Interaction of homo-aza-steroidal ester of [*p*-[bis-(2-chloroethyl) amino]phenyl]acetic acid (ASE) with DNA of Ehrlich ascites tumor cells

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A cytostatic, homo-aza-steroidal ester of [*p*-[bis-(2-chloroethyl) amino]phenyl]acetic acid (ASE) was reduced with NaB^3H_4 and [^{3}H]ASE-treated DNA prepared in vitro. We found that: (1) ASE reacts preferentially with purines; (2) ASE decreases the thermal stability of the double helix upon binding to DNA; (3) [^{3}H]ASE binding sites are clustered along the DNA molecules; (4) ASE binding sites probably represent oligo- or polypurine sequences.

Homo-aza-steroidal ester *Nitrogen mustard* *Ehrlich ascites tumor cell* *Hydroxyapatite* *S_1-nuclease* *Reduction with sodium borohydride*

1. INTRODUCTION

The compound 3β-hydroxy-13-amino-13,17-seco-5-androstan-17-oic-13,17-lactam [*p*-(bis(2-chloroethyl)amino phenyl]acetate (ASE) [1] has shown excellent antitumor activity in mice P388-leukemia and satisfactory effect in L1210 leukemia [2]. It is also active against B16-melanoma in $C_{57}B_1$ mice and T_8-Guerin tumor in rats [3].

Preliminary experiments show that the ASE inhibits irreversibly the incorporation of [^{3}H]thymidine into DNA of Ehrlich ascites tumor cells (EAT) in tissue culture conditions and that ASE, when incubated with EAT DNA, causes a shift of the DNA band towards higher densities in CsCl density gradients. We considered these data as evidence of binding of ASE to DNA.

Here we present experiments on the in vitro interaction of ASE with DNA. A radioactive analog of ASE was prepared by reduction of ASE with sodium boro[^{3}H]hydride. The data characterize ASE as preferentially reacting with purines. It was found also that the target sites of ASE-binding are irregularly distributed (clustered) along DNA.

2. MATERIALS AND METHODS

2.1. *Cells and isolation of DNA*

Ascites tumors were produced in A mice by i.p. inoculation of $5–7 \times 10^6$ Ehrlich ascites tumor cells. The ascitic fluid was aspirated on day 7 after inoculation and the cells were washed twice with 0.01 XSSC (1 XSSC = 0.15 NaCl–0.015 M trisodium citrate, pH 7.0). DNA was isolated by the MUP method as in [4].

2.2. Origin of ASE and radioactive labelling

Synthesis of ASE was as in [1]. Labelling of ASE was performed by reduction of ASE with tritiated sodium borohydride (NaB^3H_4). ASE (10 mg) was dissolved in 1 ml absolute ethanol and mixed with 1.1 mol of NaB^3H_4 (spec. act. 7.1 Ci/mmol, Amersham). The reaction was carried out overnight at room temperature and terminated by adding 2 ml of acetone. The alcohol–acetone mixture was evaporated under vacuum and the residue was dissolved in 5 ml chloroform. The chloroform was extensively washed with 0.5 M Tris–HCl (pH 7.0) dried with Na_2SO_4 and evaporated under vacuum.

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The dry residue was dissolved in acetone and crystallized from acetone–hexane. The final yield of the reduced ASE was 40% with a specific radioactivity of 41 200 cpm/μg.

2.3. *In vitro treatment of DNA with [$_3$H]ASE*

Taking into account the factors affecting the in vitro interaction of ASE with DNA (see section 3), the following conditions were accepted as standard for alkylating DNA with [^{3}H]ASE. DNA (0.5–1 mg/ml in 0.01 XSSC) was mixed with [^{3}H]ASE (stock solution 40 mg/ml in dry dioxan) in appropriate ratios, vortexed and incubated at 37°C for 2 h. The DNA–ASE complex was purified from unbound ASE by the following procedure: NaCl was added to the mixture (0.2 M) before ethanol precipitation (−20°C for at least 1 h). The precipitate was collected by centrifugation and dissolved in 0.01 M NaCl. The salt concentration was brought to 0.2 M and precipitated as above. This procedure was repeated several times until no radioactivity was registered in the ethanol. Three precipitation steps were usually sufficient. The final precipitate representing the [^{3}H]ASE–DNA complex was finally dissolved in 0.01 M NaCl.

