$ for 5, 10 and 15 min. The mitomycin C–DNA complex was separated from unreacted mitomycin C on a Sephadex G-50 (2.5 × 30 cm) column equilibrated with 0.15 M NaCl containing 0.2 M sodium citrate (pH 7.4).

Estimation of the binding ratio of mitomycin C–DNA complex. The binding ratio was defined here as the molar ratio of DNA nucleotide to mitomycin C. The molar concentration of DNA was estimated by measuring A_{260} in 0.15 M NaCl–0.02 M sodium citrate (pH 7.4) on the basis of the extinction coefficient (ε) of 7900 at 260 nm [11]. Bound mitomycin C was determined by u.v. absorption of the complex at 310 nm on the basis of ε_{310} = 11,500 [11].

RESULTS

Inhibition of DNA polymerases α and β and TdT by mitomycin C–DNA complex. Three kinds of mitomycin C–DNA complexes (binding ratios: 3.1, 7.2 and 20.6 molecules of DNA nucleotides per 1 molecule of mitomycin C) were obtained by changing the incubation time as described in Materials and Methods. These mitomycin C–DNA complexes were poorly used by DNA polymerases α and β as template-primers or as initiators by TdT (less than 9 per cent of the control). Furthermore, it was found that mitomycin C–DNA complex is a potent inhibitor for DNA polymerizing reactions.

As shown in Fig. 1A, the reaction of DNA polymerase α was less sensitive to the mitomycin C–DNA complex than the DNA polymerase β was. In the presence of 160 μg/ml of activated DNA template-primer, DNA polymerase α was inhibited 38 per cent by 3.17 μg/ml of mitomycin C–DNA complex (binding ratio, 3), and DNA polymerase β was inhibited 51 per cent by the same concentration of the complex. It is shown in Fig. 1B that the mitomycin C–DNA complex containing the larger amounts of mitomycin C inhibited the reaction more strongly. TdT was inhibited by mitomycin C–DNA complex to an extent similar to that of DNA polymerase β. DNA polymerase I of *Escherichia coli* was also inhibited by mitomycin C–DNA complex to an extent similar to that of DNA polymerase α (data not shown). The free form of mitomycin C, added directly to the reaction mixtures of DNA polymerase α and β, did not inhibit the reactions at concentrations up to 4 mM (Fig. 1A).

Mitomycin C–DNA complex prepared from native calf thymus DNA inhibited the reactions of DNA polymerases α and β to exactly the same extent as

