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Polyclonal Antibodies to Quantitate *Cis*-Diamminedichloroplatinum(II)—DNA Adducts in Cancer Patients and Animal Models

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***cis*-Diamminedichloroplatinum (II) (*cis*-DDP), the antitumor drug, is cytotoxic *in vitro* primarily by binding to DNA and disrupting its normal functions. We have studied *cis*-DDP modification of DNA in nucleated peripheral blood cells (buffy coat cells) of testicular and ovarian cancer patients receiving *cis*-DDP chemotherapy, and of untreated controls. Using a highly sensitive enzyme-linked immunosorbent assay (ELISA) with an antiserum specific for the bidentate intrastrand N^7-deoxyguanosine adduct, blood cell DNA was assayed at multiple times during courses of *cis*-DDP treatment. A total of 138 samples were analyzed from 54 individuals. Of these, all samples from 18 untreated controls were negative, while 44 out of 120 samples from *cis*-DDP patients were positive. Testicular and ovarian cancer patients receiving chemotherapy on the first course, and given *cis*-DDP in 21- or 28-day cycles (five days of drug infusion followed by two or three drug-free weeks) accumulated *cis*-DDP–DNA adducts in blood cell DNA as a function of dose. Patients receiving their first course of *cis*-DDP on 56-day cycles and those given high doses of this drug after failing other chemotherapy showed much slower adduct accumulation than patients receiving their first course on 21- or 28-day cycles. Adduct accumulation, in positive patients, occurred both as a function of total cumulative dose and with increasing cycle number, suggesting that adduct removal took at least a month in these patients. Disease response data, available on 33 of the 45 *cis*-DDP patients, suggested that individuals who failed *cis*-DDP chemotherapy were less likely to form measurable *cis*-DDP–DNA adducts than those who responded. Characteristics of *cis*-DDP–DNA adduct formation and removal in rat tissues have indicated that both the dose-response phenomenon and slow adduct removal occur in rats exposed to *cis*-DDP. It is anticipated that studies with patients and animal models will lead to a greater understanding of the mechanisms underlying *cis*-DDP tumoricidal activity and may lead to more effective and less toxic drug treatment methods.**

Introduction

cis-Diamminedichloroplatinum (II) (*cis*-DDP) is one of the most effective tumoricidal drugs currently available. In combination with other drugs, it is responsible for cure rates of approximately 80% in testicular carcinoma (*1*) and results in clinical complete response rates of approximately 65% in advanced ovarian cancer patients (*2*). Although the mechanism of therapeutic efficacy is not completely understood, the cytocidal activity of *cis*-DDP has long been associated with effects on DNA (*3–5*), and damage in the form of specific adducts has been characterized chemically (*6–8*). This includes primarily mono-adducts on deoxyguanosine (dG), intrastrand adducts between two dGs with or without an intervening base, and interstrand DNA–DNA cross-links. Thus, it appeared possible that adducts might form in cancer patients treated with *cis*-DDP, and that determination of such *cis*-DDP–DNA adducts might provide a useful model in which to investigate the biological consequences of adduct formation in humans. Such a study would be attractive for a variety of reasons: drug dosages and exposure times would be known precisely, the DNA of peripheral blood would be readily obtainable, measurable adducts could be correlated with clinical course and the ensuing information might assist the clinician in management of the neoplastic disease. In addition, since secondary tumors may eventually occur among the individuals currently undergoing *cis*-DDP-induced cures, such data might be useful in exploring the role of DNA adduct formation during human tumorigenesis.

Recently, the utilization of antibodies specific for carcinogen-DNA adducts has made possible the development of sensitive immunoassays able to detect a few

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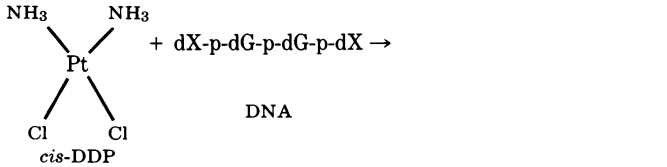
hundred adducts per mammalian genome, or about one adduct in 10^8 nucleotides (*9*). The highly sensitive and specific nature of this technology, coupled with the lack of a requirement for a radioactive sample, has made it an attractive candidate for attempting to monitor DNA adducts in the human population. A major adduct formed *in vivo* subsequent to the interaction between *cis*-DDP and DNA is an intrastrand bidentate adduct on adjacent deoxyguanosines [Eq. (1)], where linkage is through N^7 positions by displacement of the *cis*-chlorides (d[GpG]-N^7-diammineplatinum) (*7*). An antiserum elicited against



