Volume 12 Number 14 1984 Nucleic Acids Research

Immunological and spectroscopic studies of poly(dG-dC).poly(dG-dC) modified by cis-diamminedichloroplatinum(II).

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Received 14 June 1984; Accepted 29 June 1984

SUMMARY

The conformational changes induced by the binding of cis-diamminedichloroplatinum(II) to poly(dG-dC).poly(dG-dC) have been studied by reaction with specific antibodies, by circular dichroism and ^{31}P nuclear magnetic resonance. Polyclonal and monoclonal antibodies to Z-DNA bind to platinated poly(dG-dC).poly(dG-dC) at low and high ionic strength. Antibodies elicited in rabbits immunized with the platinated polynucleotide bind to double stranded polynucleotides known to adopt the Z-conformation. At low and high ionic strength the circular dichroism spectrum of platinated poly(dG-dC).poly(dG-dC) does not resemble that of poly(dG-dC).poly(dG-dC) (B or Z conformation). At low ionic strength, the characteristic ^{31}P nuclear magnetic resonance spectrum of the Z-form is not detected. It appears only at high ionic strength, as a component of a more complex spectrum.

INTRODUCTION

The antitumoral activity of cis-diamminedichloroplatinum(II) (cisPt) is now well-recognized (1) but its mechanism of action is not yet understood. Numerous studies have shown that *in vivo* and *in vitro* cisPt binds to DNA. Several adducts are formed and some of them have been identified (2-7, and references therein).

Knowledge of the conformational changes induced by the adducts might help to understand the mechanism of action of cisPt. In a recent work (8), we showed that in the reaction of cisPt and poly(dG-m^5dC).poly(dG-m^5dC), the nature of the adducts depends upon the conformation of the polynucleotide. An intrastrand bidentate adduct between two guanine residues separated by a methyl cytosine residue is formed when cisPt is added to the polymer in the B conformation. A monodentate adduct is formed when cisPt is added to the polymer in the Z conformation. The monodentate adduct stabilized the Z conformation while the bidentate adduct induces a conformational change from the B conformation towards a distorted Z conformation. A distorted Z conformation was proposed because the antibodies to Z-DNA bind to the platinated polymer but the circular dichroism spectrum is different from that of poly(dG-m^5dC).

poly(dG-m^{5}dC) in the Z conformation.

The antibodies to Z-DNA bind also to platinated poly(dG-dC).poly(dG-dC)(8). In this paper, we report some more results on this conformational change induced in the reaction of cisPt and poly(dG-dC).poly(dG-dC), cisPt having been mixed with the polymer in the B conformation. We show that antibodies elicited in rabbits immunized with platinated poly(dG-dC).poly(dG-dC) bind to double stranded polynucleotides known to be in the Z conformation. On the other hand, as circular dichroism and ^{31}P nuclear magnetic resonance do not give direct evidence for the existence of a Z conformation in the platinated polynucleotide, a question is raised about the mechanism of interaction between the anti Z-DNA antibodies and these polynucleotides.

MATERIALS AND METHODS

Calf thymus DNA from Boehringer, poly(dG-dC).poly(dG-dC) from PL Biochemicals, poly(dG-br^{5}dC).poly(dG-br^{5}dC) synthesized as previously described (9) were treated with phenol and then precipitated with ethanol. Bromination of poly(dG-dC).poly(dG-dC) was prepared using the method of Lafer *et al.* (10).

The reaction of cisPt and nucleic acids, the synthesis of [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC) and [8-^{3}H]poly(dG-dC).poly(dG-dC) and the radioimmunoassays have been already described (8,9,11,12).

The Sepharose-denatured calf thymus DNA affinity column was prepared as described for the Sepharose-poly(A).poly(U) column (13). About 0.5 mg dDNA was linked per ml wet Sepharose.

For NMR experiments, poly(dG-dC).poly(dG-dC) in SSC was sonicated for 10 minutes at 4°C and then fractionated on an ACA 34 (Ultrogel) column to yield fragments having an average chain length of 200 ± 100 base pairs, as measured by Agarose gel electrophoresis.

Immunodiffusion was performed in Agarose gel (1 %) in the presence of 0.15 M NaCl, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Precipitation lines were stained with coomassie blue.

Immunization of rabbits with poly(dG-dC)cisPt(0.10) and brominated poly (dG-dC).poly(dG-dC) was performed as previously described (9). The results reported here were obtained with the antisera of the bleeding a week after the first booster.

