CARCINOGENS INHIBIT DNA SYNTHESIS WITH ISOLATED DNA POLYMERASES FROM *ESCHERICHIA COLI*

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1. Introduction

The carcinogens *N*-acetoxy-2-acetylaminofluorene ($(Ac)_2$ONFln) and *N*-methyl-*N*-nitrosourea (MeNOUr) bind covalently to duplex DNA in vivo and in vitro [1,2]. MeNOUr predominantly methylates guanine bases in the N-7 (60%) and O^6 (8%) position, adenine in the N-3 (8%) position and phosphorus oxygen (25%) [3]. $(Ac)_2$ONFln substitutes H-8 of guanine by the *N*-2-fluorenylacetamido residue and to a minor extent N^2–H of guanine by the 2-acetylaminofluorenyl residue [1]. Substitution of H-8 forces the base to rotate from the normal *anti* to the *syn* conformation thus causing steric distortions and localized denaturation of the double helix [4].

The above alterations have been shown to affect the biological activity of nucleic acids by inducing mutation and malignant cell transformation [1,5,6], termination of transcription [7] or inactivation of phage ϕX-DNA for transfection [8].

Single-stranded DNA is particularly prone to the attack of carcinogens as functional groups required for hydrogen bonding are exposed in addition to the nucleophilic sites of the helix. Therefore, when tested for template activity, carcinogen-modified single-stranded DNA should show diminished ability for base pairing, which could result in a block of DNA synthesis.

An in vitro DNA-replicating system [9] which uses RNA-primed single-stranded phage fd DNA and *E. coli* DNA polymerase III holoenzyme or DNA polymerase I appeared to be sensitive enough to prove this point. The present study will show that the carcinogen $(Ac)_2$ONFln is capable of drastically inhibiting DNA synthesis. This inhibition is less pronounced, when single-stranded DNA is chemically modified in the presence of the DNA binding protein I. MeNOUr also causes inhibition, although at higher concentrations. DNA binding protein I is less effective in protecting the DNA template against methylation than against arylamidation.

Abbreviations: $(Ac)_2$ONFln, *N*-acetoxy-2-acetylaminofluorene; MeNOUr, *N*-methyl-*N*-nitrosourea; DNA binding protein I is from *E. coli* and was formerly named DNA unwinding protein [14,15]

2. Materials and methods

N-acetoxy-2-acetylaminofluorene (MW 281) [10] and *N*-methyl-*N*-nitrosourea (MW 103) [11] were prepared according to published methods. DNA polymerase III holoenzyme was partially purified by a modification of the first steps in [12], DNA polymerase I by the modified procedure in [13]. The purification of *E. coli* DNA binding protein I [14] was published [15].

The carcinogens were freshly dissolved and diluted in dimethylsulfoxide (Me_2SO), further diluted in buffer C100 [9] which is 10% glycerol, 20 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, 1 mM mercaptoethanol and 100 mM NaCl. Aliquots were immediately added to RNA-primed single-stranded phage fd DNA (400 pmol deoxynucleotides). Unless stated otherwise, the mixture (15 μl) was incubated for 30 min ($(Ac)_2$ONFln) or 10 min (MeNOUr) at 30°C, and then DNA polymerase, deoxynucleoside triphosphates (at 50 μM; the α-^{32}P-labelled dTTP at 25 μM), $MgCl_2$

at 5 mM, spermidine at 3 mM or DNA binding protein I (2 μg/assay) were added to final vol. 20 μl. Incubation was continued for 10 min (DNA polymerase III holoenzyme) or 30 min (DNA polymerase I), and the mixture precipitated with 7% perchloric acid, filtered and the nitrocellulose filter counted in a gas flow counter. About 80 pmol deoxynucleotides were incorporated on the template in the control experiment without inhibition.

3. Results

Binding of $(Ac)_2$ONFln to single-stranded DNA may cause an obstacle for DNA synthesis. This was investigated for DNA polymerase I and III holoenzyme from *E. coli*. The reaction of $(Ac)_2$ONFln with RNA-primed single-stranded fd DNA is a time-requiring process (fig.1). Thus inhibition of DNA polymerase III holoenzyme was found to be 85% when RNA-primed single-stranded fd DNA was preincubated with the carcinogen for 1 h as compared to 12%, when carcinogen, DNA polymerase III holoenzyme and deoxynucleoside triphosphates were added simultaneously.