The same procedure was also applied when DNA was treated with unlabelled ASE.

2.4. *Hydroxyapatite (HAP) thermal chromatography of DNA*

Control or ASE-treated DNA in 0.12 M phosphate buffer (PB) (pH 6.8) was sonicated so that fragments with a length of 400 base pairs on the average were obtained. The material was loaded on a water-jacketed HAP column equilibrated with 0.12 M PB at 50°C. The temperature was elevated in steps of 4°C intervals to 98°C and the single-stranded DNA at each temperature was eluted with 0.12 M PB. The thermal stability of the DNA was followed by measuring the absorbance at 260 nm or the ^{3}H-radioactivity of the effluents.

2.5. *S_1-nuclease treatment*

DNA in a buffer containing 0.2 M NaCl, 0.03 M CH_3COONa (pH 4.5) and 2×10^{-4} M $ZnSO_4$ was treated with S_1-nuclease for 15 min at 50°C. The amount of the S_1-nuclease sensitive material (single-stranded DNA regions) was estimated by precipitation in 0.5 N $HClO_4$ (PCA) final concentration. The radioactivity and the absorbance at 260 nm of the supernatant was also measured.

2.6. *In vitro treatment of free deoxyribonucleotides with [^{3}H]ASE*

One mg of each of the four 5′-deoxyribonucleotide-monophosphates (dGMP, dAMP and dTMP, Sigma) dissolved in 1 ml of different concentrations of SSC (see section 3) was mixed with 50 μg [^{3}H]ASE (stock solution 40 mg/ml in dry dioxan) and incubated at 37°C for 2 h. The [^{3}H]ASE-bound nucleotides were separated from the unbound free [^{3}H]ASE by thin-layer chromatography on silicagel (Merck) with a liquid phase of chloroform:methanol, 9:1. Under these conditions the free [^{3}H]ASE had an R_f of 0.75, whereas the [^{3}H]ASE bound to the nucleotides had an R_f of 0.0. The nucleotide spots were cut under UV light at 254 nm and their radioactivity was measured as follows: to each strip of silicagel containing one spot, 0.5 ml of 1 N KOH was added, shaken for 2–3 min and centrifuged at 12000 × g. The supernatant (0.4 ml) was neutralized with 0.2 ml 2 N $HClO_4$ and the radioactivity was measured by liquid scintillation counting.

3. RESULTS AND DISCUSSION

Our previously reported results characterize ASE as a compound with a strong cytostatic effect towards several transplantable tumors [2,3] probably due to alkylation of DNA. A more detailed study of the molecular mechanisms of this effect requires application of radioactively labelled ASE with high specific radioactivity. Since the total synthesis of radioactive ASE proved to be very difficult, we prepared a ^{3}H-labelled analog of ASE by its reduction with NaB^3H_4. Our preliminary analysis (IR-spectroscopy, elementary analysis and alkylating ability test [5]) showed that the reduction performed affects the lactam group in the steroid part of the compound (unpublished). The high cytostatic activity of the ^{3}H-labelled ASE (80% of that of ASE) and the preserved alkylating ability of the compound justified the use of the reduced form of ASE instead of the original compound in studying the mechanisms of interaction of ASE with DNA.

3.1. *Factors affecting the binding of ASE to DNA*

To standardize the experimental conditions for the in vitro treatment of DNA with [^{3}H]ASE the following factors affecting the alkylation reaction were studied: salt concentration, DNA:ASE ratio and duration of incubation.