The circular dichroism spectra were recorded with a Roussel Jouan III dichrograph. Proton decoupled ^{31}P spectra were recorded on an AM 300 WB Bruker spectrometer operating at a frequency of 121.5 MHz.

For sake of clarity, we will write poly(dG-dC)cisPt(0.20) a sample of po-

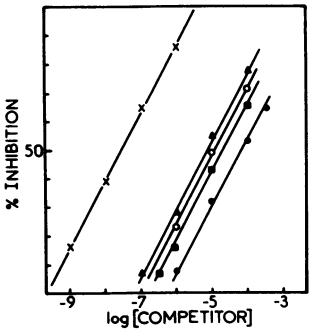

Figure 1 - Inhibition of tracer-antibody binding by poly(dG-dC)cisPt in competitive RIA. Tracer [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC), C = 4 x 10^{-9} M. Antiserum dilution 1/2 x 10^4. Competitors (×) poly(dG-br5dC).poly(dG-br^{5}dC) ; poly (dG-dC)cisPt, r_b = 0.20 (▲), r_b = 0.15 (○), r_b = 0.10 (■), r_b= 0.05 (●). Solvent 0.2 M NaCl, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Temperature 4°C.

ly(dG-dC).poly(dG-dC) complexed with cisPt at r_b = 0.20, r_b being the molar ratio of cisPt per nucleotide residue.

RESULTS

Interaction with antibodies to Z-DNA

Poly(dG-dC).poly(dG-dC) has the B conformation in 0.1 M NaCl and the Z conformation in 3 M NaCl (14). Poly(dG-br^{5}dC).poly(dG-br^{5}dC) has the Z conformation in 0.1 M and 3 M NaCl (9). The affinity of the anti Z-DNA antibodies towards platinated poly(dG-dC).poly(dG-dC) has been studied by RIA, the tracer being [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC). As shown in figure 1, in 0.1 M NaCl, the four platinated samples (r_b = 0.05, 0.10, 0.15 and 0.20) are recognized by the antibodies to Z-DNA. In figure 1, the concentrations of the competitors are expressed in moles of nucleotide residues. When expressed in moles of platinum residues, the four samples behave similarly.

The competition was then performed in 3 M NaCl. The molar ratios (in nucleotides residues) poly(dG-dC)cisPt over poly(dG-br^{5}dC).poly(dG-br^{5}dC) at 50 % inhibition of tracer precipitation are equal to 40, 160, 290 and 700 for r_b = 0.05, 0.10, 0.15 and 0.20, respectively. The relative affinity of the antibodies towards the platinated polynucleotides decreases as the value of r_b increases.

The antibodies to Z-DNA were elicited in rabbits immunized with a poly (dG-dC).poly(dG-dC) modified by the monofunctional platinum derivative chlorodiethylenetriaminoplatinum(II) chloride (dienPt). The competition experiments were also performed with antibodies to Z-DNA elicited in rabbits immunized

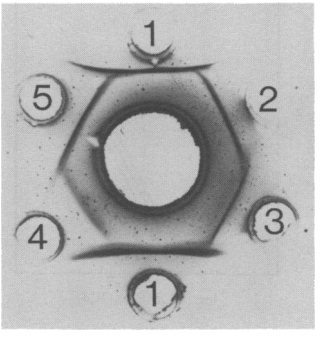

Figure 2 - Double diffusion of antibodies to poly(dG-dC)cisPt(0.10). Wells 1 : poly(dG-dC)cisPt(0.10) ; well 2 : poly(dG-br^{5}dC).poly(dG-br^{5}dC) ; well 3 : poly(dG-dC).dienPt(0.12) ; well 4 : denatured calf thymus DNA ; well 5 : brominated poly(dG-dC).

with a chemically brominated poly(dG-dC).poly(dG-dC) and with monoclonal antibodies (D-11, a gift from Dr. Pohl). Both polyclonal and the monoclonal antibodies recognize poly(dG-dC)cisPt (not shown).

Antiserum to poly(dG-dC)cisPt(0.10)

Two rabbits were immunized with poly(dG-dC)cisPt(0.10). The specificity of the antisera was first analysed by double diffusion. Both antisera gave similar results. As shown in figure 2, a strong positive reaction was found with poly(dG-dC)cisPt(0.10). Under these experimental conditions (0.15 M NaCl), poly(dG-dC)dienPt(0.12), brominated poly(dG-dC).poly(dG-dC) and poly(dG-br^{5}dC).poly(dG-br^{5}dC) have the Z conformation (9,10) and they bind to the antibodies. A faint line of precipitation is obtained with denatured calf thymus DNA.

