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Reactivity of the Antibodies to DNA Modified by the Carcinogen *N*-Acetoxy-*N*-acetyl-2-aminofluorene†

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ABSTRACT: Rabbits were immunized with native DNA modified by the carcinogen *N*-acetoxy-*N*-acetyl-2-aminofluorene. The interactions between the purified antibodies to nDNA-AAF (or the Fab fragments) and several ligands have been studied. By radioimmunoassay, nDNA-AAF, dDNA-AAF, and GMP-AAF were found to bind to the antibodies with about the same affinity. GMP-AF interacts slightly less, and GMP and *N*-OH-AAF do not interact. The values of the association constants deduced from fluorescence measurements for the binding of the Fab fragments to nDNA-AAF, dDNA-AAF, and GMP-AAF, in 50 mM NaCl, pH 7.5, are of the same order of magnitude. The values of the association constants with nDNA-AAF and dDNA-AAF depend upon salt concentration. From this variation, it is deduced that 1–1.5 phosphate groups interact by charge–charge interactions with the Fab fragments. The absorption and circular dichroism spectra of GMP-AAF, nDNA-AAF, and dDNA-AAF bound to the Fab fragments show that the Fab fragments induce similar perturbation to the three ligands. These results lead to the conclusion that the immunodeterminant group is the dGMP-AAF residue.

It is now well established that the carcinogen *N*-acetoxy-*N*-acetyl-2-aminofluorene (AAAF)[1] can react in vitro with native DNA. Two main adducts have been identified (for a general review, see Kriek, 1974). One is *N*-(2′-deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dGuo-AAF) and results from the covalent binding of the carcinogen on the C(8) of guanine residues. The other is 3-(2′-deoxyguanosin-N^2-yl)-2-(acetylamino)fluorene and results from the covalent binding of the carcinogen on the amino group of guanine residues. The relative amounts of the two adducts after in vitro modification are about 80–90 and 10–20%, respectively (Kriek, 1972; Fuchs, 1978). There is evidence that the geometry of the double helix is modified by the reaction of the carcinogen on the C(8). It has been proposed that the acetylaminofluorene residues are inside the double helix while the guanine residues are outside (for general reviews, see Weinstein, 1977, and Daune & Fuchs, 1977). These modifications of the conformation may be a critical step in the carcinogenic process.

We have undertaken a study of these conformational changes of the DNA using antibodies as probes. Antibodies to nucleosides were found to react with DNA modified by AAAF (Bases et al., 1976; Sage & Leng, unpublished experiments). On the other hand, recently we have shown that native DNA after reaction with AAAF was immunogenic (Leng et al., 1978a,b). Injections in rabbits of the modified DNA led to synthesis of antibodies which react with the modified DNA and not with the unmodified DNA. The specific antibodies have been purified by affinity chromatography. Some evidence was presented, showing that the antibodies recognize the dGMP-AAF residues in the modified DNA. It seemed to us of interest to study in more detail the reactivity of the purified antibodies.

In this paper, we report complementary results which confirm the fact that the dGMP-AAF residues are the immunodeterminant groups. The experiments have been done with the purified antibodies and the Fab fragments by use of several techniques, i.e., radioimmunoassay, fluorescence, absorption, and circular dichroism.

Material and Methods

Double-stranded calf thymus DNA ($M \simeq 5 \times 10^5$) and heat-denatured samples were modified by reaction with AAAF according to a procedure already published (Fuchs & Daune, 1972). The percentage of bound AAF was determined from the analysis of the ultraviolet absorption spectrum. We will note respectively nDNA-AAF or dDNA-AAF for double-stranded and heat-denatured DNA which have been reacted with AAAF. The percentage of modified bases will be given in parentheses.

N-(Guanosin-8-yl)-2-(acetylamino)fluorene (Guo-AAF) was prepared by the reaction of guanosine (Sigma) and AAAF as described by Kriek (1969). Guo-AAF was isolated by

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[1] Abbreviations used: AAAF, *N*-acetoxy-*N*-acetyl-2-aminofluorene; AAF, *N*-acetyl-2-aminofluorene; AF, 2-aminofluorene; *N*-OH-AAF, *N*-hydroxy-*N*-acetyl-2-aminofluorene; nucleotide-AAF or AF, nucleotide substituted on C(8) with AAF or AF; DNA-AAF, DNA modified covalently with AAAF; RIA, radioimmunoassays.

