Monoclonal antibodies to DNA modified with cis- or trans-diaminedichloroplatinum(II)

(platinum antitumor drugs/cisplatin/ELISA/DNA crosslinking)

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ABSTRACT Murine monoclonal antibodies that bind selectively to adducts formed on DNA by the antitumor drug cis-diaminedichloroplatinum(II), cis-DDP, or to the chemotherapeutically inactive trans isomer trans-DDP were elicited by immunization with calf thymus DNA modified with either cis- or trans-DDP at ratios of bound platinum per nucleotide, (D/N)_b of 0.06–0.08. The binding of two monoclonal antibodies to cis-DDP-modified DNA was competitively inhibited (50% control) in an enzyme-linked immunosorbent assay (ELISA) by 4–6 nM concentrations (600–900 fmol) of cis-DDP bound to DNA, (D/N)_b = 0.031. Similar concentrations (4–6 nM) of cis-DDP-modified poly(dG)poly(dC) also inhibited antibody binding, whereas higher concentrations (17–36 nM) of cis-DDP-modified poly[d(A)]poly[d(T)] were required for inhibition. Adducts formed by cis-DDP on other synthetic DNA polymers did not inhibit antibody binding to cis-DDP-DNA. The biologically active compounds [Pt(en)Cl_2], [Pt(dach)Cl_2], and [Pt(NH_3)_2(cbdca)] (where en is ethylenediamine, dach, 1,2-diaminocyclohexane, and cbdca is cyclobutane-1,1-dicarboxylate) all formed antibody-detectable adducts on DNA, whereas the inactive platinum complexes trans-DDP and [Pt(dien)Cl]Cl (dien, diethyleneetriamine) did not. The monoclonal antibodies therefore recognize a bifunctional Pt-DNA adduct with cis stereochirality in which platinum is coordinated to two adjacent guanines or, to a lesser degree, by adjacent adenine and guanine. A monoclonal antibody raised against trans-DDP-DNA was competitively inhibited in an ELISA by 40 nM trans-DDP bound to DNA, (D/N)_b = 0.022. This antibody crossreacted with unmodified, denatured DNA. Its binding to trans-DDP-DNA was selectively inhibited by trans-DDP-modified poly(dG)poly(dC) at 0.5% inhibition at 1 nM bound trans-DDP. The recognition of cis- or trans-DDP-modified DNAs by monoclonal antibodies thus parallels the known modes of DNA binding of these compounds and may correlate with their biological activities.

The cytotoxicity of the antineoplastic drug cis-diaminedichloroplatinum(II), cis-DDP (Fig. 1), is believed to result from covalent coordination to the bases in DNA and subsequent inhibition of replication (for reviews, see refs. 1 and 2). Since both cis-DDP and the inactive isomer trans-DDP bind to DNA in vivo and in vitro, differences in the structures of their DNA adducts probably account for their different biological activities. Both compounds exhibit a strong preference for coordination at the N7 position of guanine nucleotides in DNA. Intrastrand crosslinks at d(GpG) and d(ApG) sequences account for 50% and 28%, respectively, of the adducts formed by cis-DDP on DNA in vitro (3). The DNA adduct profile for trans-DDP has not yet been as extensively quantified (26). Unlike cis-DDP, however, trans-DDP does not, for stereochemical reasons, crosslink two adjacent nucleotides (4, 5). Replication mapping experiments show a number of possible trans-DDP-DNA adducts on single-stranded DNA, the most frequent of which involve intrastrand crosslinking of two guanines separated by an intervening nucleotide (6). Such trans-DDP-d(GpNPg) adducts have been chemically synthesized and characterized by 1H NMR spectroscopy (7, 8).

The success of cis-DDP in anticancer chemotherapy has stimulated the synthesis and testing of several analogs. In general, two labile sites in a cis geometry are required for activity, although other properties may be changed with retention of biological potency. For example, [Pt(dach)Cl_2] is active despite the steric bulk and hydrophobicity of the inert 1,2-diaminocyclohexane ligand, and [Pt(NH_3)_2(cbdca)] is as active as cis-DDP despite its much slower DNA binding kinetics.

