ACLACINOMYCIN A-INHIBITION OF PHAGE ϕX174 DNA SYNTHESIS *IN VITRO*

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Aclacinomycin A inhibited the *in vitro* conversion of phage ϕX174 single-stranded DNA to the replicative form DNA. DNA synthesis was inhibited by 50% in the presence of 15 μM aclacinomycin A. The inhibition was competitive with respect to template DNA (K_i=13 μM) and was reversed by addition of *Escherichia coli* cell extracts. Short complementary strands approximately one-third of unit length molecule were synthesized in the presence of 15 μM aclacinomycin A. The data suggest that aclacinomycin A may inhibit the process of ϕX174 DNA chain elongation by a direct interaction with the *E. coli* host enzymes.

Aclacinomycin A, a novel anthracycline antitumor agent isolated from *Streptomyces galilaeus* MA144-MI,[1] is potentially and clinically important because of its low cardiotoxicity[2] and of no mutagenicity in the AMES' test.[3]

Previous studies have shown that aclacinomycin A inhibited both DNA and RNA synthesis, and that the binding of aclacinomycin A to template DNA may interfere the association of the polymerizing enzymes[4] with template DNA. TANAKA *et al.*[5] recently reported that other anthracycline antibiotics, daunomycin and adriamycin, strongly inhibited the reaction of both DNA polymerases α and β from calf thymus by competing with the template primers. This inhibition appears to be due to the direct action of these anthracycline antibiotics with DNA polymerases.

We reinvestigated the target of the aclacinomycin A in DNA synthesis using ϕX174 *in vitro* replication system. Although ϕX174 DNA is synthesized by a complex process in which many proteins are involved, the replication of ϕX174 single-stranded (SS) DNA to the duplex replicative form (RF) DNA is the best characterized among *in vitro* systems for DNA synthesis.[6] This system depends solely upon *E. coli* host enzymes and includes the following processes: prepriming, priming, chain elongation, gap filling and ligation. Furthermore, as the *in vitro* system of DNA synthesis proceeds with the cell extracts from an *E. coli polA* mutant, DNA polymerizing activity measured with the extracts predominantly reflects DNA polymerase III activity. Using this *in vitro* system of ϕX174 DNA synthesis, we have found that aclacinomycin A inhibited the chain elongation by a direct interaction with *E. coli* cell extracts.

Materials and Methods

Bacterial and Phage Strains

Escherichia coli H560[7] (*polA, endA, thyA*) was used for the preparation of enzyme. Phage ϕX174 *am*3, a lysis defective mutant of ϕX174, was used.

Chemicals

Unlabeled deoxyribonucleoside triphosphates (dNTPs) and ribonucleoside triphosphates (rNTPs)

were obtained from Boehringer Mannheim GmbH. [*Methyl*-^{3}H]dTTP (78 Ci/mmole) was purchased from New England Nuclear Corporation. Aclacinomycin A hydrochloride was generously given by Sanraku Ocean Co., Ltd. Other chemicals were purchased from Nakarai Chemicals Co., Ltd.

Preparation of Phage DNA

Phage ϕX174 SS DNA was prepared by the procedure of SEDAT and SINSHEIMER.[8]