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chromatography on Sephadex LH-20. The same procedure was used to prepare [^{3}H]Guo-AAF ([5′-^{3}H]guanosine, 24.6 Ci/mmol, was bought from New England Nuclear). *N*-(Guanosin-8-yl 5′-monophosphate)-2-(acetylamino)fluorene (GMP-AAF) was prepared according to the procedure of Kapuler & Michelson (1971). *N*-(Guanosin-8-yl 5′-monophosphate)-2-aminofluorene (GMP-AF) was obtained by the incubation of GMP-AAF (1 mg/mL) in 10 mM NaOH at 60 °C during 90 min (Kapuler & Michelson, 1971).

The purity of Guo-AAF, GMP-AAF, and GMP-AF was checked by paper chromatography (the system solvent was ammonium acetate (1 M)–ethanol, 30:70 v/v). The ultraviolet spectra were in agreement with those of the literature (Kriek et al., 1967; Kapuler & Michelson, 1971). The antibodies to nDNA-AAF were purified on a Sepharose–dDNA-AAF (23%) column as already described (Leng et al., 1978a,b). The antiserum was applied on the column [3 mL of antiserum/mL of wet Sepharose; 0.4 mg of dDNA-AAF (23%) was linked per milliliter of wet Sepharose]. The column was washed with 0.5 M NaCl, 5 mM Tris-HCl, pH 7.5, until the absorbance of the effluent was less than 0.04 at 280 nm. The antibodies were eluted with 2 M acetic acid, in the cold. After neutralization, they were applied on a Sepharose–dDNA column and then on a Sephadex G-200 column. The purified antibodies were IgG.

The Fab fragments were prepared by reaction of the IgG with papain. They were purified on the Sepharose–dDNA-AAF (23%) column (Leng et al., 1978a,b).

Radioimmunoassays. The antibodies and the inhibitor were mixed. After 20 min, [^{3}H]Guo-AAF (or DNA-[^{3}H]AAF) was added. After 1 h, 10 μL of the antiserum anti-IgG (Miles; 11 mg/mL) was added. The total volume was 70 μL; the concentration of the antibodies was 2.2×10^{-6} M and that of [^{3}H]Guo-AAF was 1.3×10^{-6} M (13 000 cpm), the solvent being 30 mM NaCl, 5 mM Tris-HCl, pH 7.5. After 12 h, the mixture was centrifuged. The precipitate was dissolved in 0.1 mL of NaOH (0.1 M). The radioactivities of the supernatant and of the precipitate were determined in a Beckman scintillation counter.

All the other techniques (double diffusion, precipitin assays, absorption, circular dichroism, and fluorescence) have been already described in detail (Lavayre & Leng, 1977; Guigues & Leng, 1976).

Immunization. Random-bred rabbits were injected with a mixture of equal weights of nDNA-AAF and of methylated bovine serum albumin according to the procedure of Nahon-Merlin et al. (1973). The mixture (250 μg/injection), emulsified in complete Freund's adjuvant, was injected into the toe pads and into the back at weeks 0, 1, 2, and 3. At week 4, the same mixture without adjuvant was injected intravenously. An intravenous booster was done at week 8.

Results

Reactivity of the Antiserum. Several rabbits were immunized with nDNA-AAF. Two were immunized with nDNA-AAF (5.7%), two with nDNA-AAF (7%), and two with nDNA-AAF (2.5%). The rabbits were bled 1 week after the intravenous injection. All the antisera reacted with nDNA-AAF (5.7%) and dDNA-AAF (23%) as found by double diffusion in agarose (1%). The antisera also precipitated dDNA but not nDNA. There was no more precipitation of dDNA in 0.5 M NaCl. This effect of ionic strength was confirmed by quantitative precipitation (Figure 1).

Qualitatively, by immunodiffusion, similar results were found with the antisera obtained 1 week after the intravenous booster.

