EFFECT OF THE ANTITUMOR ANTIBIOTIC NEOCARZINOSTATIN ON DNA SYNTHESIS *IN VITRO*

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The effect of neocarzinostatin on *in vitro* DNA synthesis by DNA polymerase I prepared from *Escherichia coli* was investigated. DNA synthesis *in vitro* by *Escherichia coli* polymerase I was inhibited in the presence of neocarzinostatin. The inhibition increased when the template was preincubated with the antibiotic. Synthesis directed by poly d(A-T) was more sensitive to the antibiotic than synthesis directed by calf thymus DNA. The melting temperature of DNA decreased when it was incubated with neocarzinostatin *in vitro*. From these results it was suggested that the effect of neocarzinostatin on DNA synthesis *in vitro* could be caused by a direct interaction of the antibiotic with the template.

Neocarzinostatin is an antitumor antibiotic obtained from the culture filtrate of *Streptomyces carzinostaticus*[1]. A highly purified preparation of this antibiotic has been characterized as an acidic polypeptide with a molecular weight of about 9,000 having alanine and asparagine as its N- and C-terminal amino acids, respectively[2]. The cytotoxic effect of this compound against several malignant cells such as mouse[3] and rat[4] ascites tumors, Sarcoma 180[5], L-1210[6] and HeLa[7] cells has already been reported. The cytomorphological characteristics of HeLa[7] and Sarcoma 180[5] cells treated with neocarzinostatin were a sudden decrease of mitotic index which was followed by various chromosomal abnormalities and enlargement of the cells.

The mechanism of the action of this antibiotic, however, has not been clarified. In whole-cell experiments, ISHIDA and his coworkers reported[8] that neocarzinostatin selectively inhibited DNA synthesis in the susceptible bacterium, *Sarcina lutea*, without affecting RNA and protein biosyntheses and that in the neocarzinostatin-treated cells, DNA was degraded into free bases and the degradation seemed to be caused by an induction of a new enzyme protein[9].

We have examined the effects of this antibiotic on DNA synthesis *in vitro* using a highly purified DNA polymerase from *Escherichia coli* and describe the results in this communication.

Materials and Methods

Chemicals

Unlabeled deoxyribonucleoside 5′-triphosphates and highly polymerized calf thymus DNA (type I) were purchased from Sigma Chemical Company. ^{3}H-TTP (specific activity 9.8 C per m mole) was obtained from Schwarz BioResearch. Poly d(A-T) (alternating

copolymer of deoxyriboadenylate and thymidylate) was purchased from Miles Laboratories, Inc. Neocarzinostatin was prepared by Kayaku Antibiotics Research Company Ltd. Tokyo.

DNA Polymerase

E. coli DNA polymerase was purified from *E. coli* strain B according to JOVIN *et al.*[10], the final specific activity of the enzyme was 13,000 units/mg protein (1 unit will incorporate 10 mμ moles of nucleoside triphosphate into acid-insoluble product per 30 minutes).

Polymerization Reaction

The assay of DNA polymerase measured the conversion of the radioactively labeled deoxyribonucleoside triphosphate into an acid-insoluble product. The standard reaction mixture (S.R.M.: 0.15 ml) contained 70 mM Tris-HCl buffer (pH 7.2), 7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 140 μM* "activated" calf thymus DNA[11], 35 μM each of dATP, dGTP, dCTP and ^{3}H-TTP (specific activity 10 μC per μmole), and 0.05 to 0.1 unit of enzyme, the range in which linear synthesis of DNA has been observed[11]. The incubation was performed at 37°C for various time intervals and the reaction was stopped by chilling the tubes. An aliquot of 100 μl was removed from the tubes and placed on a Toyo Roshi No. 514 filter paper disk which was previously washed with 0.2 M sodium pyrophosphate to diminish the non-specific adsorption of radioactive material[12]. After washing the disks three times with cold 5 % trichloroacetic acid, radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrophotometer. In the reaction where 20 μM of poly d(A-T) was used as template instead of calf thymus DNA, dGTP and dCTP were omitted.

Preincubation of Neocarzinostatin with each Component of the DNA Synthesis System *in vitro*

To determine possible interactions of neocarzinostatin with any component of the DNA synthesizing system, the antibiotic was separately preincubated at various concentrations with DNA polymerase, template or deoxyribonucleoside triphosphates in the presence of 70 mM Tri-HCl buffer (pH 7.2), 7 mM $MgCl_2$ and 1 mM 2-mercaptoethanol (total volume 70~110 μl) at 37°C for 30 minutes (Figs. 1b and 2). DNA synthesis was started by adding the missing components. After incubation at 37°C for additional various time intervals, the incorporation of radioactivity into acid-insoluble product was determined.