Preparation of *E. coli* Ammonium Sulfate Fraction

An *E. coli* ammonium sulfate fraction capable of catalyzing the conversion of ϕX174 RF DNA from SS DNA was prepared by the method of WICKNER *et al.*[9] with some modifications. *E. coli* H560 was grown to the cell density of 0.6 absorbance unit at 610 nm in L-broth medium supplemented with 50 μg/ml of thymine. The cells were collected by centrifugation at 20°C and resuspended in 0.002 volume of 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5) containing 10% sucrose and frozen in a dry ice-acetone bath. Cells were thawed in an ice-water bath and then incubated with 0.2 mg/ml lysozyme, 1 mM dithiothreitol (DTT), 20 mM EDTA, 100 mM KCl for 5 minutes at 0°C. The suspension was further incubated with 0.1% Brij 58 for 25 minutes at 0°C. The lysate thus prepared was centrifuged at 2°C for 45 minutes at 24,000 rpm in an RPS25 rotor of a Hitachi ultracentrifuge. The supernatant was adjusted to a final concentration of 4% with a solution of 20% streptomycin sulfate and centrifuged at 10,000$\times g$ for 5 minutes. This supernatant was adjusted to 43% saturation with ammonium sulfate and centrifuged at 10,000$\times g$ for 15 minutes at 2°C. The pellet was suspended in 0.1 volume (of the supernatant obtained after ultracentrifugation) of 0.01 M Tris-HCl (pH 7.5), 1 mM DTT, 0.1 mM EDTA and 15% glycerol, and dialyzed against the same buffer for 2 hours at 2°C. This ammonium sulfate fraction was stored in small portions at −20°C.

Assay Conditions for DNA Synthesis

The standard reaction mixture (50 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM DTT, 1 mM ATP, 1 mM NAD^+, 40 μM each of dATP, dCTP, dGTP and [*methyl*-^{3}H]dTTP (200 cpm/pmole), 100 μM each of CTP, UTP and GTP, 2 mM spermidine·3HCl, 0.3 nmole (as nucleotides) of ϕX174 SS DNA and 400 μg proteins of ammonium sulfate fraction. The reaction mixture was incubated at 30°C for 30 minutes. The reaction was stopped by the addition of 1.5 ml of 0.1 M sodium pyrophosphate-10% trichloroacetic acid. The precipitates formed were collected onto Whatman GF/C glass fiber discs. The discs were dried and counted for radioactivity. When the reaction product was characterized by the sedimentation analysis, the reaction was stopped by the addition of EDTA and Sarkosyl to final concentrations of 24 mM and 2%, respectively, followed by incubation at 42°C for 10 minutes as described by SUMIDA-YASUMOTO *et al.*[10]

Sedimentation Analysis

The reaction product was analyzed (a) in a 5 to 20% neutral sucrose gradient containing 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl and 3 mM EDTA at 37,000 rpm for 4.5 hours at 2°C in an RPS 40T-2 rotor of a Hitachi ultracentrifuge, or (b) in a 5 to 20% alkaline sucrose gradient containing 0.5 M NaCl, 3 mM EDTA, 0.1% Sarkosyl and 0.3 N NaOH on a cushion of 0.2 ml of 55% CsCl solution at 37,000 rpm for 7.5 hours at 2°C in an RPS 40T-2 rotor of the same ultracentrifuge

Results

Inhibition of DNA Synthesis by Aclacinomycin A

Fig. 1 shows the effect of aclacinomycin A on the *in vitro* conversion of ϕX174 SS DNA to RF DNA. The DNA synthesis was inhibited proportional to the amount of aclacinomycin A added. DNA synthesis was reduced to 50% by 15 μM aclacinomycin A and inhibited completely by 30 μM of the antibiotic. When aclacinomycin A was added to the ongoing reaction at different times after the initiation of DNA synthesis, the incorporation of [^{3}H]dTMP ceased immediately upon addition of the antibiotic. No [^{3}H]dTMP was incorporated when aclacinomycin A was added at the time of the initi-

Fig. 1. Concentration dependency of the inhibition of DNA synthesis by aclacinomycin A.

Reaction was carried out as described in Materials and Methods. Aclacinomycin A was added at the indicated concentrations. The activity of DNA synthesis was measured by [^{3}H]dTMP incorporation into acid-insoluble materials. 100% activity (drug-free control) corresponds to 12 pmole of [^{3}H]dTMP incorporation into acid-insoluble materials.