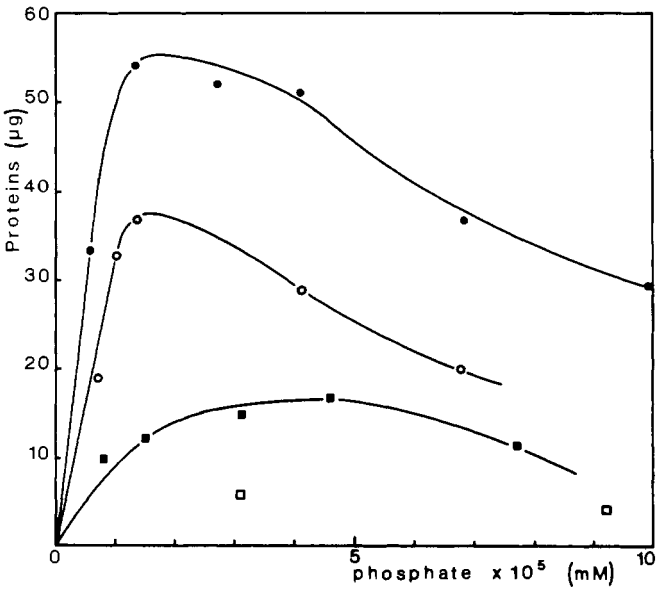

FIGURE 1: Quantitative precipitin assays. Reaction between 100 μL of the antiserum [from a rabbit immunized with nDNA-AAF (5.7%)] and nDNA-AAF (5.7%) (● and ○) or dDNA (■ and □). The total volume was 300 μL obtained by addition of 0.15 M NaCl (● and ■) or 0.5 M NaCl (○ and □) plus 5 mM Tris-HCl, 0.1 mM EDTA, pH 7.5.

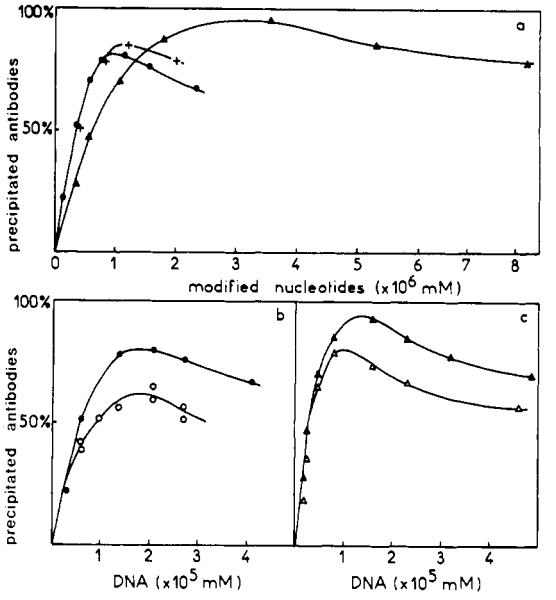

FIGURE 2: Precipitin curves. (a) Precipitation of the antibodies by nDNA-AAF (5.7%) (●), dDNA-AAF (5.3%) (+), and dDNA-AAF (23%) (▲). The solvent was 0.15 M NaCl. (b) Precipitation of the antibodies by nDNA-AAF (5.7%) in 0.15 M NaCl (●) or in 0.5 M NaCl (○). (c) Precipitation of the antibodies by dDNA-AAF (23%) in 0.15 M NaCl (▲) or 0.5 M NaCl (△). In all three cases, the amount of antibodies was 52 μg, the total volume was 120 μL, and the salt concentration was as indicated plus 5 mM Tris-HCl, pH 7.5. The temperature was 34 °C. In (b) and (c) the concentration of DNA is expressed in nucleotide residues.

Reactivity of the Purified Antibodies. This study was done with the antibodies of a rabbit immunized with nDNA-AAF (5.7%). After the intravenous booster, the rabbit was bled and the antibodies were purified by affinity chromatography (see Materials and Methods and Leng et al., 1978a,b). The reactivity of the antibodies was studied by quantitative precipitation and radioimmunoassays (RIA).

The antibodies were precipitated by DNA-AAF. The shape of the precipitin curve depended upon the amount of modified bases (Figure 2a). Almost the same curve was obtained with

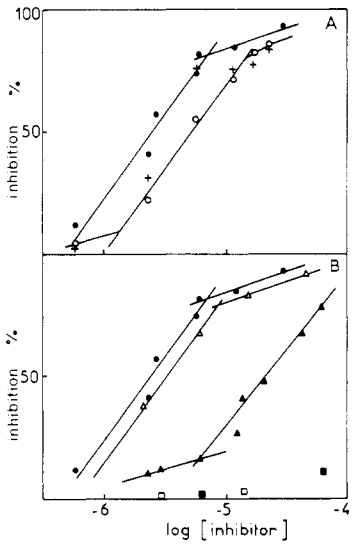

FIGURE 3: Inhibition experiments. Inhibition (%) of Guo-AAF fixation as a function of the logarithm of the inhibitor concentration. The conditions are given under Materials and Methods. (A) (●) GMP-AAF, (○) nDNA-AAF (5.7%), (+) dDNA-AAF (5.3%); (B) (●) GMP-AAF, (△) Guo-AAF, (▲) GMP-AF, (□) GMP, (■) *N*-OH-AAF.