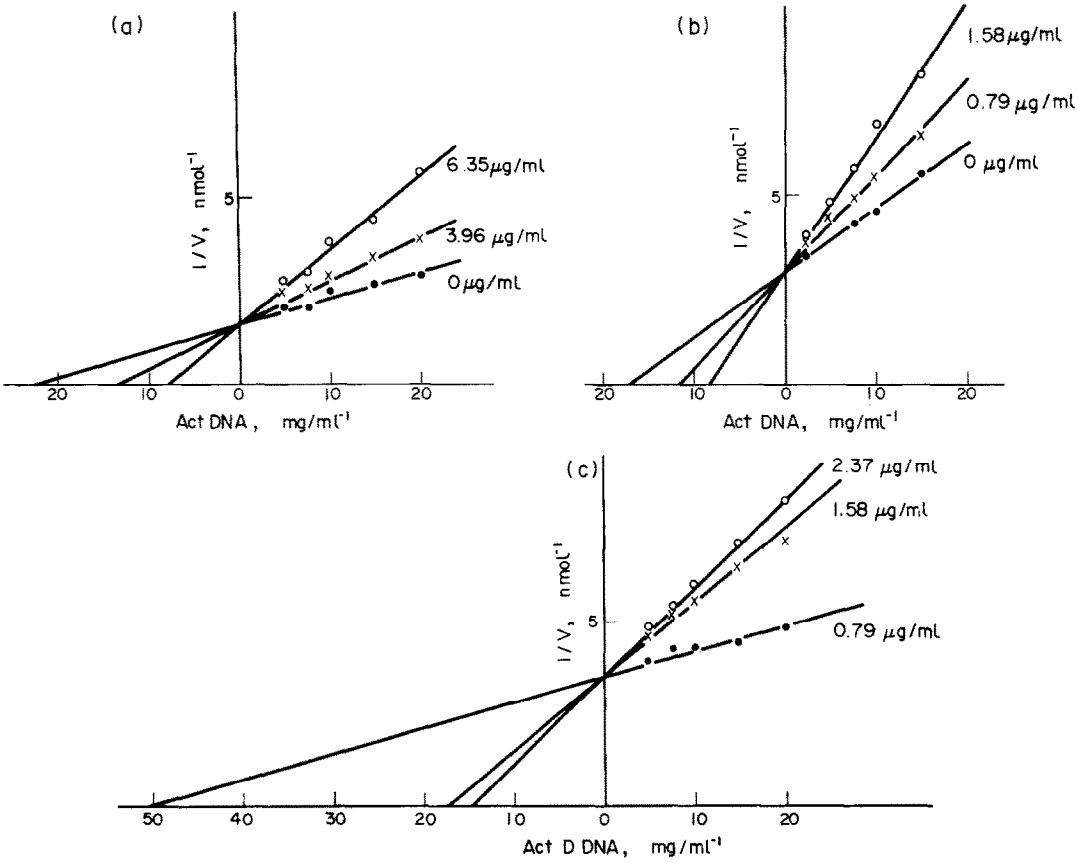

Fig. 2. Competitive inhibition of DNA polymerases α and β and of TdT by mitomycin C–DNA complex. The assays were performed with DNA polymerase α (Panel A), DNA polymerase β (panel B) and TdT (panel C) as described in Materials and Methods, except that activated DNA was added to the reaction mixture at the concentrations indicated. Mitomycin C–DNA complex with a binding ratio of 3, prepared from activated calf thymus DNA, was added to the reaction mixture at the concentrations indicated in the figure. Data were plotted according to Lineweaver and Burk.

the complex prepared from activated DNA when complexes with the same binding ratio were compared (Fig. 1A).

Mode of inhibition. As shown in Fig. 2 (panels A and B), mitomycin C–DNA complex inhibited the reactions of DNA polymerases α and β competitively with respect to the template-primer, activated DNA. The inhibition was reversed by increasing the concentration of activated DNA in the reaction mixture. The inhibition by mitomycin C–DNA complex was not affected by increasing the concentration of the enzyme or of dNTPs in the reactions of either DNA polymerases α or β (Table 1). TdT was also inhibited competitively by mitomycin C–DNA complex with respect to initiator DNA (single-stranded) (Fig. 2C).

K_i *value.* K_i values of 6.9, 5.7 and 2.6 μg/ml for mitomycin C–DNA complex (binding ratio, 3) were obtained from Dixon plots for DNA polymerases

Table 1. Effect of increased concentration of enzyme or dNTPs on the inhibition of DNA polymerases α and β by mitomycin C–DNA complex*

Treatment	Inhibition (%) produced by 4.18 μg/ml of mitomycin C–DNA complex (binding ratio, 3) DNA polymerase α	DNA polymerase β
(A) Enzyme 0.75 units, dNTPs 0.1 mM	42.1	64.1
Enzyme 1.5 units, dNTPs 0.1 mM	43.0	62.2
Enzyme 3.0 units, dNTPs 0.1 mM	46.7	64.8
(B) Enzyme 1.5 units, dNTPs 0.1 mM	43.0	62.2
Enzyme 1.5 units, dNTPs 0.2 mM	46.0	63.1
Enyzme 1.5 units, dNTPs 0.3 mM	44.4	65.9

* Assays of DNA polymerases α and β were performed as described in Materials and Methods.

Table 2. K_i values of DNA polymerases α and β and TdT for mitomycin C–DNA complex*

Mitomycin C–DNA complex	DNA polymerase α (μg/ml)	DNA polymerase β (μg/ml)	TdT (μg/ml)
Binding ratio, 3	6.98	5.71	2.65
Binding ratio, 7	9.02		
Binding ratio, 21	24.60		

* K_i values were obtained from Dixon plots. Assays of DNA polymerases α and β and TdT were performed as described in Materials and Methods. K_m values for activated calf thymus DNA were 40.0, 58.8 and 14.3 μg/ml for DNA polymerases α and β and TdT respectively.

α and β and TdT respectively. The relationships of the K_i values for DNA polymerase α to the binding ratios of the mitomycin C–DNA complex are shown in Table 2.

The K_i values were a function of the molar binding ratio; inhibition, therefore, was related to the number of molecules of mitomycin C bound to DNA. These K_i values for the mitomycin C–DNA complex are markedly lower than the K_m values for activated DNA template-primer of DNA polymerase α (40.0 μg/ml), β (58.8 μg/ml) and TdT (14.3 μg/ml).