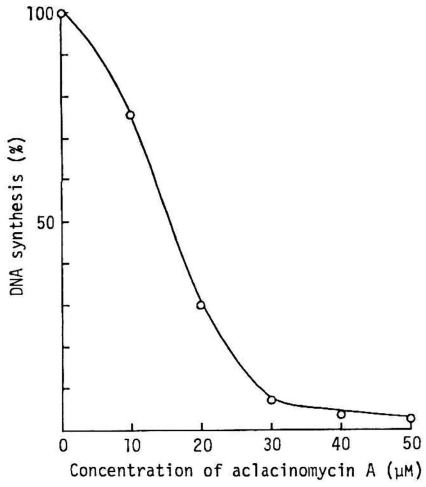
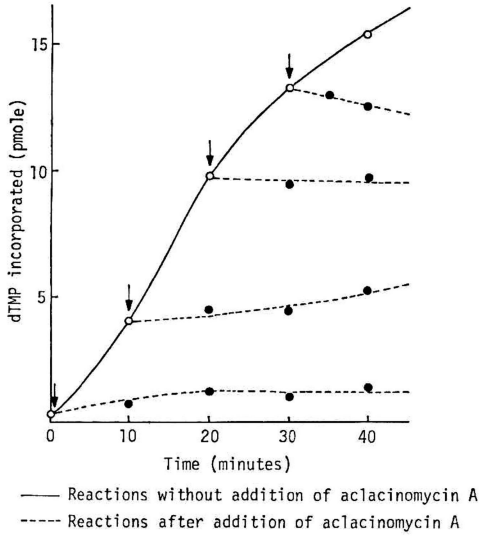


Fig. 2. Effect of aclacinomycin A on the time course of DNA synthesis.

Aclacinomycin A was added at a final concentration of 35 μM at the indicated times after the initiation of reaction. Other conditions were the same as described in Materials and Methods. Arrows indicate the times of adding the antibiotic.



ation of DNA synthesis (Fig. 2). These results suggest that DNA chain elongation was inhibited by aclacinomycin A.

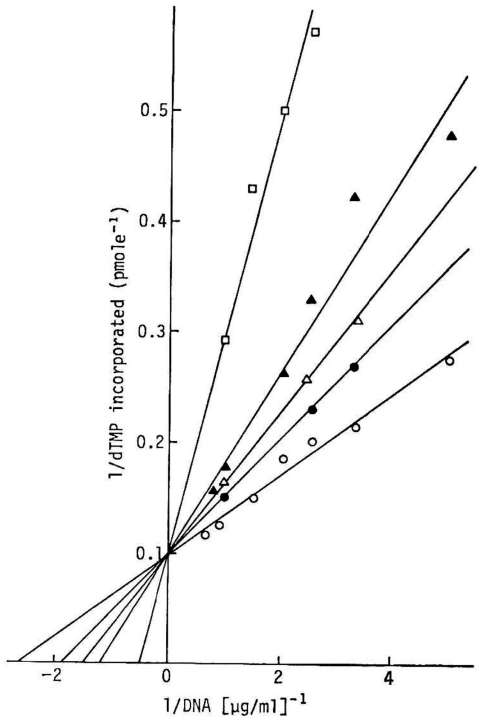
Characterization of Aclacinomycin A-Inhibition

The nature of the inhibition of DNA synthesis by aclacinomycin A was further characterized by examining the extent of the inhibition in the presence of increasing concentration of the template DNA. Fig. 3 shows double-reciprocal plots of the inhibition of DNA synthesis by aclacinomycin A. Aclacinomycin A inhibited the conversion of ϕX174 SS DNA to RF DNA competitively with respect to the template DNA. K_i value for aclacinomycin A was calculated to

Fig. 3. Double reciprocal plots of DNA polymerase activity *versus* template DNA concentration.

Reaction was carried out as described in Materials and Methods. The concentration of the template SS DNA was changed as indicated in the Figure.

Concentration of aclacinomycin A; □: 20 μM, ▲: 15 μM, △: 10 μM, ●: 5 μM, ○: 0 μM.



be 13 μM. This kinetic data indicates that aclacinomycin A binds to a template binding site in *E. coli* cell extracts, and in turn inhibits DNA chain elongation.