nDNA-AAF (5.7%) and dDNA-AAF (5.3%), while a different curve was obtained with dDNA-AAF (23%). The molar ratios of modified bases/IgG in the precipitate, in the regions of antibody excess, were 2.6 for dDNA-AAF (5.3%) and nDNA-AAF (5.7%) and 3.7 for dDNA-AAF (23%).

The amount of precipitated antibodies by nDNA-AAF or dDNA-AAF depended upon the ionic strength, decreasing as the ionic strength was increased (Figure 2b,c). On the other hand, almost no effect of temperature, in the range of 5–35 °C, was found.

The interactions between the antibodies and several ligands were studied by RIA. Results of the competition between the antibodies, [^{3}H]Guo-AAF, and several ligands are shown in Figure 3. Within the accuracy of the experiments, the affinity of the antibodies to GMP-AAF, dDNA-AAF, and nDNA-AAF is identical. GMP-AF and Guo-AAF interact slightly less than GMP-AAF, while GMP, AMP, or *N*-OH-AAF (*N*-hydroxy-*N*-acetyl-2-aminofluorene) do not interact. Also, nDNA and dDNA do not interact (results not shown).

Some competitions were also done between the antibodies, dDNA-[^{3}H]AAF (5%), and the two ligands (GMP-AAF and GMP-AF). The results were in agreement with the previous ones.

Reactivity of the Fab Fragments. Several techniques have been used to study in solution the interactions between the Fab fragments and some ligands.

(*1*) *Fluorescence.* The Fab fragments are fluorescent in solution. In the presence of Guo-AAF (and several ligands) the fluorescence intensity is quenched and reaches a limit value in the presence of an excess of ligand. If the amount of fluorescence quenching is assumed to be proportional to the amount of bound ligand, one can calculate r and c (r is the molar ratio of bound ligand over Fab fragments and c is the concentration of the free ligand) and deduce the mean value of the association constant. In Table I, these values and the values of the fluorescent quenching at ligand saturation are given.

The association constants for the binding of the Fab fragments to DNA-AAF were found to be almost independent of temperature. On the other hand, they depend upon the salt concentration (Table II). The variation of log K as a function

Table I: Association Constants for the Binding of the Fab Fragments to Several Ligands[a]

ligands	Q_m (%)	K (M^{-1})
dDNA-AAF (8.2%)	37	8×10^6
dDNA-AAF (4.4%)	37	4×10^6
dDNA-AAF (2.2%)	36	5×10^6
nDNA-AAF (5.7%)	36	4×10^6
GMP-AAF	26	2×10^6
dGMP-AAF	26	2×10^6

[a] Q_m is the maximal quenching of fluorescene; $Q_m = (I_0 - I_m)/I_0$, where I_0 and I_m are the fluorescence intensities of the Fab fragments in the absence and at saturation of ligand, respectively. Solvent was 50 mM NaCl, 5 mM Tris-HCl, pH 7.5. The temperature was 15 °C.

Table II: Effect of Salt on the Values of the Association Constants[a]

	15 mM NaCl	50 mM NaCl	0.15 M NaCl	0.5 M NaCl
dDNA-AAF (8.2%)	3×10^7	8×10^6	2×10^6	8×10^5
nDNA-AAF (5.7%)		4×10^6		6×10^5

[a] Solvent was as indicated in the table plus 5 mM Tris-HCl, pH 7.5. The temperature was 5 °C.