Preincubation of RNA-primed single-stranded fd

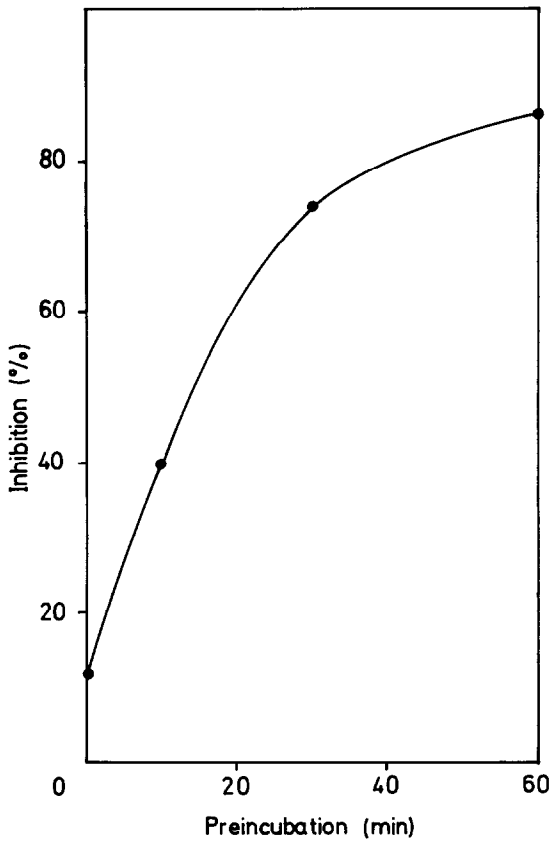

Fig.1. Time-dependent interaction of $(Ac)_2$ONFln (60 μM; 20 μg/ml) with RNA-primed fd-DNA: after incubation of the DNA with carcinogen at 30°C for the time indicated DNA polymerase III holoenzyme and deoxynucleoside triphosphates were added and the mixture incubated for additional 10 min.

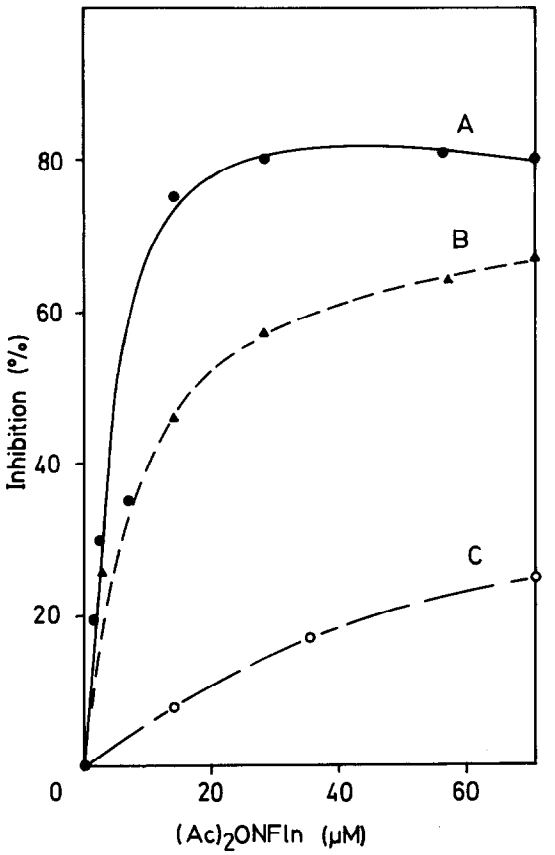

Fig.2. Inhibition of DNA synthesis with increasing amounts of $(Ac)_2$ONFln. RNA-primed fd DNA was preincubated with the carcinogen for 30 min at 30°C. Then DNA synthesis in the presence of spermidine (3 mM) with DNA polymerase III holoenzyme (A: •—•) or DNA polymerase I (B: ▲—▲) was allowed for 10 or 30 min, respectively. For the lower curve (C: ○—○) 2 μg DNA binding protein I were added first, followed by the preincubation with carcinogen for 30 min, and finally 10 min DNA synthesis with DNA polymerase III holoenzyme was allowed.

DNA with increasing concentrations of $(Ac)_2ONFln$ resulted in increasing inhibition of DNA synthesis (fig.2). An inhibition of 50% could be achieved for DNA polymerase III holoenzyme at 5 μM $(Ac)_2ONFln$ in the incubation mixture (curve A). DNA polymerase I was less inhibited at the same carcinogen concentration (curve B). Complex formation of fd DNA with the DNA-binding protein I prior to the addition of $(Ac)_2ONFln$ (15 μM) led to reduction of DNA synthesis of only 7% compared to 75% in the assay with free DNA (curve A,C).

MeNOUr was also capable of slowing down the rate of DNA synthesis. The extent of inhibition depended upon the MeNOUr concentration fd DNA was exposed to (fig.3). Preincubation of the RNA-primed DNA template in a 10 mM solution of MeNOUr for 10 min led to 70% inhibition, whereas 26% inhibition was measured, when DNA polymerization was allowed to proceed immediately after addition of the alkylating carcinogen. Extension of the incubation time beyond 10 min did not result in markedly increased inhibition of DNA synthesis (data not shown). DNA polymerase III holoenzyme (fig.3, curve A) is slightly more sensitive to MeNOUr than DNA polymerase I (curve B).

As judged from the rate of DNA synthesis, complexation of the template with the DNA binding protein I prior to the application of MeNOUr was little effective in preventing methylation (fig.3, curve C).

Methylation of guanine at O^6 and fluorenylation at N^2 interfere with base pairing. $(Ac)_2ONFln$, however, causes an additional inhibitory effect on DNA synthesis, since arylamidation of C-8 of guanine sterically alters single-stranded DNA to an extent as to make formation of a normal double-helical structure impossible. It is thus not surprising that 50% inhibition of the DNA polymerases by $(Ac)_2ONFln$ was found at an about 1000-fold lower concentration than required for MeNOUr (fig.2,3).