The antiserum was applied to an affinity column (Sepharose-denatured calf thymus DNA, 0.5 ml wet Sepharose per ml antiserum). The reactivity of unbound antibodies with [^{3}H]poly(dG-dC)cisPt(0.10) and [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC) was assayed by direct binding. As shown in figure 3, 50 % binding to [^{3}H]poly(dG-dC)cisPt(0.10) occurred at a 1/1000 dilution and to [^{32}P] poly(dG-br^{5}dC).poly(dG-br^{5}dC) at a 1/5000 dilution (it has to be noted that the concentration of the tracers are not the same in the two experiments).

Competition experiments in 0.2 M NaCl were performed with [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC) as a tracer. The results are shown in figure 4. Poly(dG-dC)dienPt(0.12), brominated poly(dG-dC) and poly(dG-dC)cisPt(0.10) interact with the antibodies, the affinity being slightly smaller than that of poly(dG-br^{5}dC).poly(dG-br^{5}dC). Native and denatured calf thymus DNA, nDNA-cisPt(0.10)

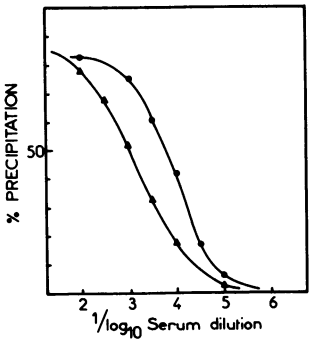

Figure 3 - Binding of antibodies to poly(dG-dC)cisPt(0.10) to [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC), C = 2 x 10^{-9} M (○) and to [^{3}H]poly(dG-dC)cisPt(0.10), C = 2 x 10^{-7} M (△). Solvent 0.2 M NaCl, 5 mM NaCl, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Temperature 4°C.

and poly(dG-dC).poly(dG-dC) are not recognized by the antibodies (the concentration of the polynucleotides is expressed in moles of nucleotide residues).

Z-DNA was detected in negatively supercoiled plasmids through the use of antibodies to poly(dG-dC)cisPt(0.10). The antibodies were precipitated three times with $(NH_4)_2SO_4$ (0.27 g/ml) and then exhaustively dialyzed against 50 mM NaCl, 5 mM Tris-HCl, pH 7.5. It had been shown that negatively supercoiled pBR322 plasmid DNA in presence of antibodies to Z-DNA is retained on nitrocellulose filter, linear DNA is not (15). As shown in figure 5, similar results are obtained with the antibodies to poly(dG-dC)cisPt(0.10).

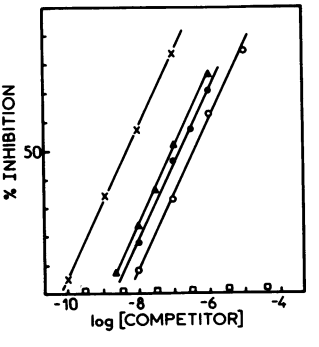

Figure 4 - Inhibition of tracer antibody binding by various DNA in competitive RIA. Tracer [^{32}P]poly(dG-br^{5}dC).poly(dG-br^{5}dC), C = 2 x 10^{-9} M ; antiserum dilution 1/5000. Competitors : (×) poly(dG-br^{5}dC).poly(dG-br^{5}dC), (▲) brominated poly(dG-dC).poly(dG-dC), (○) poly(dG-dC)dienPt(0.12), (●) poly(dG-dC)cisPt(0.10), (□) native or denatured calf thymus DNA, calf thymus DNA cisPt(0.10) or poly(dG-dC).poly(dG-dC). Solvent 0.2 M NaCl, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Temperature 4°C.

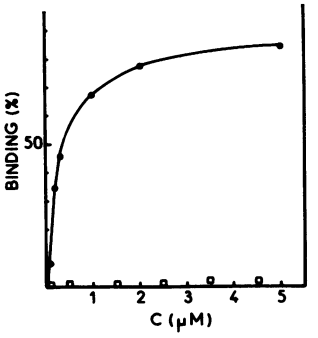

Figure 5 - The binding of increasing concentrations (μM) of anti poly(dG-dC) cisPt(0.10) antibodies to negatively supercoiled pBR322 at a negative superhelical density (-σ) of 0.063 (o) and to linear pBR322 (□). Binding is measured by trapping plasmid-antibody complexes on nitrocellulose filters. Solvent 20 mM NaCl, 30 mM EDTA, 5 mM Tris-HCl pH 7.5.