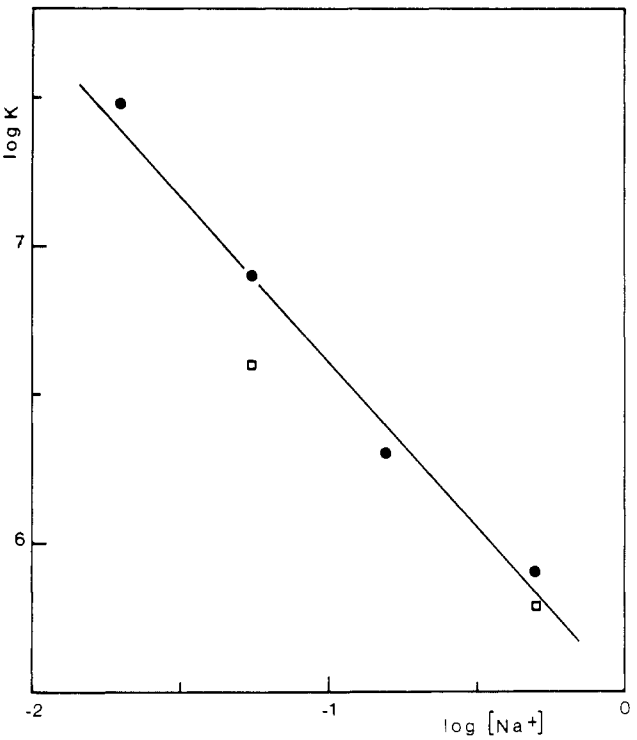

FIGURE 4: Effect of salt upon the association constants. Variation of log K as a function of log [Na^+]. (●) dDNA-AAF (8.2%); (□) nDNA-AAF (5.7%). The temperature was 34 °C.

of log [Na^+] is linear (Figure 4), and the slope is 1.1 for DNA-AAF. There are several causes of errors in the estimation of the association constants (errors on the maximal quenching, errors on the composition of modified DNAs, experimental errors, and photosensitivity of the ligands). The values of the association constants are not known at better than ±25%, and thus the same graph is obtained for the variation of log K (nDNA-AAF) or log K (dDNA-AAF) as a function of log [Na^+].

(*2*) *Absorption.* In the fluorescent experiments, the excitation wavelength was 295 nm. The screening effect of the ligands has been corrected (Guigues & Leng, 1976).

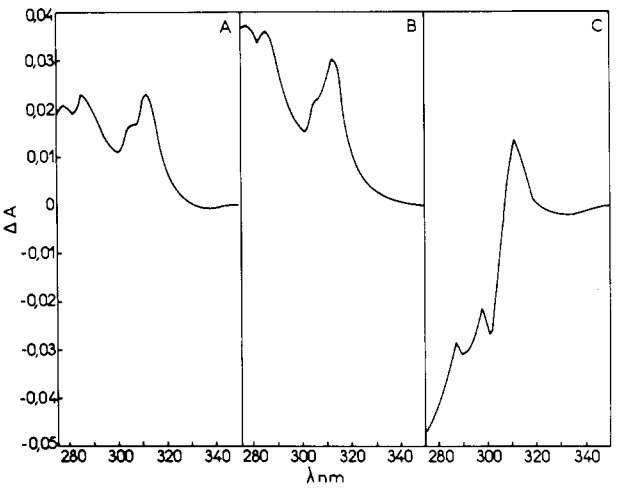

FIGURE 5: Ultraviolet difference spectra. Difference spectra between the Fab fragments–ligand mixture and Fab fragments plus ligand. Fab fragments' concentration was 8×10^{-6} M, and GMP-AAF or DNA-AAF (in modified nucleotide residues) concentration was 8×10^{-6} M. Solvent was 10 mM NaCl, 5 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The temperature was 23 °C. (A) nDNA-AAF (5.7%); (B) dDNA-AAF (12.5%); (C) GMP-AAF.

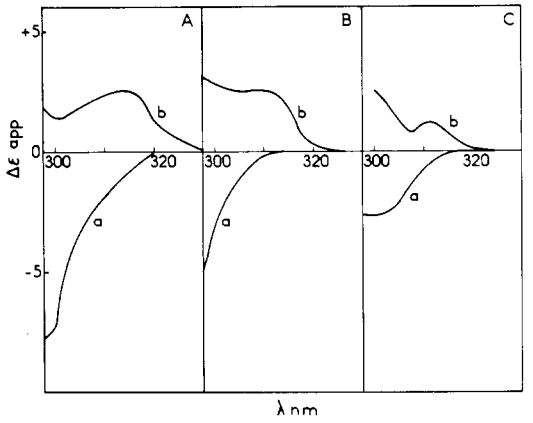

FIGURE 6: Circular dichroism of AAF residues free (a) and bound (b) to Fab fragments. (A) nDNA-AAF (5.7%); (B) dDNA-AAF (12.5%); (C) GMP-AAF. Solvent was 10 mM NaCl, 5 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The temperature was 23 °C.

