ANTIBODIES TO DNA MODIFIED BY THE CARCINOGEN *N*-ACETOXY-*N*-2-ACETYLAMINOFLUORENE

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1. Introduction

Several studies have shown that the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene (AAAF) reacts in vivo and in vitro with native DNA and that the DNA contains a major (80%) adduct *N*-(deoxyguanosin-8-yl)-acetylaminofluorene (dGuo-8-AAF) and a minor (20%) adduct 3-(deoxyguanosin-N^2-yl)-acetylaminofluorene (dGuo-N^2-AAF) (reviewed [1,2]). It has been shown that the major alteration induced by the fluorene ring is the creation of locally disorganized regions inside the double helical structure [3–8].

Methods sensitive enough to assay the regions of DNA modified by a carcinogen at the levels of modification occuring in the 'in vivo' carcinogenesis experiments would be of great value. The immunological method has already been shown to be able to detect small modifications in DNA (see e.g. [9,10]). On the other hand, the study of the specificity of the antibodies can bring some knowledge on the conformation of the antigen. For these reasons, we have undertaken a study of the immunogenicity of native DNA after reaction with AAAF.

In this paper, we show that native DNA slightly modified by AAAF can induce in rabbits the synthesis of specific antibodies which selectively recognize AAF-substituted DNA. A methods of purification of these antibodies is described. Also, the association constants for the binding of the antibodies and several ligands are reported.

2. Materials and methods

Native calf thymus DNA (mol. wt $\sim 5 \times 10^5$) and denatured samples were modified by the reaction with AAAF according to the procedure in [4]. The percentage of bound AAF were determined from the analysis of the ultraviolet spectra. We will write nDNA-AAF or dDNA-AAF for native or denatured DNA which has been reacted with AAAF and the percentage of modified bases will be given into brackets. No large denaturation of native DNA occured by the reaction with AAAF because the absorbances did not significantly change between 20°C and a temperature (T_m −20°C).

GMP-AAF was prepared according to the procedure in [6].

2.1. *Affinity column*

dDNA-AAF was linked to Sepharose 4B through a spacer, the 6-aminohexanoic acid. Activation of the Sepharose 4B and the reaction with 6-aminohexanoic acid, has been described [11]. dDNA-AAF was reacted with the 6-aminohexanoic acid, at pH 5, in presence of carbodiimide as already described for the linkage of oligo(A) or poly(A)·poly(U) [11,12]. About 0.4 mg dDNA-AAF (23%) were linked per ml wet Sepharose.

Fab fragments were prepared by the reaction of the IgG with papain [13]. They were purified on the Sepharose dDNA-AAF column. The elution of the

bound Fab fragments was done with 1 M acetic acid. They were further purified by gel filtration on a Sephadex G-200 column. Immunodiffusion in agarose and precipitin assays were performed as in [11].

Fluorescence measurements were performed with a spectrofluorimeter Farrand MKI [11].

2.2. *Immunization*

Two random-bred rabbits were injected with a mixture of equal weights of nDNA-AAF (5%) and methylated bovine serum albumin according to the procedure in [14]. The rabbits were bled a week after the intravenous booster.

3. Results

3.1. *Reactivity*

The reactivity of the antisera was first analyzed by double diffusion in agarose (1%). The antisera reacted with nDNA-AAF (5%), dDNA-AAF (23%), dDNA but not with nDNA. There was no more precipitation with dDNA in 0.5 M NaCl while nDNA-AAF and dDNA-AAF were still precipitated.

3.2. *Purification of the antibodies*

The antibodies were purified by affinity chromatography on a Sepharose–dDNA-AAF column. The serum was applied on the column equilibrated with 0.15 M NaCl, 5 mM Tris–HCl, pH 7.5, 0.1 mM EDTA (buffer 1). The column was washed with buffer 1 until the effluent A_{280} was < 0.04, then with 0.5 M NaCl pH 7.5 and then with 1 mM acetic acid (2 column vol.). The antibodies were eluted with 2 M acetic acid. They were neutralized by dialysis against 0.1 M Tris–HCl, pH 8, and then against buffer 1.