Polyclonal antibodies have been employed to probe the structure of adducts formed by DNA by cis-DDP (3, 9–15). Here we report the preparation of monoclonal antibodies raised against cis- and trans-DDP-modified DNA. These antibodies allow a comparison of the antigenic determinants formed upon platination of double-stranded DNA by the two isomers and provide a rapid and structurally revealing probe of adducts formed on DNA by different biologically active and inactive platinum compounds.

MATERIALS AND METHODS

Platinum Compounds. [Pt(NH_3)_2(cbdca)] (carboplatin) and K_2PtCl_4 were gifts from L. S. Hollis of Engelhard Corp. The compounds cis-DDP, trans-DDP, [Pt(dien)Cl]Cl, [Pt(en)Cl_2], and [Pt(dach)Cl_2] were synthesized as described (3).

Abbreviations: cis- and trans-DDP, cis- and trans-diaminedichloroplatinum(II); en, ethylenediamine; dach, 1,2-diaminocyclohexane; dien, diethyleneetriamine; cbdca, cyclobutane-1,1-dicarboxylate; (D/N)_b, formal and bound drug-to-nucleotide ratios; cis-DDP-DNA, DNA modified with cis-DDP at specified (D/N)_b ratio; BSA, bovine serum albumin.

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and [Pt(dach)Cl₂] were prepared by following literature procedures (13, 16-18).

 cis- and trans-DDP-DNA Antigens. Native calf thymus DNA was modified with cis- and trans-DDP, as previously described (15). DNA concentrations and bound Pt levels were determined by UV and atomic absorption spectroscopy. DNA modified at a bond drug/nucleotide ratio [(D/N)b] = 0.06-0.08 was used for immunization. Six-week-old female BALB/c and C57BL/6 mice (The Jackson Laboratory) were immunized with 150 μg of Pt-DNA electrostatically coupled to 150 μg of methylated bovine serum albumin (mBSA) in an emulsion with complete Freund’s adjuvant. Mice were given three booster injections, at 2-week intervals, with 150 μg of Pt-DNA/mBSA in incomplete Freund’s adjuvant. Five days after the last booster injection, the mice were bled and the serum antibody titer was assayed by direct antibody binding to immobilized Pt-DNA in an ELISA. In all cases, C57BL/6 mice had higher antibody titers and were used to produce hybridomas.

Hybridoma Production. Mice used in fusion studies were immunized as described above except that the third booster injection occurred 4 days prior to fusion and mice were subsequently boosted daily with 50 μg of Pt-DNA/mBSA in incomplete Freund’s adjuvant (i.p.) and 50 μg of Pt-DNA (i.v.). Washed murine spleen cells (≈1.5 × 10⁶ cells per two spleens) were fused with 8-azaguanine-resistant SP2/0 myeloma cells (10:1 ratio of splenocytes to SP2/0 cells), using 45% (wt/vol) polyethylene glycol (PEG) (19). PEG-treated cells were fused with basic medium [Dulbecco’s modified Eagle’s medium (DME-M) supplemented with 2 mM L-glutamine, 125 units of penicillin per ml, 125 units of streptomycin per ml, and 1 mM sodium pyruvate] and incubated in prewarmed basic medium supplemented with 15% fetal bovine serum. After 24 hr, these cells were transferred to 96-well tissue culture plates (10²–10⁶ cells per well) with a murine spleen-cell feeder layer. Hybrids were selected using azaserine (1 μg/ml) and hypoxanthine (0.1 mM) in basic medium supplemented with 15% fetal bovine serum. After 2 weeks, most wells showed positive growth (74/925 and 172/260 for cis-DDP-DNA and 650/650 for trans-DDP-DNA). Supernatants were screened for antibody binding selectivity to Pt-modified DNA over unmodified DNA in a direct binding ELISA, and positive hybrids were cloned by limiting dilution (<30% growth) at least three times until all progeny produced antibody. Once stabilized, cells were grown in 1-liter spinner flasks and culture supernatants were used in further investigations. All antibodies described are of the IgM class, as determined by Ouchterlony immunodiffusion using class- and subclass-specific rabbit anti-mouse antisera (Litton Bionetics).