Moreover, one has to take into account the fact that the absorption spectra of the Fab fragments–ligand mixtures are different from the sum of the spectra of the two components (Figure 5). At 295 nm, the differences are small (10–15%) and have been neglected. On the other hand, in three cases—GMP-AAF, dDNA-AAF, and nDNA-AAF—there is a positive band centered at 312 nm. In that region, only the AAF residues absorb, and there is no absorption by the Fab fragments or by the DNA (results not shown).

(*3*) *Circular Dichroism.* In Figure 6, we have reported the circular dichroism spectra of GMP-AAF, dDNA-AAF (12.5%), and nDNA-AAF (5.7%) bound to the Fab fragments. It has been assumed that the circular dichroism spectra of the free and bound Fab fragments are the same (in the range 300–320 nm, the contributions of the Fab fragments are small or null). It has been assumed also that all the ligands were bound. Thus, we wrote $\Delta\epsilon_{app} = [\Delta A(\text{complex}) - \Delta A(\text{Fab fragments})]/lC_{\text{ligand}}$, where ΔA is the difference in absorption between left and right circularly polarized light at wavelength λ and l is the cell path length. In all three cases, the circular dichroism spectra of the free ligands have a negative band in the 320–300-nm region, while the circular dichroism spectra of the bound ligands have a positive band.

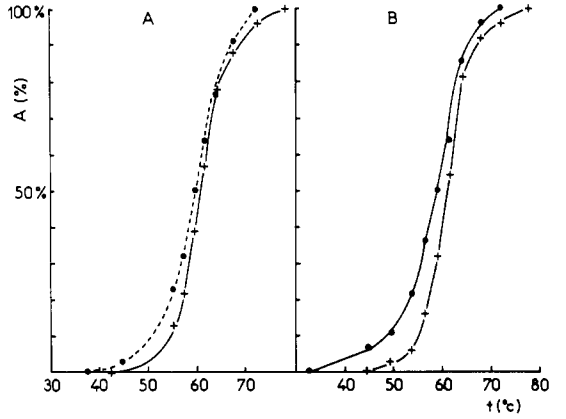

FIGURE 7: Thermal stability of nDNA-AAF. Absorbance (in percent) as a function of temperature. (+) nDNA-AAF (5.7%) and in the presence of Fab fragments—modified bases/Fab fragments equal to 1 (●---●) (A) and to 0.58 (●—●) (B). Solvent was 10 mM NaCl, 5 mM sodium cacodylate, pH 7. Concentration of DNA (in modified nucleotide residues) was 3×10^{-6} M^{-1}.

(*4*) *Thermal Stability of nDNA-AAF.* The thermal stability of nDNA-AAF in the presence and in the absence of Fab fragments is shown in Figure 7. Even in presence of an excess of Fab fragments (the ratio of modified bases/Fab fragments is equal to 0.6), the decrease of T_m is no more than 2–3 °C.

Discussion

Six rabbits were immunized with native DNA substituted by AAF. The sera of the six rabbits reacted with nDNA-AAF. The reactivity of the purified antibodies (and Fab fragments) has been studied in detail in order to characterize the immunodeterminant group.

The affinity of the antibodies (or Fab fragments) toward several ligands was studied by several techniques, i.e., precipitation assays, radioimmunoassays, fluorescence, and melting curves. The purified antibodies do not precipitate dDNA, and they do not bind GMP or *N*-OH-AAF. Thus the immunodeterminant group is not the fluorene ring, dGMP, or an oligonucleotide. The antibodies have about the same affinity for GMP-AAF, dDNA-AAF, and nDNA-AAF. Thus, the immunodeterminant group is dGMP-AAF.

The association constants K(GMP-AAF) and K(dGMP-AAF) are the same. The 2′-OH group of GMP-AAF does not prevent the binding; thus, the 2′-H of dGMP-AAF is probably not in close contact with the amino acid residues of the binding sites.