The amount of [^{3}H]ASE bound to DNA as a function of the Na^+ concentration (in the range 0.01–1 XSSC) is given in fig.1. The results clearly show that the increase of Na^+ in the reaction mixture from 0.002 M (0.01 XSSC) to 0.2 M (1 XSSC) reduces >5-times the binding of [^{3}H]ASE to DNA. This is probably due to the suppression of the formation of the ethylenimmonium ion [6]. In all subsequent experiments an ionic strength of 0.002 M Na^+ (0.01 XSSC) was used.

The dependence of binding of [^{3}H]ASE to DNA as a function of the ratio DNA to ASE was also studied in the range of 5:1 to 20:1. It was found that the amount of the radioactivity bound to DNA increased upon increasing the concentration of ASE in the reaction mixture. No saturation level was reached (not shown).

Fig.2 shows the time-dependence of the binding of [^{3}H]ASE to DNA. The reaction of [^{3}H]ASE with DNA at 37°C is completed within 2 h. In all subsequent experiments incubations of ASE with isolated DNA were carried out for 2 h.

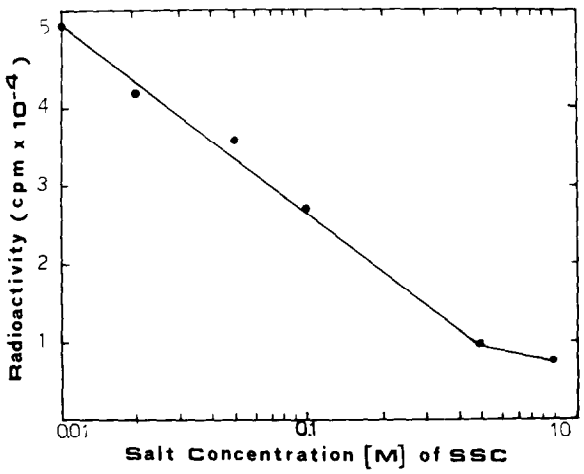

Fig.1. Salt-concentration dependence of the interaction of [^{3}H]ASE with DNA. EAT DNA was treated with [^{3}H]ASE in various SSC concentrations and the amount of the ^{3}H-radioactivity bound to DNA was measured as in section 2.

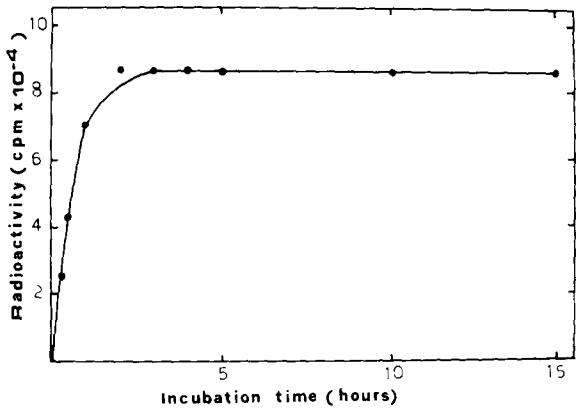

Fig.2. Binding of [^{3}H]ASE to DNA as a function of the incubation time. EAT DNA was treated with [^{3}H]ASE for various periods of time and the ^{3}H-radioactivity bound to DNA was measured as in section 2.

3.2. *Nature of the DNA–ASE bond*

Some experiments on the nature of the DNA–ASE bond were also carried out. The results presented in table 1 show that treatment with alkali (1 N KOH, 37°C, 2 h) renders 23% of the radioactivity acid-soluble, while heating for 15 min at 98°C or treatment with cold 5% trichloroacetic acid releases < 1% of the DNA-bound radioactivity. Therefore, the DNA–ASE bond is acid- and thermo-resistant. The latter gave us reason to apply the HAP thermal chromatography for studying the thermal stability of the ASE-treated DNA and the distribution of the target sites of ASE among the DNA fractions.