Inhibition of the synthetic template-primer dependent reaction. To confirm the inhibition of DNA polymerases by the mitomycin C–DNA complex, the initiated deoxyhomopolymers were used as template. As shown in Fig. 3, poly(dT)·oligo(rA)-dependent poly(dA) synthesis by DNA polymerase α was strongly inhibited by the mitomycin C–DNA complex, whereas control DNA slightly stimulated the reaction, for some unknown reason. Since the Dixon plot was not linear in this system (Fig. 3B), the K_i value was estimated from the extrapolation of the curves. The K_i value, 3.3 μg/ml, was lower than that obtained with activated DNA(Table 2). Unlike the activated DNA-dependent system, the synthetic homopolymer-dependent reaction of DNA polymerase β is strongly inhibited by either native or activated natural DNAs [8]. For this reason, it was difficult to measure the inhibition of DNA polymerase β by the complex with synthetic homopolymers.

DISCUSSION

The formation of covalent cross-links involving DNA has been suspected of being responsible for the potent cytotoxic and anti-tumor actions of bifunctional alkylating agents, including mitomycin C. Covalent cross-linking of the paired strands of the DNA helix has been studied extensively with bifunctional nitrogen-mustard, and it is clear that such lesions, if unrepaired, would interfere with DNA replication. Cross-linking of the paired DNA strands may hamper strand separation as the replication fork, and the transcription process, proceed. This has been thought to be the main mechanism for the cytotoxicity of mitomycin C.

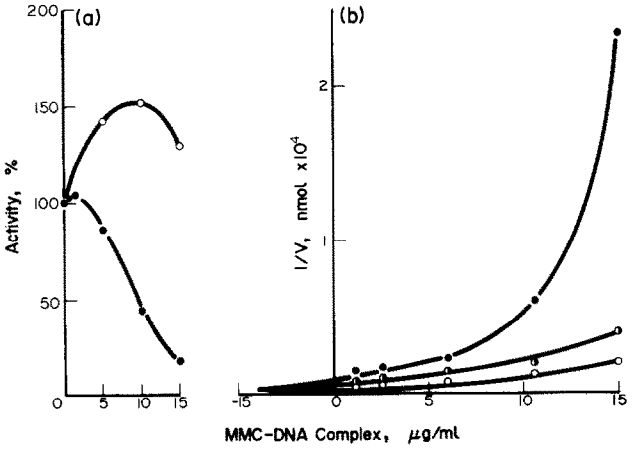

Fig. 3. Inhibition of poly(dT)·oligo(rA)-dependent activity of DNA polymerase α by mitomycin C–DNA complex. The reaction mixture (62.5 μl) contained 80 mM Tris–HCl (pH 7.5), 20 mM KCl, 4 mM 2-mercaptoethanol, 80 μg/ml bovine serum albumin, 0.5 mM $MnCl_2$, 100 μM dATP, poly(dT)·$(rA)_{10}$ (base ratio T/A = 10), DNA polymerase α (1.5 units) and the inhibitor. Incubation was carried out at 30° for 1 hr. Panel A: Reactions were carried out in the presence of mitomycin C–DNA compled (●), or control activated calf thymus DNA (○), at the concentrations indicated. Panel B: Dixon plot. Reactions were carried out with 0.032 (●), 0.08 (◑) and 0.16 (○) mg/ml of poly(dT)·$(dA)_{10}$ in the presence of mitomycin C–DNA complex at the concentrations indicated.

Here we have observed that DNA lost its template activity for DNA polymerizing reactions, by interacting with mitomycin C. Furthermore, it was found that the mitomycin C–DNA complex strongly inhibited DNA polymerizing enzymes in the presence of a large excess of template-primer. The inhibitory action of mitomycin C–DNA complex depended on the amount of mitomycin C bound to DNA. Interestingly, free mitomycin C did not inhibit the DNA polymerase reaction even at high concentrations. Therefore, the molecular structure of mitomycin C, itself, may not be important, but the peculiar conformational change(s) in DNA, produced by cross-linking, may be responsible for the inhibition. Kinetic study indicated that the mitomycin C–DNA complex binds to the site of DNA polymerases where the template-primer is expected to bind. It is conceivable that the conformation around cross-linking may mimic that of a replication fork or that of a site undergoing excision repair, to which DNA polymerase may have higher affinity.

It is remarkable that TdT is also strongly inhibited by the complex, in the same way as the DNA polymerases are. TdT is an enzyme that has a unique capacity to add mononucleotides to a 3′-OH end of a single-stranded DNA initiator in the absence of a template strand, but the biological meaning of this is unknown. The high affinity of TdT for the mitomycin C–DNA complex (double-stranded) observed here suggests that TdT is also able to recognize the double-stranded structure of DNA under special conditions.

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