The association constants for the binding of the Fab fragments to nDNA-AAF and dDNA-AAF decrease as the ionic strength is increased. The variation of log K as a function of log $[Na^+]$ is linear. The slope of such a graph yields the number of charge interactions formed between the Fab fragments and the DNA-AAF [$\delta \log K_{obsd}/\delta \log [Na^+] = -m'\psi$, where m' is the number of ion pairs formed and ψ is the fraction of counterions thermodynamically bound per phosphate; ψ is equal to 0.88 and 0.71 for native DNA and single-stranded DNA, respectively (Record et al., 1976)]. In the case of DNA-AAF, $m'\psi$ is equal to 1.1 and thus 1–1.5 ion pairs are formed.

The precipitin curves are qualitatively in agreement with this effect of ionic strength. The amounts of precipitated antibodies decrease as the salt concentration is increased, which can be explained by a decrease of the association constants. A similar effect has been found on the complexes between the anti-poly(I)·poly(C) antibodies and double-stranded polyribonucleotides (Leng et al., 1978b).

The melting curves confirm the fact that the antibodies do not recognize an oligonucleotide in a given conformation (in nDNA-AAF). If it were the case, one would expect an increase of T_m of nDNA-AAF in the presence of the antibodies [because the antibodies would interact preferentially with nDNA-AAF (McGhee, 1976; Sage & Leng, 1977)].

GMP-AF was found to react slightly less than GMP-AAF. This is in favor of some interactions between the acetyl group and the binding site, but this group is not essential in the formation of the complex (this small difference between the reactivities of GMP-AAF and GMP-AF might also be due to some differences in the conformations of the nucleotides).

At this point, it should be noted that we do not know whether the antibodies can cross-react with guanine residues substituted on the amino group in nDNA-AAF. Work is in progress in our laboratory to elucidate this point.

In the complex between the antibodies and GMP-AAF (or DNA-AAF), direct interaction between the fluorene ring and the antibodies has not been demonstrated, but the difference spectra and the circular dichroism spectra show that the fluorene ring is close to the amino acid residues. In the difference spectra with the three ligands (GMP-AAF, dDNA-AAF, and nDNA-AAF) there is a positive band centered at 312 nm. In that region, only the fluorene ring absorbs and this band is due to some changes in the surrounding of the chromophore. Also, a positive band is observed in the circular dichroism spectra of the three bound ligands, while the spectra of the free ligands have a negative band in the 320–300-nm region. There are some differences in the intensities of the bands, but these experiments indicate that the antibodies induce the same sort of perturbation to the fluorene ring in GMP-AAF, dDNA-AAF, and nDNA-AAF. These results strongly suggest that the fluorene ring is located in the binding site of the antibodies. It can be added that similar circular dichroism spectra were found with GMP-AAF (and oligonucleotides modified by AAF) bound to antibodies to Guo-AAF. In these cases, there is experimental evidence showing that the fluorene residues interact with the binding site (Guigues & Leng, unpublished experiments).

About the same values of the association constants were found for the binding of the antibodies to GMP-AAF and to both nDNA-AAF and dDNA-AAF. The accessibility of the immunodeterminant group must be the same in single-stranded and double-stranded helixes, and therefore the modified regions in nDNA-AAF and dDNA-AAF have the same conformation. These results are not in disagreement with the base-displacement model (Nelson et al., 1971; Weinstein & Grunberger, 1974) or the insertion–denaturation model (Fuchs & Daune, 1971,1972; Fuchs et al., 1976; Fuchs, 1975). In these models, the covalent binding of the AAF residue to the C(8) of the guanine residue in nDNA introduces a conformational distortion of the nucleic acid. It is assumed that the fluorene ring is stacked with the neighboring bases, that the guanine residue is outside of the double helix, and that some base pairs are disrupted. Experiments with the antibodies indicate that some part of the fluorene ring is not completely buried in the double helix and/or that the dGMP-AAF residues can rotate. These results seem more in favor of a dynamic model in which the dGMP-AAF residues have some freedom. This is also in agreement with the reactivity of formaldehyde with nDNA-AAF (Fuchs & Daune, 1974).

Finally, these results have to be compared to those recently reported by Poirier et al. (1977). They found that antibodies to Guo-AAF can be elicited in rabbits immunized with the bovine serum albumin–Guo-AAF conjugate. These antibodies react with Guo-AAF and, to a much less extent, with nDNA-AAF. The assumption of Poirier et al. (1977) is that these antibodies do not react with nDNA-AAF. On the other hand, we show that specific antibodies to nDNA-AAF can be obtained.

Acknowledgments

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