(1)

calf-thymus DNA modified with *cis*-DDP *in vitro* (4.4 adducts per 100 nucleotides) has been shown to have specificity directed primarily towards this intrastrand bidentate adduct and to recognize DNAs from cultured cells and tissues of intact animals exposed to *cis*-DDP (*10,11*). The present study demonstrates that the intrastrand bidentate adduct is present in the DNA of nucleated peripheral blood cells of cancer patients given *cis*-DDP chemotherapy, and in tissues of rats to *cis*-DDP by intravenous injections.

Methods

cis-DDP Administration to Patients and Rodents

Individuals studied were being treated for either ovarian or testicular cancer by the Medicine Branch of the National Cancer Institute. These patients were given *cis*-DDP therapy as part of approved experimental protocols (see Table 1). *cis*-DDP dose varied with protocol and was between 10 mg/m^2/day × 5 and 40 mg/m^2/day × 5. Control groups were untreated normal volunteers and patients on nonplatinum combination chemotherapy for non-Hodgkin's lymphoma. In addition some of the patients were studied for adducts prior to receiving any *cis*-DDP or other chemotherapy and therefore served as their own controls.

cis-DDP chemotherapy was given in courses comprised of three to five cycles. In each cycle the drug was administered as a 30-min daily intravenous infusion on each of five consecutive days, and no *cis*-DDP was given for the remainder of the cycle. On the morning following and infusion, 35 to 50 mL of blood was obtained via venipuncture, centrifuged (20 min, 5000*g*, 4°C), and the nucleated cells (the buffy coat) were aspirated and frozen at -20°C until DNA isolation.

Female Sprague-Dawley rats weighing 175–200 g were housed five to a cage in clear plastic cages with hardwood bedding and given free access to Purina Lab chow and tap water *ad libitum*. *cis*-DDP intravenous (IV) injections of 6 or 30 mg/kg were given at 0.9% NaCl, at a 1 mg/mL solution concentration, within 30 min of preparation. Injections were given to animals under light ether anesthesia, and sacrifice was with ether overdose 4 hr after injection. Kidneys were excised and frozen at -20°C until DNA isolation.

DNA Preparation and ELISA

DNA was extracted from buffy coat cells and rat kidneys by CsCl gradient centrifugation (*12*) within 1 month of the time the sample was taken. This DNA was dialyzed against water and quantitated by absorbance at 260 nm. Samples were assayed for *cis*-DDP–DNA adducts by ELISA as previously reported (*10,11*) with modifications. Poly(vinyl chloride) U-bottom microtiter plate wells (Dynatech, Inc., Alexandria, VA) were coated sequentially with DEAE–dextran and 0.5 ng of calf thymus DNA, either unmodified or modified to 4.3 % with *cis*-DDP. In separate tubes, rabbit antiserum specific for *cis*-DDP–DNA (diluted 1:60,000) was reacted with standard or sample DNAs for 30 min at 37°C. Subsequently, the mixture was added to the microtiter plates so that remaining free antibody could bind *cis*-DDP–DNA coated on the wells. After 90 min of further incubation, unbound antigen–antibody complexes were removed by three washes with PBS-Tween. Excess alkaline phosphatase conjugate (goat–anti-rabbit-IgG, Sigma, Inc., St. Louis, MO) was then added to the wells and incubated 90 min to interact with the bound anti-*cis*-DDP–DNA antiserum. After washing again, excess *p*-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 8.6, was added to each well, and the extent of hydrolysis to *p*-nitrophenol was proportional to the quantity of bound anti-*cis*-DDP–DNA. A Titertek Multiskan colorimetric microtiter plate reader was used at 405 nm to determine *p*-nitrophenol. The ELISA standard curve 50% inhibition, under the assay conditions described, was at 10 ± 4 fmole (mean ± range) of platinum (atomic absorption spectroscopy performed by S.J. Lippard) in standard immunogen *cis*-DDP–DNA. DNA samples were assayed as native, with 30 to 35 μg of sample DNA per well and 35 μg of unmodified calf thymus DNA in the standard curve wells. Each unknown DNA sample was assayed two or three times by ELISA and not scored as positive unless it produced greater than 20% inhibition in the assay.