Circular dichroism

In 0.1 M $NaClO_4$ (or NaCl), the CD spectrum of poly(dG-dC).poly(dG-dC) presents a first positive band (maximum at 275 nm) and then a negative band centered at 250 nm (14). The binding of cisPt to poly(dG-dC).poly(dG-dC) modifies the shape of the CD spectrum. As r_b increases, the intensity of the first positive band decreases and then becomes slightly negative while the absolute value of the negative band decreases (figure 6A).

In 3 M $NaClO_4$ (or NaCl), the CD spectrum of poly(dG-dC).poly(dG-dC) is nearly an inversion of the spectrum in 0.1 M Na^+ (14). The spectra of poly(dG-dC)cisPt(0.05 and 0.10) are also largely modified by addition of salt (figure

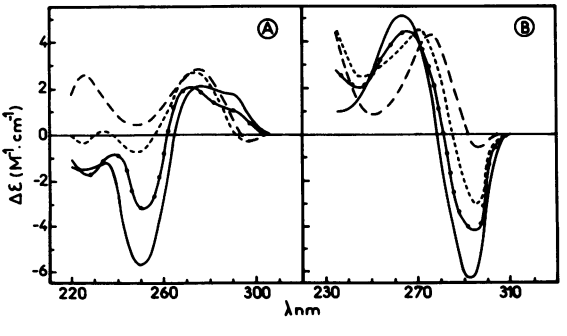

Figure 6 - Circular dichroism : Ⓐ poly(dG-dC)cisPt : r_b = 0 (——), r_b = 0.05 (—o—), r_b = 0.10 (----), r_b = 0.20 (— — —). Solvent 0.1 M $NaClO_4$, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Temperature 20°C. Ⓑ Same samples as in Ⓐ. Solvent 3 M $NaClO_4$, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Temperature 20°C.

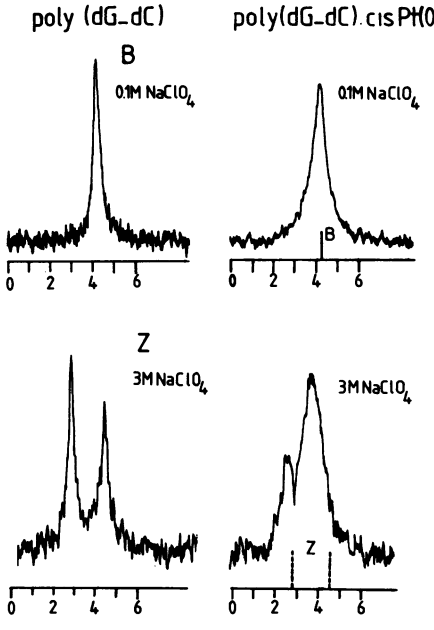

Figure 7 - Proton noise decoupled 121.5 MHz ^{31}P NMR spectra of poly (dG-dC). poly(dG-dC) and poly(dG-dC) cisPt(0.10), C = 3 x 10^{-3} M, in 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA and D_2O in low and high ionic strength. Temperature 25°C. Chemical shifts are relative to internal trimethylphosphate.

6B). In 3 M $NaClO_4$, they present a first negative band centered at 295 nm and a positive band centered at 267 nm (r_b = 0.05) and at 270 nm (r_b = 0.10). The absolute values of the intensity at 295 nm are smaller than that of poly(dG-dC).poly(dG-dC). The spectra are the same in 3 M and 4 M $NaClO_4$ (not shown). On the other hand, the spectra of poly(dG-dC)cisPt(0.20) are almost the same at low and high ionic strength. The main difference is that the first positive band is slightly more intense and red-shifted in 3 M Na^+.

^{31}P nuclear magnetic resonance

Typical ^{31}P NMR spectra of poly(dG-dC).poly(dG-dC) in the B or the Z conformation are obtained in 0.1 and 3 M $NaClO_4$ respectively (16,17) (figure 7). The spectrum of poly(dG-dC)cisPt(0.10) in low ionic strength is a single broad and slightly disymmetric line, the maximum of which coincides with that of poly(dG-dC).poly(dG-dC) (B form). This spectrum is the sum of the components corresponding to the different conformations existing in the complex. An accurate decomposition could not be achieved. Nevertheless by subtraction of the spectrum of the B form, one gets a small component located at about 0.3 ppm at lower field, the right intensity and the shape of which could not be specified. A two lines component characteristic of the Z conformation was undetectable. On the other hand, such a Z component appears in the spectrum re-

corded in 3 M $NaClO_4$ and can be roughly estimated to be about 40 % of the total spectrum.