None of the carcinogen-modified templates used in the above experiments showed strand breaks when analysed by velocity sedimentation in neutral sucrose.

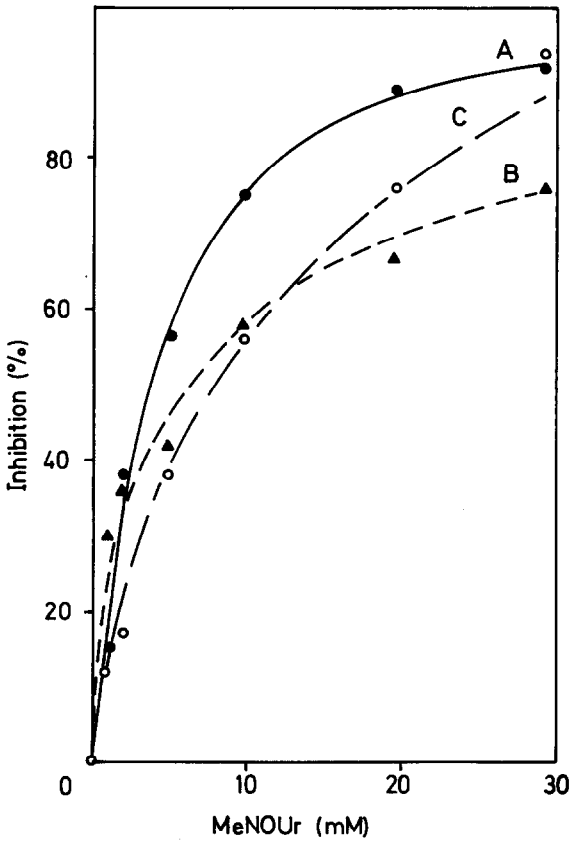

Fig.3. Inhibition of DNA synthesis by MeNOUr. The carcinogen was incubated with RNA-primed fd DNA for 10 min at 30°C, and DNA polymerase, spermidine or DNA binding protein I were added as described in fig.2. In controls Me_2SO was added in the same concentration as required for the carcinogen. Some inhibition of DNA polymerase III holoenzyme was found at concentrations of more than 10% Me_2SO. A (●–●), DNA polymerase III holoenzyme; B (▲–▲), DNA polymerase I; C (○–○) DNA binding protein I and DNA polymerase III holoenzyme.

4. Discussion

Possibly one of the initiating steps in chemical carcinogenesis is transfer of electrophilic groups from carcinogens to DNA. The replication fork is especially exposed as target for mutagens [16]. Eukaryotic DNA polymerases lack fidelity in the presence of carcinogens [17]. Chemical alterations of the helix as well as steric distortions resulting from covalent fixation of carcinogenic residues have been shown to give rise to repair [10,11]. Persistence of the DNA damage during replication may cause the newly synthesized strand to be terminated at the site of the damage and to be resumed at an initiation site beyond, leaving a post-replication gap [18]. Indeed, both

carcinogens caused DNA polymerase to discontinue DNA synthesis. Filling of the post-replication gap could result in insertions of non-complementary nucleotides thus giving rise to mutations. Such a mechanism leading eventually to transformation of a normal into a malignant cell can be postulated for MeNOUr [5] and for $(Ac)_2$ONFln [1].

Pronounced sensitivity of DNA synthesis towards $(Ac)_2$ONFln was found (fig.2). A concentration range of 4–40 μM during preincubation proved to be sufficient for effective inhibition. Reversion of *his* mutants of *Salmonella* could be achieved at similarly low concentrations [19], and single-stranded ϕX174-DNA was inactivated for transfection in the same range using carcinogens of comparable reactivity [8]. By contrast, DNA repair in ether-permeabilized *E. coli* cells required a 10-fold higher concentration of $(Ac)_2$ONFln [10].

MeNOUr had to be applied at a concentration of 10 mM for maximal DNA repair [11] and also for more than 50% inhibition of DNA synthesis. This high level of MeNOUr compared to $(Ac)_2$ONFln might be due to differences in reactivity of the carcinogens towards DNA or due to the different potencies of the respective modified DNA sites to inhibit DNA polymerases. Thus, MeNOUr proved to be less biologically active by a factor of 1000. This difference may also be explainable by the fact that only 10% (O^6 of guanine and apurinic sites) of the total DNA methylation interfere with base pairing, whereas every arylamidated or fluorenylated site in single-stranded DNA is likely to make base pairing impossible.

Carcinogens are especially potent on histone-free DNA [20]. Also phage fd DNA was protected against $(Ac)_2$ONFln by DNA binding protein I (fig.2) which indicates that carcinogens predominantly attack open DNA structures.

Note added in proof

Recently Hsu et al. (Proc. Natl Acad. Sci. USA (1977) 74, 3335–3339) have shown that the carcinogen benzo[α]pyrene-7,8-dihydrodiol-9,10-epoxide inhibits the conversion of single-stranded phage ϕX 174 DNA to replicative form DNA in soluble cell extracts.

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