Preparation of Platinated DNAs. Calf thymus DNA (Sigma) and synthetic DNA polymers (P-L Biochemicals) were extracted with 24:1 chloroform/isooamyl alcohol three times, precipitated with ethanol twice, and dialyzed against low-salt buffer (3 mM sodium chloride/1 mM sodium phosphate, pH 7.4) prior to reaction with Pt complexes. Native DNA was used except where noted. Heat-denatured DNA was prepared by boiling for 10 min and cooling rapidly to 4°C. DNA samples were incubated for 48 hr at 37°C with Pt compounds at several formal drug/nucleotide [(D/N)b] ratios in order to achieve similar levels [(D/N)b ≈ 0.04] of bound Pt. In general, 70–100% of the added Pt complexes bound to DNA under these conditions. An exception was [Pt(NH₃)₂(cdbca)], for which only 2% of added Pt was bound. For this compound, platination at a (D/N)b ratio of 1.0 for 96 hr at 37°C was required to achieve (D/N)b = 0.032. Unbound Pt was removed by dialysis against saline-PBS buffer at 4°C for 24 hr. [Pt(NH₃)₂(cdbca)] DNA was dialyzed against ethanol prior to dialysis. DNA concentrations were determined by UV spectroscopy (20). The amount of Pt bound to the DNA was measured by atomic absorption spectroscopy on a Varian 1475 spectrophotometer equipped with a GTA 95 graphite-tube atomization system and a programmable sample dispenser.

ELISA Protocol. Enzyme-linked immunosorbent assays (ELISA) were performed as described (15). cis- or trans-DDP-DNA at specified (D/N)b ratios was bound to poly(l-lysine) (Sigma)-coated Microtiter plates (Dynatech, Alexandria, VA). Unbound sites on the plates were blocked with poly(l-glutamate) (Sigma) and 1% BSA (Sigma). Unbound reagents were removed by successive washes with 0.15 M NaCl/0.01 M Tris, pH 7.2. Antibody solutions were either added directly to Microtiter wells (direct binding ELISA) or preincubated for 60 min with DNA competitors (competitive ELISA). Unbound antibody was removed by three washes with 0.2% Tween 20 in phosphate-buffered saline, and the amount of bound antibody was measured with an alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antibody (Sigma). The latter antibody was detected by measurement of absorbance at 405 nm after addition of the substrate p-nitrophenyl phosphate (1 mg/ml in 50 mM sodium carbonate/2 mM magnesium chloride, pH 9.5). Antibodies were titrated and used at concentrations that gave absorbances of 1–2 at 405 nm after 60 min at 37°C in the absence of inhibitor.

RESULTS

Immune Response to cis- or trans-DDP-Modified DNA. Immunization with either cis- or trans-DDP-DNA [(D/N)b = 0.06–0.08] resulted, by week 7, in dramatic increases in serum antibody titers over nonimmune controls (Fig. 2). Two mice immunized with cis-DDP-DNA showed measurable antibody binding at serum dilutions of 1:25,000 (A₄₀₅ > 0.16) in an ELISA, a response similar to that previously obtained for rabbits immunized with cis-DDP-DNA (9). Two mice immunized with trans-DDP-DNA gave comparable levels of

![Fig. 2. Serum antibody binding to Pt-modified DNA in an ELISA. Antibody binding was measured by colorimetric assay (see Materials and Methods) and is shown as absorbance at 405 nm. (a) Binding of serum antibody from two cis-DDP-DNA-immunized mice (○, ●) and one nonimmune mouse (□) to cis-DDP-DNA (D/N)b = 0.066. (b) Binding of serum antibody from two trans-DDP-DNA-immunized mice (○, ▲) and one nonimmune mouse (□) to trans-DDP-DNA (D/N)b = 0.081.)](image-url)
antibody binding to trans-DDP-modified DNA at serum dilutions of 1:6000 and 1:8000, respectively. With both antigens, control nonimmune sera yielded \( A_{05} < 0.02 \) at dilutions of 1:1600 and greater.

**Monoclonal Antibodies to cis-DDP-Modified DNA.** Hybridomas derived from immunized mice were screened for production of antibodies that bound to cis-DDP-DNA ([D/N]$_b$ = 0.047) over unmodified DNA in an ELISA. In two experiments, 1.5% and 1.9% (11/740 and 5/260) of the wells exhibited this reactivity. Cells from these wells were subcloned at limiting dilutions at least three times, to ensure their stability and monoclonal character.