All the antibodies which react with nDNA-AAF were bound to the Sepharose–dDNA-AAF column. By immunodiffusion, no precipitation was found between the proteins which did not bind to the column and nDNA-AAF or dDNA-AAF.

The eluted antibodies reacted with dDNA and with DNA-AAF. They were applied on a Sepharose dDNA column. About 15% of the proteins were bound to this column. The unbound proteins were further purified on a Sephadex G-200 column. Almost all the proteins were eluted in a single peak.

These proteins are IgG as shown by ultracentrifugation and immunodiffusion against goat antisera anti-IgG or anti-IgM. By gel diffusion these IgG were found to react with nDNA-AAF and dDNA-AAF but not with nDNA or dDNA.

All the IgG can be precipitated by dDNA-AAF as shown in fig.1. Moreover, the precipitation can be inhibited by GMP-AAF but not by GMP or AMP.

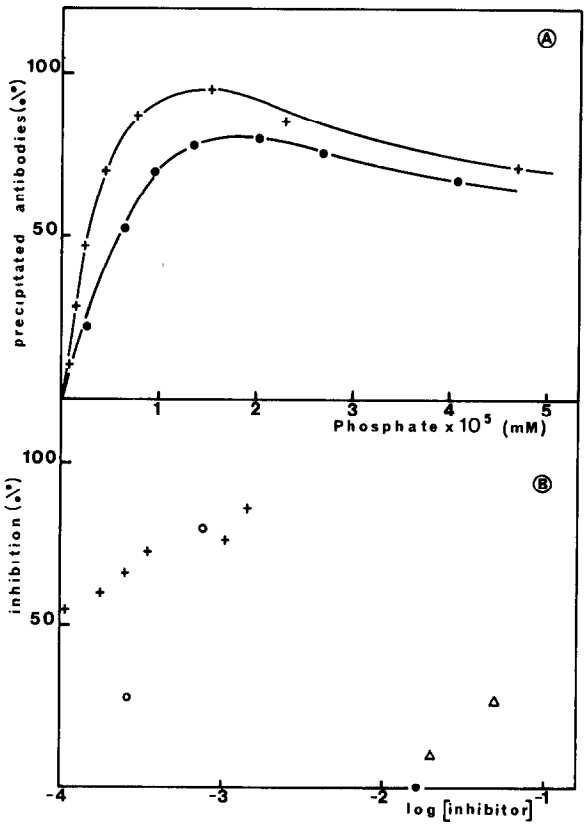

Fig.1.(A) Precipitation of DNA-AAF by the purified antibodies. Antibodies, 52 μg, were mixed with nDNA-AAF (5.7%) (•—•) or with dDNA-AAF (23%) (+—+) (B) Inhibition of DNA-AAF precipitation as a function of the logarithm of the inhibitor concentration. Inhibition of nDNA-AAF (5.7%) precipitation (○) and of dDNA-AAF (5.3%) precipitation (+) by GMP-AAF. Inhibition of nDNA-AAF (5.7%) precipitation by GMP (△) and by AMP (○). nDNA-AAF (5.7%) conc. 1.7×10^{-4} M and dDNA-AAF (5.3%) conc. 1.9×10^{-4} M (in nucleotide residues). Amount of antibodies, 52 μg in 120 μl solvent. The antibodies and the inhibitor were mixed and kept 1 h at 35°C and then DNA-AAF was added. After 24 h, the amounts of antibodies in the precipitate were determined.

3.3. *Association constants*

Solutions of Fab fragments are fluorescent. In presence of GMP-AAF or DNA-AAF, the fluorescence is quenched (λ_{exc} = 295 nm, λ_{em} = 355 nm) and tends towards a limit for large amounts of ligand. Assuming the same quenching for all the bound Fab fragments, one can calculate r/c and r, where r is the molar ratio bound GMP-AAP over Fab fragments and c the concentration of free GMP-AAF. The variation of r/c as a function of r is shown in fig.2. The mean value of the association constant for the binding of Fab fragments to GMP-AAF is 2×10^6 M^{-1} [15,16].