Thermal Denaturation of DNA Treated with Neocarzinostatin

The melting profiles of DNA were determined after incubation of DNA with increasing amounts of neocarzinostatin at various incubation times. The concentration of reactants in the incubation mixture were the same as those in the polymerization reaction. Thus the mixture contained 70 mM Tris-HCl buffer (pH 7.2), 7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 μg/ml of highly polymerized calf thymus DNA in total volume of 4 ml. The antibiotic was added in the concentration range used in the inhibition experiments (Fig. 1). After incubation at 37°C for various time intervals, the absorbance at 260 mμ was measured as the temperature was increased according to MANDEL and MARMUR[13]. Absorbance at 260 mμ given in Fig. 3 was obtained by subtraction of $A_{260\,m\mu}$ at 25°C as blank value from the observed values.

Results

Inhibition of *in vitro* DNA Synthesis by Neocarzinostatin

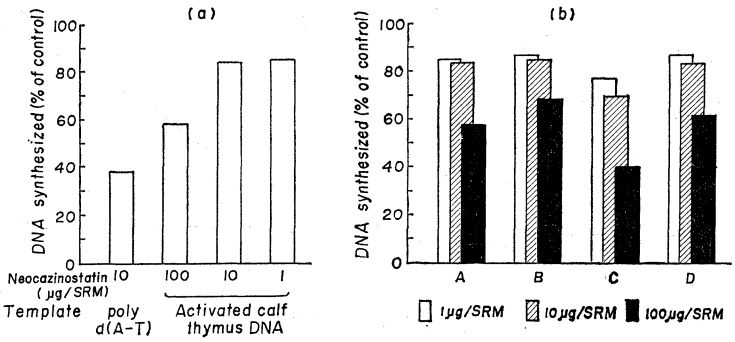
The effect of neocarzinostatin on DNA synthesis *in vitro* was examined using two kinds of template, *i.e.* calf thymus DNA and poly d(A-T). In both cases the pH of the reactions did not change after the addition of neocarzinostatin. The results are shown in Figs. 1a and 1b. DNA synthesis was inhibited in both cases by addition of the antibiotic. A higher level of inhibition was observed for poly d(A-T) than for

* Molarity of DNA refers to concentration of nucleotide residues.

Fig. 1. Inhibition of *in vitro* DNA synthesis by neocarzinostatin.

(a) The inhibition of *in vitro* DNA synthesis by *E. coli* DNA polymerase was examined using two kinds of template *i.e.* activated calf thymus DNA and poly d(A-T). The reaction mixtures contained the reagents described in the text and amounts of neocarzinostatin given in the abscissa of the figure. After incubation for 60 minutes at 37℃, the reaction was stopped and acid-insoluble radioactivity was determined as described in Materials and Methods. Under these experimental conditions, the acid-insoluble radioactivity was proportional to the amount of enzyme used and incubation time. Controls where neocarzinostatin was omitted showed an incorporation of about 1.4 and 0.8 mμ moles of deoxyribonucleotides for poly d(A-T) and activated calf thymus DNA primed reactions, respectively.

(b) Influence of the preincubation of neocarzinostatin with the component in the DNA polymerase reaction was examined using activated calf thymus DNA as a template. The antibiotic was separately preincubated with DNA polymerase (B), template (C), and deoxyribonucleoside triphosphates (D) at 37℃ for 30 minutes at the antibiotic levels of 1 μg/S.R.M., 10 μg/S.R.M. and 100 μg/S.R.M. After the preincubation, the reaction was started by adding the missing component at 37℃. The reaction was stopped after 60 minutes by cooling at 0℃ and the amount of DNA synthesized under these conditions was compared with that of the standard reaction which was performed without the preincubation (A). Controls where neocarzinostain was omitted showed an incorporation of about 0.8 mμ moles of deoxyribonucleotides for these activated calf thymus DNA primed reactions.



the "activated" calf thymus DNA used as template (Fig. 1a). With calf thymus DNA as template, inhibition increased with increasing amount of the antibiotic from 10 to 100 μg/S. R. M. (S. R. M.: volume of the standard reaction mixture; see "Materials and Methods"). No increase was observed when the concentration of the antibiotic was increased from 1 to 10 μg/S.R.M. (Fig. 1a).

The enzyme or each of the other components contained in the reaction system were separately preincubated with neocarzinostatin at 37°C for 30 minutes and then the reaction was started by addition of the missing components. As is shown in Fig. 1b, at all of the antibiotic concentrations tested, the inhibition of DNA synthesis by neocarzinostatin was most marked when the antibiotic was preincubated with the template DNA.