 Supernatants from positive hybridomas were tested in an ELISA for competitive inhibition of binding to cis-DDP-DNA by native DNA, denatured DNA, and cis-DDP-DNA ([D/N]$_b$ = 0.031). Two antibodies inhibited by the lowest concentrations of cis-DDP-modified DNA, CPT1 and CPT2, were selected for further study. Both antibodies were inhibited selectively by cis-DDP-DNA but not by native or denatured DNA (Fig. 3). Fifty percent inhibition of antibody binding was observed with average competitor concentrations of 4–6 nM bound Pt (600–900 fmol per well) (Table 1). At lower ([D/N]$_b$) levels ([D/N]$_b$ = 10$^{-2}$–10$^{-4}$]M, competitive inhibition of antibody binding is still observed; however, higher Pt concentrations are required for equivalent inhibition. We attribute this effect to multivalent antibody binding at higher levels of DNA modification.

 Antibodies not studied further fell into two general categories. Binding of five such antibodies to cis-DDP-DNA was inhibited by unmodified denatured DNA, cis-DDP-DNA, and, to a lesser extent, native DNA. These antibodies thus appear to bind to denatured regions on the DNA exposed by cis-DDP binding (15). For nine antibodies, binding to the immunogen was inhibited selectively by cis-DDP-DNA, but at higher concentrations than those required for inhibition of the best antibodies. These antibodies were similar in specificity to those studied in more detail but were probably of lower affinity.

**Crossreactivity with Other Pt-DNA Complexes.** In order to assess the contribution of ligands in the metal coordination sphere to the antigenic determinant, a series of different Pt complexes were bound to DNA and assayed for competitive inhibition of antibody binding to cis-DDP-DNA. The Pt complexes cis-DDP, trans-DDP, [Pt(en)Cl$_2$], [Pt(dach)Cl$_2$], [Pt(NH$_3$)$_2$(cbda)], and [Pt(dien)Cl]Cl were bound to calf thymus DNA at similar ([D/N]$_b$) ratios (0.032–0.041). Binding of both monoclonal antibodies to immobilized cis-DDP-DNA was 50% inhibited by 3–10 nM Pt concentrations of DNA modified at ([D/N]$_b$) ratios of 0.032–0.041 with cis-DDP, [Pt(en)Cl$_2$], [Pt(dach)Cl$_2$], and [Pt(NH$_3$)$_2$(cbda)], as revealed by the competitive ELISAs (Fig. 4). In contrast, DNA modified with [Pt(dien)Cl]Cl or trans-DDP did not inhibit antibody binding, demonstrating that a DNA adduct with bifunctional cis coordination of Pt is necessary for antibody recognition.

 Only a small decrease in antibody binding was observed when the inert amine substituents on Pt were varied from ammonia to 1,2-diaminocyclohexane (Table 1). This result suggests that the amine ligands are not a major component of the antigenic determinant and demonstrates that cis-DDP, [Pt(en)Cl$_2$], and [Pt(dach)Cl$_2$] all make stereochemically similar adducts on DNA.

**Base-Sequence Specificity of the Immunologically Reactive Pt Adducts.** Synthetic polymers of known sequence were modified by cis-DDP and tested for competitive inhibition of antibody binding to cis-DDP-DNA. For both monoclonal antibodies, cis-DDP-poly(dG)+poly(dC) ([D/N]$_b$ = 0.032] was the best competitor (Fig. 5). This modified polymer is the only one studied in which cis-DDP can crosslink adjacent guanines. Inhibition by 50% occurred at 4–6 nM bound Pt concentrations, which implies that similar antigenic determinants are recognized on both cis-DDP-DNA and cis-DDP-poly(dG)+poly(dC).

 For both antibodies, cis-DDP-poly(dAG)+poly(dTC)] ([D/N]$_b$ = 0.036] also competitively inhibited antibody binding to cis-DDP-DNA, although higher concentrations were required for 50% inhibition. This polymer allows the formation of the second most common cis-DDP adduct on DNA, a d(ApG) intrastand crosslink. CPT1 and CPT2 differed somewhat in their crossreactivity to cis-DDP-poly[d(AG)]+poly-[d(TC)] (Table 1).