Preincubation Effect of Aclacinomycin A on the Activity of DNA Synthesis

The kinetic analysis (Fig. 3) showed that the reaction of DNA synthesis was inhibited competitively by aclacinomycin A. To examine whether aclacinomycin A reacts directly with *E. coli* cell extracts or template DNA, preincubation experiment was carried out. The *E. coli* cell extracts or template ϕX174 SS DNA was preincubated with aclacinomycin A at 0°C for 30 minutes, then other ingredients were added and the reaction was performed as usual. When the template DNA was preincubated with aclacinomycin A, the extent of inhibition was the same as that without preincubation (Fig. 4a). When the template DNA was preincubated at 30°C for 15 minutes, the extent of inhibition was also the same as that without preincubation (data not shown). However, the *E. coli* cell extracts preincubated with aclacinomycin A showed less DNA polymerizing activity than that without preincubation (Fig. 4b).

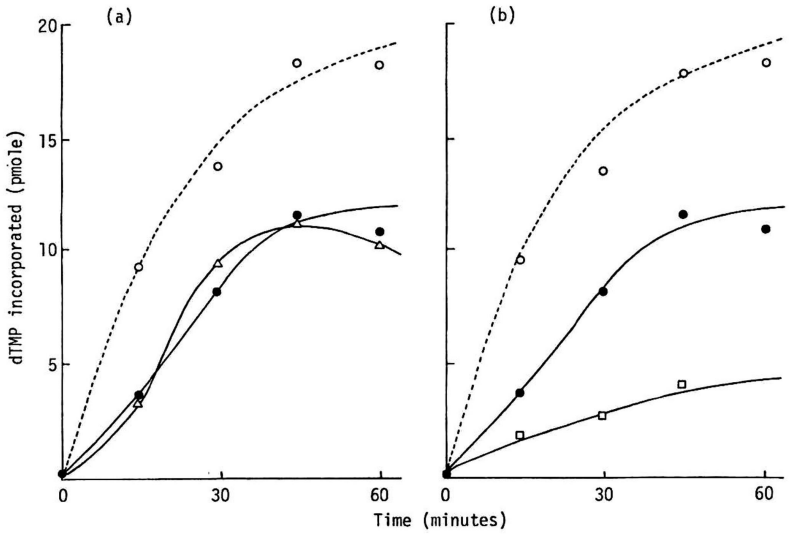
These results suggest that the inhibition of DNA synthesis by aclacinomycin A was caused by the interaction of the antibiotic with the *E. coli* cell extracts rather than with template DNA.

Reversibility of the Inhibitory Effect of Aclacinomycin A

Reversibility of aclacinomycin A-inhibition was investigated by addition of either the *E. coli* cell extracts or template DNA to the reaction mixture containing aclacinomycin A. Addition of *E. coli*

Fig. 4. Preincubation effects of aclacinomycin A on DNA synthesis.

Prior to starting the reaction, various reaction constituents were preincubated at 0°C for 30 minutes. The results of preincubating the template DNA with the antibiotic is shown in panel (a); and the *E. coli* cell extracts with the antibiotic in panel (b); DNA synthesis after preincubation of template SS DNA (0.3 nmole) and aclacinomycin A (30 μM) △; after preincubation of *E. coli*, cell extracts (400 μg) and aclacinomycin A (30 μM) □; with complete reaction mixture including 15 μM aclacinomycin without preincubation ●; and with the drug-free control (the complete reaction mixture was preincubated at 0°C for 30 minutes) ○: The other ingredients were added to the reaction mixture after preincubation and the complete reaction was incubated for 60 minutes a 30°C. The final concentration of aclacinomycin A in samples was 15 μM.



cell extracts was more effective in reversing the inhibition of aclacinomycin A than that of template DNA. (Table 1)

These results also indicate that aclacinomycin A interacts directly with the *E. coli* cell extracts.

Table 1. Aclacinomycin A-inhibition of *in vitro* ϕX174 DNA synthesis.