DISCUSSION

In the reaction between cisPt and poly(dG-dC).poly(dG-dC) (or poly(dG-m^5dC).poly(dG-m^5dC)) the main adduct arises from an intrastrand crosslink between two guanines separated by a cytosine (4,8). It results in a large conformational change which has been characterized by binding to specific antibodies, circular dichroism and ^{31}P nuclear magnetic resonance.

Antibodies to Z-DNA elicited in rabbits immunized with poly(dG-dC).poly (dG-dC) chemically modified by dienPt bind to poly(dG-dC)cisPt at low and high ionic strength. In 0.1 M NaCl, poly(dG-dC).poly(dG-dC) has the B conformation and it is not recognized by the antibodies to Z-DNA (9). The relative affinity of the antibodies towards poly(dG-dC)cisPt at various r_b has been determined by RIA. When the concentration of poly(dG-dC)cisPt is expressed in moles of bound platinum residues, the affinity of the antibodies is proportional to r_b, increasing as r_b increases. In 3 M NaCl, poly(dG-dC).poly(dG-dC) has the Z conformation and it interacts with the antibodies (9). When the concentration of poly(dG-dC)cisPt is expressed in moles of nucleotide residues, the relative affinity of the antibodies decreases as r_b increases, but poly(dG-dC)cisPt(0.20) is still well-recognized by the antibodies. As judged by the antibodies to Z-DNA, the conformation of poly(dG-dC)cisPt presents some specific elements of the Z conformation.

It is not yet known what is recognized by the antibodies to poly(dG-dC) dienPt. The platinum residues are not involved in the antigenic determinant. These antibodies do not bind to thymus DNA modified by dienPt and they have the same affinity for poly(dG-dC)dienPt and poly(dG-dC).poly(dG-dC) (Z form) (9). The recognition of poly(dG-dC)cisPt by these antibodies to Z-DNA is not due to an interaction with platinum. This is confirmed by the results with the antibodies to Z-DNA elicited in rabbits immunized with brominated poly(dG-dC) and with the monoclonal antibodies (D-11). The polyclonal and monoclonal antibodies bind to poly(dG-dC)cisPt and behave as the antibodies to poly(dG-dC)dienPt.

We have shown that each binding site of the antibodies to poly(dG-dC)dienPt covers about 4 nucleotide residues, that the complexes are stabilized by electrostatic interactions (the binding constant is smaller in 3 M Na^+ than in 0.1 M Na^+) and that some groups of the bases can interact with the amino-acid residues of the antibody binding site (9). Similar properties were re-

ported for the antibodies to brominated poly(dG-dC) (18). However a detailed picture of the mechanism of recognition between these antibodies and nucleic acids cannot be drawn from these data.

Poly(dG-dC)cisPt(0.10) is a strong immunogen in rabbits. A qualitative analysis of the antiserum by immunodiffusion indicates that there are several families of antibodies. A faint precipitin line is formed with denatured calf thymus DNA. These antibodies were removed by passage on a Sepharose dDNA column. The affinity of the unbound antibodies was determined by direct precipitation and by competition. In 0.2 M NaCl, the antibodies recognize poly(dG-br^{5}dC).poly(dG-br^{5}dC), poly(dG-dC)dienPt and brominated poly(dG-dC) but they do not cross-react with poly(dG-dC).poly(dG-dC) (B form), native and denatured calf thymus DNA. These antibodies bind to poly(dG-dC)cisPt but not to native calf thymus DNA-cisPt(0.10) (in this DNA, the bidentate adduct d(GCG)cisPt is formed (4)). Again, the platinum residue is not the main antigenic determinant and in the recognition by these antibodies the conformation of the nucleic acid is an important parameter. Since the antibodies to Z-DNA bind to poly(dG-dC)cisPt and the antibodies to poly(dG-dC)cisPt bind to poly(dG-dC)cisPt and to double stranded polynucleotides known to be in Z conformation, we are lead to the conclusion that the binding of cisPt to poly(dG-dC).poly(dG-dC) (or to poly(dG-m^{5}dC).poly(dG-m^{5}dC) (8)) induces a conformational change from the B conformation to a conformation which has some similarities with the Z conformation.