The association constant for the binding of Fab fragments to nDNA-AAF (5.6%) and to dDNA-AAF (4.4%) were also determined, the concentration of DNA being expressed in mol modified nucleotide residues. It was assumed that all the modified bases were equivalent and sufficiently far apart so that two modified bases were not covered by one Fab fragment. This assumption is justified because in the precipitin assays, in the region of antibodies excess, the molar ratio modified nucleotide residues over IgG in the precipitate is ~ 2 for nDNA-AAF (5.7%) (see fig.1) and for dDNA-AAF (4.4%) (results not shown). The association constants for the binding of Fab fragments to nDNA-AAF or dDNA-AAF are the same (4×10^6 M^{-1}) within the experimental error.

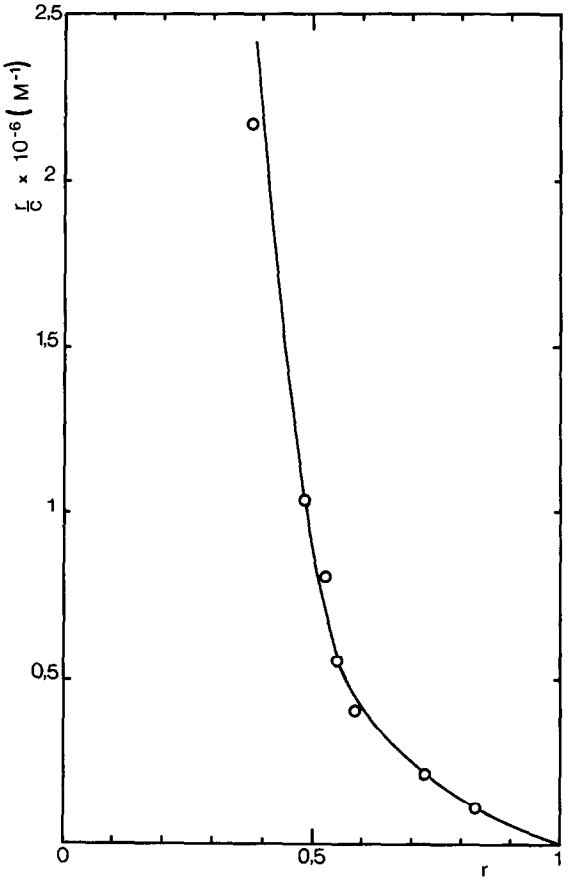

Fig.2. Scatchard plot for the binding of GMP-AAF to Fab fragments. Solvent 50 mM NaCl, 5 mM Tris–HCl, 0.1 mM EDTA, pH 7.5, temp. 15°C. Fab fragments conc. 4.8×10^{-7} M (λ_{exc} = 290 nm, λ_{em} = 355 nm).

4. Discussion

These results show that injections in rabbits of native DNA slightly modified by the reaction with AAAF can induce the synthesis of antibodies which react with the modified DNA. The antibodies have been purified by affinity chromatography on a Sephadex–dDNA-AAF column. It is possible that some specific antibodies to nDNA-AAF were lost. In the limit of sensitivity of double diffusion, all the antibodies to nDNA-AAF were bound to the Sepharose dDNA-AAF column. After passage on a Sepharose–dDNA column, the antibodies only react with DNA-AAF.

The purified antibodies do not precipitate DNA. The precipitation of DNA-AAF can be inhibited by GMP-AAF and almost not by GMP. Thus guanosine residues or oligonucleotides are not the immunodeterminant group while AAF is involved. On the other hand, the association constants for the binding of the Fab fragments to GMP-AAF, dDNA-AAF and nDNA-AAF are of the same order of magnitude. These results imply that the accessibility of the antigenic determinant (dGMP-AAF residues) is the same in single stranded and double stranded conformations.

In conclusion, it is shown that highly purified antibodies to DNA-AAF can be obtained. These antibodies react with nDNA-AAF. Therefore they can be used as probes to detect AAF linked to DNA without having to degrade the modified DNA. This is interesting as compared to the antibodies to guanosine-AAF. It has been recently found that they react poorly with nDNA-AAF [17]. Finally, since the antibodies

have the same affinity to GMP-AAF, nDNA-AAF and dDNA-AAF, this shows that if the AAF-residues lie inside the DNA structure [2,4,8], they must be sufficiently free to be as reactive as free GMP-AAF.

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