Time-dependent Inhibition of *in vitro* DNA Synthesis by Neocarzinostatin

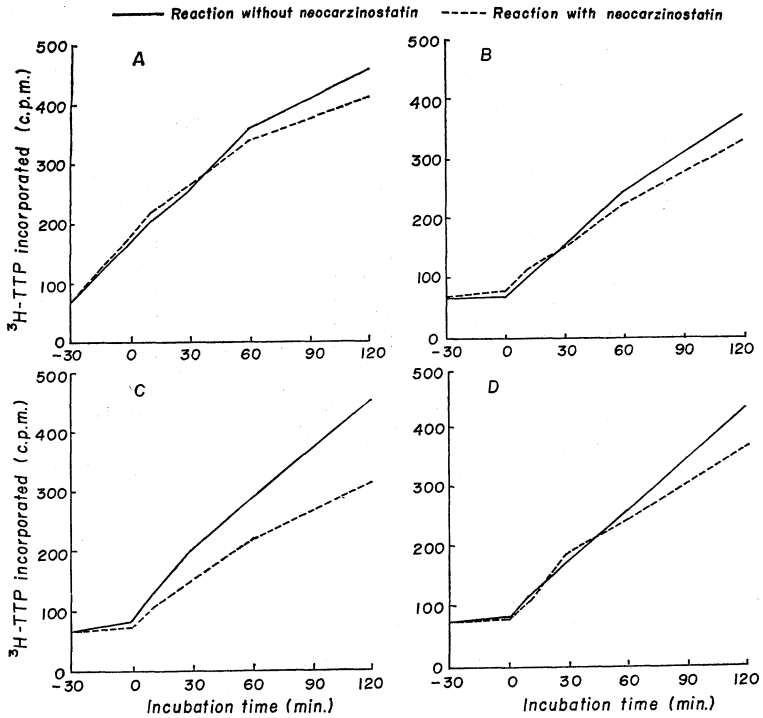
To confirm the effect of neocarzinostatin on DNA synthesis, the polymerization reaction was assayed at various time intervals and the results are shown in Fig. 2. In this case also the largest inhibition was observed when neocarzinostatin was preincubated with the template calf thymus DNA (Fig. 2-C). No such increase of the inhibition was observed when the antibiotic was preincubated with the *E. coli* DNA polymerase or deoxyribonucleoside triphosphates (Fig. 2-B, or 2-D). These results suggest that neocarzinostatin interacts with template DNA and lowers its template activity in the reaction mixture, but no such interaction of the antibiotic occurrs with the polymerase or deoxyribonucleoside triphosphates.

Effect of Neocarzinostatin on DNA as Estimated by Melting Profiles

The melting profiles were determined for DNA which was preincubated with

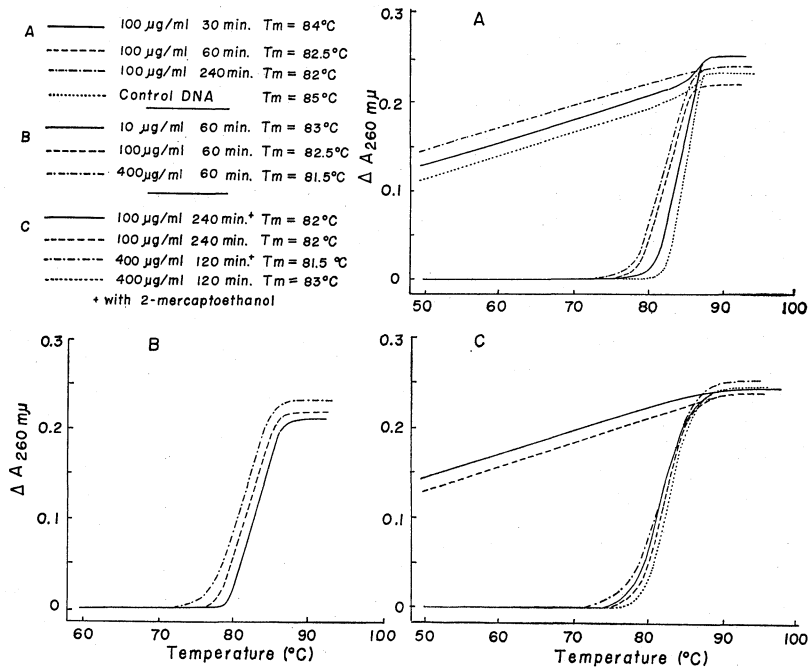
Fig. 2. Inhibition of DNA synthesis by neocarzinostatin at different incubation time.