3.3. *Thermal stability of [^{3}H]ASE-treated DNA*

It is well known that different kinds of chemical modifications of the bases in DNA result in a decrease of the thermal stability of the DNA double helix [7]. On the average, modification of 1% of the bases lowers the T_m by 1°C. It could therefore be expected that if [^{3}H]ASE alkylates DNA at random, the sigmoid shape of the curve will be preserved, although shifted to lower temperatures. The use of the labelled analog of ASE enabled us to follow simultaneously both the melting of the ASE-treated DNA as a whole (A_{260}) and the thermal stability of the [^{3}H]ASE containing sequences (radioactivity). The results of this experiment are presented in fig.3. As seen, the shape of the melting curve and the T_m of both control

Table 1

Properties of the [^{3}H]ASE–DNA bond

[^{3}H]ASE–DNA	cpm	%
(a) Heating	130	0.7
(b) Alkali	4500	24.3
(c) Acid	120	0.6
(d) Control	18500	100.0

Ehrlich ascites tumor cell DNA was treated with [^{3}H]ASE under standard conditions (2 h, 37°C) and the DNA was purified from the unbound [^{3}H]ASE as described in section 2. Equal amounts of [^{3}H]ASE–DNA were treated as follows: (a) heating for 15 min at 98°C, cooling and precipitation with 0.5 N trichloroacetic acid; (b) incubation in the presence of 0.5 N KOH for 1 h at 37°C, cooling, neutralization and precipitation with 0.5 N trichloroacetic acid; (c) incubation in the presence of 0.5 N trichloroacetic acid at 4°C for 15 min. The control sample (d) represents the same amount of [^{3}H]ASE–DNA as in (a), (b) and (c), completely hydrolysed by incubation in 0.5 N trichloroacetic acid at 85°C for 30 min. All DNA samples were centrifuged at 4°C and the ^{3}H-radioactivity in the supernatants was measured

and ASE-treated DNA when 260 nm absorbance was measured were almost identical. These two parameters however were quite different when melting was followed by radioactivity. As seen in fig.3b, the T_m of the ^{3}H-radioactivity melting curve is about 10°C lower than that of the total ASE-treated DNA. This corresponds to 10–15% alkylation of the nucleotides in the [^{3}H]ASE containing DNA sequences. As can be estimated from the derivative melting curve (fig.3a), these sequences amount to only a few percent of the total DNA: 6% of the total ASE-treated DNA is eluted as single strands up to 75°C and this material contains 50% of the total DNA-bound radioactivity. There are two possible explanations of this finding: (i) the [^{3}H]ASE–DNA bond is thermolabile; (ii) the [^{3}H]ASE binding sites are clustered along the DNA. The thermal stability of such overalkylated DNA regions would be strongly decreased. However, we had found that the [^{3}H]ASE–DNA bond is thermoresistant which strongly supports the latter explanation. Considering the spec. act. of [^{3}H]ASE, its M_r and the spec. act. of [^{3}H]ASE–DNA complexes, we have estimated that in the fraction of DNA which is

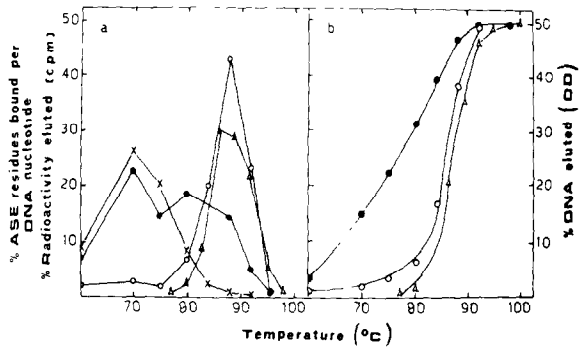

Fig.3. Thermal stability of [^{3}H]ASE-treated DNA. EAT DNA was treated with [^{3}H]ASE under standard conditions (see section 2), sonicated and melted on hydroxyapatite. The thermal elution was followed both by absorbance at 260 nm (○—○) and ^{3}H-radioactivity measurements (●—●). (Δ—Δ) absorbance of untreated DNA at 260 nm. (×—×) ASE residues bound/DNA nucleotides. (a) Derivative melting curve; (b) integral melting curve.

eluted up to 75°C (only 6% of the DNA), 20–25% of the nucleotides are bound with ASE residues. In the remaining DNA, only 1% of the nucleotides are bound to ASE, which corresponds to very small T_m difference as measured by absorbance (fig.3).