Poly(dG-dC)cisPt has been studied by circular dichroism and ^{31}P nuclear magnetic resonance.

At low and high ionic strength, the binding of platinum residues to poly (dG-dC).poly(dG-dC) strongly modified the CD spectrum. For example in 0.1 M Na^+, the intensity of the positive band decreases and even becomes slightly negative as r_b increases. Large changes are observed in the first negative band of the spectrum. In 3 M Na^+, the absolute value of the intensity at 295 nm decreases as r_b increases. At high level of platination, the spectrum is almost the same in 0.1 M and 3 M Na^+ and does not resemble that of poly(dG-dC).poly(dG-dC). Thus, the platinated regions have a similar conformation in low and high salt conditions and this conformation is not that of poly(dG-dC). poly(dG-dC) (B or Z conformation).

The ^{31}P NMR experiments give some more details. In 0.1 M Na^+ the spectrum of poly(dG-dC)cisPt(0.10) is a slightly disymmetric single line and its maximum coincides with that of poly(dG-dC).poly(dG-dC) in the B conformation. This suggests that the unplatinated regions have the B conformation and that

the platinated regions do not adopt a canonical Z conformation (19,20). However, the effect of platinum on the chemical shift of proximal phosphodiester groups is unknown. The ^{31}P NMR spectrum might be sensitive to chemical modifications of the bases as shown by a recent study of brominated poly(dG-dC) (21). In 3 M Na^+, the low field line characterizing a canonical Z conformation clearly appears in the spectrum, the second line assigned to CpG phosphodiester group (22) being superposed on a broad line. About 40 % of the nucleotide residues adopt the canonical Z conformation and thus in poly(dG-dC)cisPt(0.10) each platinated region corresponds to six nucleotide residues. The "pinching" of two guanine residues by cisPt brings these residues closer (in free cisPt the ClPtCl angle is close to 90° and the distance between cis-chloride atoms is 3.3 Å (23)) which distorts the complementary strand. Thus each bound platinum residue may disturb at least six nucleotide residues. This suggests that the junctions between the canonical Z regions and the platinated regions are very short. On the other hand, the NMR results support the conclusion that the conformation of the platinated regions is the same in low and high ionic strength. Since the conformation of platinated regions is rather insensitive to salt conditions, it seems likely that there is no important denaturation, in agreement with the finding that poly(dG-dC)cisPt is not degraded by S_I nuclease (8). Thus, under low salt conditions, the junctions between the B and the platinated regions have a different conformation than that in plasmid DNA which are sensitive to S_I nuclease (24).

In summary, the spectroscopic data show that in poly(dG-dC)cisPt, there are unplatinated regions which have the B or the Z conformation depending upon the experimental conditions and platinated regions in a conformation which is not the canonical Z conformation. The immunological studies show that in low and high ionic strength conditions, the antibodies to Z-DNA bind to poly(dG-dC)cisPt and the antibodies elicited in rabbits immunized with poly (dG-dC)cisPt recognize the Z conformation. Up to this point, we can only speculate on the conformation of the platinated regions in order to reconcile the spectroscopic data and the recognition by the antibodies to Z-DNA. We cannot exclude the possibility that the antibodies shift the conformation of the platinated regions towards a Z-like conformation. This seems to us unlikely because this shift was not observed by an increase of the salt concentration. The crosslink by cisPt between two guanine residues (through the N7 atoms) separated by a cytosine residue in a double helix implies a distortion of the double helix. When cisPt reacts with Z-DNA, the main adduct is monodentate and is stable even at 37°C. The second function of cisPt does not bind to

another guanine residue but can react with molecules in solution (8). In the oligonucleotide d(GpCpG)$_2$cisPt, much conformational freedom along the backbone angles is seen as compared to d(GpCpG). Moreover the guanine can adopt the syn conformation and the N conformational population of the sugar is favored (25). On the other hand, a transition from the Z conformation to a new conformation corresponding to a distortion of the Z double helix in which the internal phosphate groups have a more similar environment has been suggested from the study of d(m^5C-G)$_3$ as a function of temperature (26).

Work is in progress to better characterize the mechanism of recognition of Z-DNA by the antibodies to Z-DNA.

ACKNOWLEDGEMENTS

We are indebted to Dr. F.M. Pohl for his kind gift of monoclonal antibodies. This work was supported in part by la Ligue Nationale Française contre le Cancer, l'Association pour le Développement de la Recherche sur le Cancer et le PIRMED (n° 4301).

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