Additions	dTMP incorporated (pmole)[e]
None[a]	27.8
Aclacinomycin A[b]	5.4
Aclacinomycin A, template DNA[c]	9.3
Aclacinomycin A, *E. coli* cell extracts[d]	14.6

The reaction mixtures were incubated at 30°C for 15 minutes in the absence[a] or presence[b] of aclacinomycin A (20 μM). Two samples with aclacinomycin A were supplemented with either additional template DNA (0.2 nmole)[c] or *E. coli* cell extracts (200 μg).[d] The complete reaction mixtures were incubated for 15 minutes and the radioactivity in the acid-insoluble materials was determined.[e]

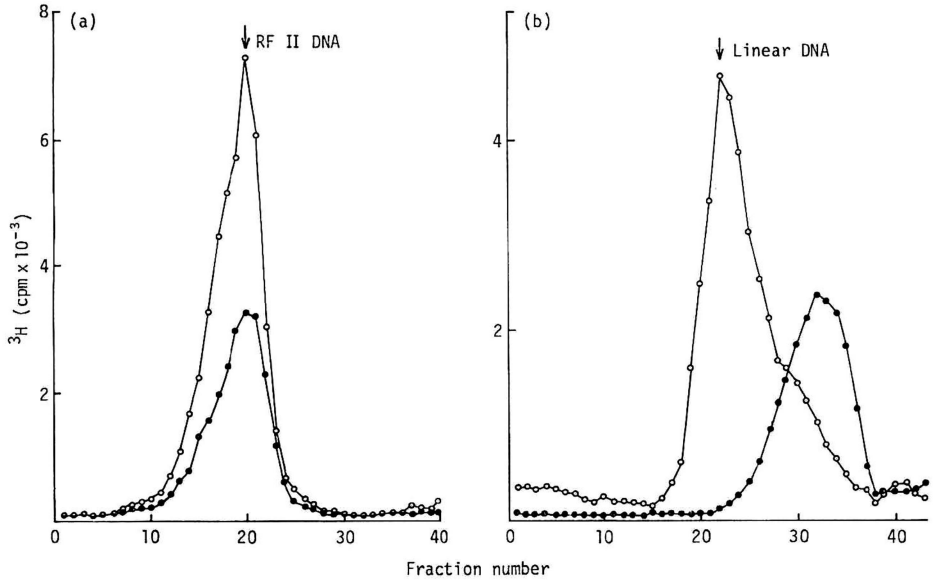
Sedimentation Analysis of the Reaction Products

The DNA product newly synthesized in the presence of 15 μM aclacinomycin A (which gave 50% inhibition) was analyzed by both neutral and alkaline sucrose gradient sedimentations (Fig. 5). In a neutral sucrose gradient (Fig. 5a) the DNA product sedimented at the position of RF II (open circular double-stranded DNA).

The DNA product sedimented at the same position when DNA synthesis was performed in the presence of the antibiotic. However, the amount of dTMP incorporated in the presence of the antibiotic was about half of that in the absence of the antibiotic. In an alkaline sucrose gradient, DNA product synthesized in the absence of the antibiotic sedimented as a single peak of full-length linear DNA, while the DNA product in the presence of 15 μM aclacinomycin A sedimented as a broad peak having a sedimentation coefficient of around 5S (Fig. 5b), and no detectable peaks were observed at the

Fig. 5. Sucrose gradient analysis of DNA synthesized *in vitro*.

The reaction mixture (250 μl) was incubated at 30°C for 40 minutes in the absence (○) or presence of 15 μM aclacinomycin A (●). Samples were layered on 5~20% (a) neutral or (b) alkaline sucrose gradients and centrifuged as described in Materials and Methods. Fractions were collected and radioactivity in acid-insoluble materials was counted. Arrows indicate the marker position of ϕX174 RF II DNA in panel (a) or single-stranded linear DNA in (b).



position of full-length linear DNA.