Data Analysis

Adduct analyses were performed on 138 samples collected from a total of 53 individuals. Patients in the treatment groups outlined above were studied one or more times during their course of chemotherapy. Individuals in the control groups were generally studied only once. Linear regression analyses of data points in

Table 1. Summary of ELISA data from 138 human peripheral blood DNA samples.

Type of cancer	Chemotherapy protocol	Drug cycle time, days	Number of individuals	Blood samples	
				Positive	Negative
Testicular	*cis*-DDP, combination 1[a]	21 or 28	11	11	23
Ovarian	*cis*-DDP, combination 2[a]	28	13	11	16
Ovarian	*cis*-DDP, combination 3[a]	56	8	8	12
Ovarian	*cis*-DDP, single high dose	28	13	14	25
None	None	—	4	0	4
Non-Hodgkin's lymphoma	Non-*cis*-DDP	—	4	0	4
Ovarian or testicular	None (prior to *cis*-DDP)	—	10	0	10

[a] Combination 1 is *cis*-DDP, velban, bleomycin, with or without epidophyllotoxin; combination 2 is cytoxan, *cis*-DDP; combination 3 is cytoxan, hexamethylmelamine, irradiation, *cis*-DDP with or without radiosensitizer.

each patient group were performed by computer. Statistical significance was calculated by the two-sided Fisher Exact Test as described by Armitage (*13*).

Results

Antibody Specificity and Assay Sensitivity

The antiserum utilized in these studies has previously been shown to be specific for adducts of *cis*- but not *trans*-DDP–DNA formed in cultured cells and intact animals (*10*). This antiserum fails to recognize *cis*-DDP alone (Fig. 1*A*), DNA alone (Fig. 1*B*), and the DNA interstrand crosslinks determined by alkaline elution (*5*). Through the use of a highly sensitive competitive ELISA (Fig. 1*A*) to which 50 μg samples of DNA can be added per microtiter plate well, it is possible to measure attomole (10^{-18} mole) quantities of *cis*-DDP-DNA adduct per microgram of DNA. A series of studies with homopolymers and heteropolymers modified with *cis*-DDP, and calf-thymus DNAs modified with various platinum drug analogs have indicated that the antibody specificity is directed towards d(GpG)-N^7-diammineplatinum, a bidentate adduct with *cis* covalent bonds on the same strand of DNA (*11*).

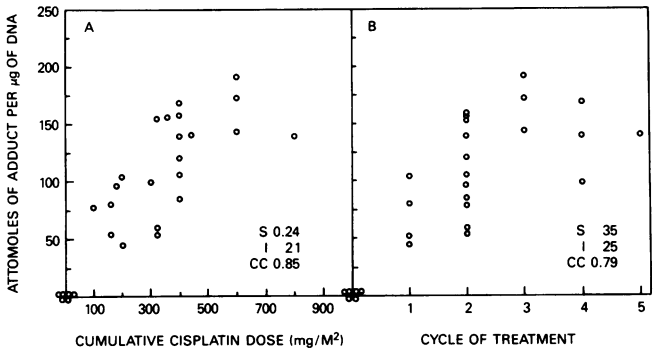


FIGURE 1. Plots of (*A*) representative of ELISA standard curve and (*B*) ELISA of patient buffy coat cell DNA. (*A*) Mixtures of anti-*cis*-DDP–DNA (diluted 1:30,000) and either immunogen *cis*-DDP–DNA (4.3% modified) (●), or *cis*-DDP (○) were assayed in competition with 0.5 ng of *cis*-DDP-DNA (modified 4.3%) coated to microtiter wells under conditions described in Methods. (*B*) DNA extracted from buffy coat cells of either an ovarian cancer patient on *cis*-DDP therapy (●) or a normal volunteer (○) was mixed with anti-*cis*-DDP–DNA (diluted 1:60,000) and assayed in competition as above. Increasing quantities of DNA (μg) are plotted on the abscissa.