The composition of reaction mixture was given in "Materials and Methods". The template used in this case was activated calf thymus DNA. At indicated time intervals, aliquots were removed from the reaction mixture and the DNA polymerase activity was measured. (A) The reaction was started at minus 30 minutes and 10 μg/S.R.M. of neocarzinostatin was added at time 0. (B) *E. coli* DNA polymerase was preincubated with 10 μg/S.R.M. of neocarzinostatin at 37℃ for 30 minutes in the presence of buffer, $MgCl_2$, and 2-mercaptoethanol and at time 0, the reaction was started by adding the template DNA (activated calf thymus DNA) and deoxyribonucleoside triphosphates. (C) Template DNA was preincubated with 10 μg/S.R.M. of neocarzinostatin and the reaction was started at time 0 by adding the enzyme and deoxyribonucleoside triphosphates. (D) Deoxyribonucleoside triphosphates was preincubated with 10 μg/S.R.M. of neocarzinostatin and the reaction was started by adding the enzyme and DNA at time 0.



neocarzinostatin. The melting temperatures of each sample are shown in the legend of each Fig. 3 A~C. When DNA was incubated with 100 μg/ml of neocarzinostatin (Fig. 3-A), the melting temperature gradually decreased with increasing incubation time, and the breadth of thermal transition of each curve increased. The samples which were separately incubated for 30 minutes, 240 minutes, and the control gave different profiles on cooling. The total decrease of the absorbance observed on cooling the control sample was larger than that of the sample incubated for 240 minutes. The melting profiles of DNA incubated with different amounts of neocarzinostatin are shown in Fig. 3-B. The melting temperature of DNA slightly decreased as the amount of the antibiotic increased. Next, the influence of 2-mercaptoethanol on the melting profiles of DNA was investigated (Fig. 3-C) since this reagent is known to stimulate the reduction of melting temperature caused by the antibiotic bleomycin[14]. There was no difference in melting and cooling profiles for DNA samples which were incubated in the presence and absence of 2-mercaptoethanol. These results indicate that 2-mercaptoethanol has no influence on the melting profiles of DNA in the

Fig. 3. Melting profiles of DNA after incubation with neocarzinostatin.
The experimental details are described in the text. The concentration of the antibiotic of each sample, the incubation time and the melting temperatures of DNA after treatment with neocarzinostatin are shown below.



presence of neocarzinostatin in contrast for that reported for bleomycin.

Discussion

It has previously been reported[8] that neocarzinostatin causes an immediate 90 % inhibition of DNA synthesis in bacteria at concentrations of 0.5 μg/ml, while it did not show any effect on *in vitro* DNA synthesis by crude DNA polymerase extracted from HeLa cells.

In our experiment, in order to determine the component affected by the antibiotic in *in vitro* DNA synthesis, a reaction system consisting of the simplest constituents of the purified DNA polymerase and templates was used, and it was found that *in vitro* DNA synthesis, was inhibited by neocarzinostatin. The extent of the inhibition of *in vitro* DNA synthesis, however, was smaller than that observed in whole cells using a similar ratio of antibiotic to DNA. Therefore the effect of neocarzinostatin observed *in vitro* may be one contributing factor to the inhibition of DNA synthesis which occurs in the cell.

The primary action of this antibiotic on the *in vitro* DNA synthesis was neither an inactivation of the DNA polymerase nor a degradation of deoxynuleoside triphosphates, but an interaction with the template DNA resulting in a change of its template activity. From the time-course study of the inhibition of DNA synthesis by neocarzinostatin and the thermal denaturation test of the template DNA after its interaction with antibiotic, the mode of action of this antibiotic in its effect on DNA synthesis was assumed to consist mainly in a change of DNA structure such as denaturation, or single strand scission. Several antibiotics such as mitomycin C[15], phleomycin[16] and bleomycin[17] have been shown to inhibit strongly the synthesis of DNA by affecting the structure of DNA. The primary action of mitomycin C[18] is the formation of cross-linkages in the complementary DNA strands, and that of phleomycin[16] is binding to the template DNA resulting in an increase of the melting temperature of DNA, while bleomycin[14,19] acts on the template DNA to induce single strand scissions which decrease the melting temperature. The results of the

present experiment suggested that the mode of action of neocarzinostatin resembles that of bleomycin but the extent of the effect on DNA structure by this antibiotic is smaller than that of bleomycin.

The finding that the inhibition of DNA synthesis directed by poly d(A-T) was larger than that directed by DNA could be explained by the greater instability of the former template. The slight increase in the synthesis of DNA which was observed between 0～30 minutes of the reaction when the reaction was started in the presence of the antibiotic (Figs. 2 A, B and D) might due to strand scission of DNA induced by the antibiotic, because it has been reported that the proper amount of strand scission stimulates the synthesis of DNA[20] by DNA polymerase. Studies on the precise structural change of the template DNA produced by neocarzinostatin are in progress.

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