 Single-stranded, cis-DDP-modified poly(dG) ([D/N] = 0.040] was also studied as a competitor, in order to test

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**Table 1.** Immunoreactivity of DNA-bound Pt complexes measured by ELISA

<table>
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<th>Antibody</th>
<th>Competitor</th>
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<tr>
<td></td>
<td>[Pt(NH$_3$)$_2$(cbda)]-DNA</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>[Pt(en)Cl$_2$]-DNA</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>[Pt(dach)Cl$_2$]-DNA</td>
<td>8</td>
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<tr>
<td></td>
<td>cis-DDP-poly(dG)+poly(dC)</td>
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<tr>
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</tr>
<tr>
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<td>4</td>
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<tr>
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<tr>
<td></td>
<td>cis-DDP-poly(dAG)+poly(dTC)</td>
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</tr>
<tr>
<td>TPT1</td>
<td>trans-DDP-DNA</td>
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<tr>
<td></td>
<td>trans-DDP-poly(dGT)+poly(dCA)</td>
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*DNA-bound Pt concentrations at 50% inhibition, derived from data in Figs. 2–5. Average values are given for experiments run in duplicate.

**Fig. 3.** Competitive inhibition in an ELISA of antibody binding to cis-DDP-DNA ([D/N]$_b$ = 0.012] by native DNA (■), heat-denatured DNA (▲), or cis-DDP-DNA ([D/N]$_b$ = 0.031] (●). Antibody binding is plotted as a percentage of antibody binding in the absence of any competitor (1% control). Competitor concentrations are given as DNA phosphate (nM). (a) Antibody CPT1 (hybridoma supernatant) at a dilution of 1:50. (b) Antibody CPT2 (hybridoma supernatant) at a dilution of 1:150.
whether cis-DDP-modified double-stranded DNA is required for antibody binding. Neither of the antibodies was competitively inhibited by cis-DDP-poly(dG) at concentrations that produced inhibition for cis-DDP-poly(dG)poly(dC) (Fig. 5). These results demonstrate that, although cis-DDP forms d(GpG) adducts on single-stranded DNA (2), both DNA strands are apparently required for antibody recognition.

Monoclonal Antibodies Raised Against trans-DDP-Modified DNA. Immunization with trans-DDP-DNA ([D/N]₀ = 0.06–0.08) elicited a response that was somewhat weaker than that observed for cis-DDP-DNA (Fig. 2b). Use of long incubation times (48 hr at 37°C) ensured that most of the trans-DDP was bifunctionally bound to DNA (21). Hybridomas were derived from the immunized mice and screened for antibodies to trans-DDP-DNA. A total of 0.5% (3/650) of the wells tested positive for antibodies that bound better to trans-DDP-DNA ([D/N]₀ = 0.078) than to unmodified DNA. In contrast to the cis-DDP-DNA antibodies, all of the trans-DDP-DNA antibodies crossreacted with heat-denatured DNA in competitive ELISA experiments. Fig. 6a shows the most selective antibody, TPT1, binding to trans-DDP-DNA in competition with native, denatured, and trans-DDP-modified DNA ([D/N]₀ = 0.037]; 50% inhibition of antibody binding occurred with >30, 2, and 0.7 μM concentrations of DNA phosphate; respectively. In analogous experiments with the two other trans-DDP-DNA antibodies, denatured DNA competitively inhibited antibody binding to trans-DDP-DNA at even lower concentrations than did trans-DDP-DNA itself.

cis-DDP-DNA, trans-DDP-DNA, and [Pt(dien)]-DNA were tested for competitive inhibition of TPT1 binding to trans-DDP-DNA, in order to assess the effect of Pt stereochemistry on antibody binding. cis-DDP-DNA and [Pt(dien)]-DNA inhibited antibody binding at levels similar to unmodified DNA, whereas a lower concentration of trans-DDP-DNA was required for inhibition of antibody binding (Fig. 6b).

![Fig. 4. Competitive inhibition of antibody binding to cis-DDP-DNA by cis-DDP-DNA (D/N)₀ = 0.038] (c), trans-DDP-DNA ([D/N]₀ = 0.034] (a), [Pt(dien)]Cl₂-DNA ([D/N]₀ = 0.038] (e), [Pt(en)Cl₂]₂-DNA ([D/N]₀ = 0.035] (c), [Pt(dach)Cl₂]-DNA ([D/N]₀ = 0.041] (c), or [Pt(NH₃)₂(μ-dca)]-DNA ([D/N]₀ = 0.032] (e). ELISA conditions are given in the caption to Fig. 3. Competitor concentrations are given as DNA-bound Pt (nM). (a) CPT1 hybridoma supernatant. (b) CPT2 hybridoma supernatant.