These results indicate that aclacinomycin A inhibits the conversion of ϕX174 SS DNA to RF DNA and the DNA synthesized was accumulated as the short fragments of approximately one-third of the complete molecule.

Discussion

Aclacinomycin A appears to inhibit the *in vitro* conversion of ϕX174 SS DNA to RF DNA during the process of chain elongation. This conclusion is derived from the following criteria; (i) DNA synthesis immediately ceased upon addition of aclacinomycin A (Fig. 2), and (ii) the DNAs formed in the presence of aclacinomycin A were short fragments (Fig. 5).

The mode of this inhibition was competitive with respect to the template DNA but not with respect to substrate deoxyribonucleoside triphosphate. This mode of inhibition represents a mixed type one class (data not shown). The distinct mode of inhibition may be due to the different binding sites of template DNA and substrates in *E. coli* host enzymes.

Since aclacinomycin A does not affect the transfection activity of ϕX174 SS DNA at the concentration used in this experiment[11], the inhibition of DNA synthesis does not involve the interaction of this antibiotic with template DNA. Preincubation experiments indicate that DNA synthesis is inhibited by the interaction of the antibiotic with *E. coli* host enzymes. Furthermore, the result that the addition of *E. coli* cell extracts was effective in reversing the inhibitory effect of aclacinomycin A strongly suggests that the site of action is in *E. coli* host enzymes and not template DNA. As many enzymes participate in the chain elongation process for ϕX174 SS DNA conversion to RF DNA (such as DNA polymerase III holoenzyme, single-stranded DNA binding protein and some other proteins) it is very difficult to determine the target enzyme of aclacinomycin A. YAMAKI *et al.*[4] reported that RNA polymerase reaction using calf thymus DNA as a template was sensitive to aclacinomycin A, but the reaction catalyzed by *E. coli* DNA polymerase I was not significantly affected. However, aclacinomycin A may affects DNA polymerase III activity since the present DNA synthesis system does not involve DNA polymerase I activity.

The effects of other anthracycline antibiotics, daunomycin and adriamycin, on DNA synthesis have been examined by several investigators. ZUNINO *et al.* reported that the DNA syntheses catalyzed by DNA polymerases from many sources such as murine sarcoma virus, rat liver and *Micrococcus luteus* were inhibited by these antibiotics.[12] They concluded that the reduction of the template activity by these antibiotics could be responsible for the inhibition of DNA polymerase reactions. TANAKA *et al.* showed the competitive inhibition of calf thymus DNA polymerases by the direct interaction of anthracycline antibiotics with the enzyme molecules.[5] GOODMAN also suggested that the competitive inhibition was observed in T4 DNA polymerase reaction by adriamycin.[13] We also investigated the effects of adriamycin and daunomycin on ϕX174 DNA synthesis *in vitro* (data not shown). These antibiotics prevented the conversion of ϕX174 SS DNA to RF DNA by competing with template DNA. The K_i values of adriamycin and daunomycin were 14 μM and 17 μM, respectively. However, since these antibiotics reduce the template activity of ϕX174 SS DNA by the formation of DNA-drug complex,[14] the direct evidence of interaction of antibiotics with *E. coli* host enzymes could not be obtained.

When DNA synthesis is blocked by aclacinomycin A, the synthesized complementary DNAs are incomplete. Therefore, why did the reaction products sediment at the position of RF II DNA under neutral sucrose gradient analysis (Fig. 5a)? One explanation is that initiation of DNA synthesis occurs but chain elongation is inhibited. The other possible explanation is that the activity of ligation in *E. coli* is reduced by aclacinomycin A and the unit length DNA of ϕX174 can not be synthesized. The DNA formed has a molecular size of approximately one-third of the unit length DNA. The template DNA hybridized with this size of DNA fragment might sediment at the position of RF II under neutral conditions. Since aclacinomycin A itself breaks neither ϕX174 SS DNA nor RF DNA,[14] the short DNA fragments are not the degradation products of the synthesized DNA.

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