The clustering of the [^{3}H]ASE along the DNA molecule is further supported by the S_1-nuclease assay.

3.4. *S_1-nuclease assay*

If the binding sites of [^{3}H]ASE in DNA were clustered, one might expect that these regions would be strongly destabilized and therefore highly sensitive to the single strand specific S_1-nuclease. It was found that upon treatment of [^{3}H]ASE-alkylated DNA with S_1-nuclease (see section 2) 55% of the radioactivity and only 4% of the *A* were released as trichloroacetic acid-soluble material.

4. BASE SPECIFICITY OF ASE–DNA INTERACTION

A plausible explanation of the clustering of the [^{3}H]ASE-binding sites in the free DNA might be that this compound reacts preferentially with some of the bases. To investigate the base specificity of the reactions 0.05 mol of each of the 4 deoxyribo-

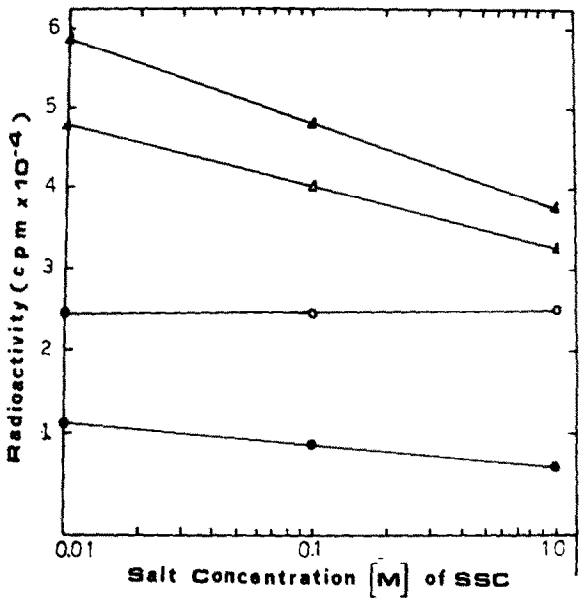

Fig.4. Base specificity and salt concentration dependence of the interaction of [^{3}H]ASE with 5'-deoxynucleoside monophosphates. dAMP, dGMP, dCMP and dTMP were incubated with [^{3}H]ASE in various concentrations of SSC and the ^{3}H-radioactivity bound to the nucleotides was measured as in section 2. (●—●) dTMP; (○—○) dCMP; (△—△) dAMP; (▲—▲) dGMP.

nucleotide-monophosphates were treated with [^{3}H]ASE at different SSC concentrations, as described in section 2, and the relative amount of [^{3}H]ASE bound to each of the nucleotides was estimated. As seen in fig.4, [^{3}H]ASE shows a well expressed specificity to purines and to guanine in particular. Fig.4 also shows that the salt concentration affects, to some extent, the alkylation of purines while it does not affect the reaction with pyrimidines. The presence of oligo- and polypurine sequences is a common feature of the native DNAs [8]. Taken together with the purine specificity of [^{3}H]ASE this may explain the existence of clustering of the binding sites for [^{3}H]ASE along the DNA.

If the polypurine nature of the [^{3}H]ASE binding sites is true and if ASE reacts with DNA by both chloroethyl groups, then intrastrand cross-links would be predominantly formed.

Here, we show unambiguously that the cytostatic compound ASE, in spite of its very low solubility in water, is capable of binding covalently to DNA under in vitro conditions. Experiments on whether ASE interacts with DNA, also under in vivo conditions, are now in progress.

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