![Fig. 5. Competitive inhibition of antibody binding to cis-DDP-DNA by cis-DDP-poly(dA)-poly(dT) ([D/N]₀ = 0.048] (c), cis-DDP-poly(dAT)-poly(dTA) ([D/N]₀ = 0.056] (e), cis-DDP-poly(dG)-poly(dC) ([D/N]₀ = 0.032] (c), cis-DDP-poly(dG)-poly(dC) ([D/N]₀ = 0.037] (e), cis-DDP-poly(dAG)-poly(dTC) ([D/N]₀ = 0.036] (c), cis-DDP-poly(dGT)-poly(dCA) ([D/N]₀ = 0.034] (a), or cis-DDP-poly(dG) ([D/N]₀ = 0.040] (e). ELISA conditions are given in the caption to Fig. 3. Competitor concentrations are given as DNA-bound Pt (nM). (a) CPT1 hybridoma supernatant. (b) CPT2 hybridoma supernatant.

trans-DDP-modified synthetic DNA polymers of varying sequence ([D/N]₀ = 0.024–0.043) were investigated as competitive inhibitors of TPT1 antibody binding to trans-DDP-DNA. The only trans-DDP-modified polymer that competitively inhibited antibody binding was trans-DDP-poly(d-GT)-poly(d-CA) (Fig. 6c). This trans-DDP-modified polymer inhibited TPT1 binding by 50% at 1.0 μM bound Pt. A 40-fold higher concentration of trans-DDP-modified, random-sequence DNA (Table 1) was required for similar inhibition, suggesting that the antigenic determinant is present at higher levels on modified poly(dGT)poly(dCA).

**DISCUSSION**

We have obtained monoclonal antibodies that bind selectively to DNA modified with the antitumor drug cis-DDP. These antibodies, like the polyclonal antibodies reported earlier (13), show strong binding to cis-DDP-poly(dG)poly(dC), implying that the major adduct formed on duplex DNA by cis-DDP, an intrastand crosslink of adjacent guanines, is also the major antigenic determinant. Crossreactivity is also seen with cis-DDP-poly(dAG)poly(dTC), which suggests that the second most common adduct formed on duplex DNA by cis-DDP, cis-[Pt(NH₃)₂(d(ApG))], shares structural features with cis-[Pt(NH₃)₂(d(GpG))]. cis-DDP adducts form on other polymers, including the single-stranded platinated polymer cis-DDP-poly(dG), are not recognized by these antibodies.

The cis-DDP-DNA antibodies exhibit a strict requirement for a Pt center with two DNA bases coordinated in a cis geometry. Antibody binding, however, is relatively unaffected by substitution of the bulky, hydrophobic 1,2-diaminocyclohexane group for the ammonia ligands in cis-DDP. Taken together, these results suggest that antibody recognition probably depends more on a Pt-induced conformational
We have also obtained a monoclonal antibody that binds preferentially to DNA modified in a bifunctional manner by trans-DDP over unmodified DNA. In contrast to the cis-DDP-DNA antibodies, this antibody shows a strong sequence specificity for trans-DDP-modified poly[d(GTAGT)]poly[d(CTAGC)]. This result is consistent with replication mapping studies (6), which suggest that trans-DDP can form d(GpGpGpG) adducts rather than d(GpG) adducts, as seen for cis-DDP.

A number of the antibodies that bind preferentially to Pt-modified DNA over native DNA were more effectively inhibited by unmodified, denatured DNA. This result suggests that these antibodies bind to denatured regions on the DNA that form upon Pt binding at these relatively high (D/N) levels. This observation is consistent with increased anti-nucleoside antibody binding to native DNA modified by cis- and trans-DDP, reported previously (15).

For the Pt compounds described here, antibody recognition correlates well with biological activity, and the observed sequence specificity suggests that all of the active compounds tested make a stereochemically similar structure by coordinating adjacent guanines, or adenine and guanine, in DNA despite differences in their inert amine ligands and their DNA-binding kinetics.

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