Assay of *cis*-DDP-DNA Adducts in Nucleated Peripheral Blood Cells of Cancer Patients

ELISA assay profiles, showing competition for specific antibody by increasing amounts of buffy coat DNA from a normal volunteer and an ovarian cancer patient receiving 40 mg/m²/day of *cis*-DDP, are shown in Figure 1*B*. It is evident that the antibody recognized the patient DNA and not the DNA from the normal volunteer. This constituted the first evidence that measurable levels of *cis*-DDP–DNA intrastrand adducts are formed in individuals receiving *cis*-DDP chemotherapy. The antibody recognition is not as complete as with the immunogen *cis*-DDP–DNA since the slope of the competition curve (Fig. 1*B*) is more shallow than the standard curve (Fig. 1*A*). This observation suggests that other types of *cis*-DDP–DNA adducts (possibly mono-or G-X-G adducts) may be formed simultaneously, resulting in lower affinity antibody binding for certain DNA regions and an overall decrease in ability to compete, as compared to the immunogen DNA.

Table 1 shows a summary of the results from ELISAs of 138 blood DNA samples obtained from 54 individuals. Most patients were studied during more than one cycle of chemotherapy, and most samples were obtained after the 5-day drug infusion (the morning of day 6). This time was chosen because early results demonstrated that the proportion of positive samples increased towards the end of the infusion. Table 1 shows that blood samples from all 18 individuals who did not receive *cis*-DDP therapy were negative. In addition, 44 samples out of 120 from *cis*-DDP-treated individuals were positive in the assay. The first three groups of cancer pa-

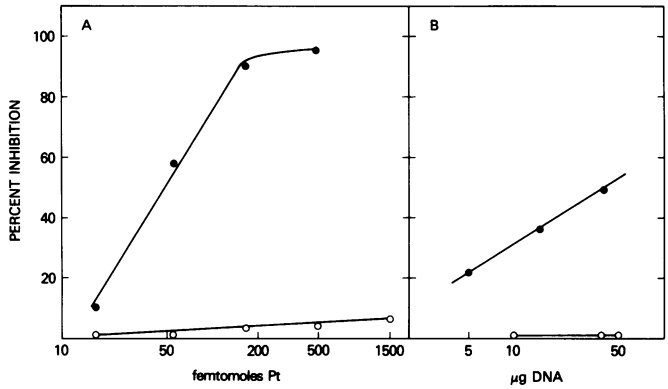


FIGURE 2. Positive *cis*-DDP–DNA adduct determination on buffy coat DNA samples from patients on their first course of 21- and 28-day cycle chemotherapy. Adduct levels (ordinate) are expressed as a function of (*A*) *cis*-DDP cumulative dose and (*B*) cycle of treatment. Both plots are of the same data. S = slope, I = intercept, CC = correlation coefficient, are from linear regression analysis (lines not drawn).

tients shown in Table 1 were individuals who received no courses of chemotherapy prior to this study. The fourth group comprised more advanced individuals who had previously failed various courses of *cis*-DDP and/or other therapy. An analysis of the ELISA data by group showed that some of the testicular and ovarian patients (combinations 1 and 2, Table 1) who were given *cis*-DDP in 21- and 28-day cycles and were followed from the inception of chemotherapy, accumulated *cis*-DDP–DNA adducts with increasing cumulative dose and cycle number. Data for the positive samples are plotted in Figure 2, and linear regression parameters (noted on the chart) indicate good linear fit. In contrast, individuals receiving their first course of chemotherapy on 56-day cycles (Table 1, combination 3) appeared not to accumulate adducts as well as those in the previous groups (data not shown, correlation coefficient = 0.48). Taken together, these data suggest that a substantial portion of the adducts formed as the result of one cycle of treatment are removed during 56 days, with a half-life for removal in the range of 28 days. The fourth patient group, individuals on "salvage" high-dose *cis*-DDP, did not accumulate adducts in a similar consistent fashion (data not shown, correlation coefficient = 0.63), suggesting that the combination of advanced disease and previous chemotherapy may somehow alter the ability of the patient to form or remove measurable *cis*-DDP–DNA adducts.

The ability to detect *cis*-DDP–DNA adduct formation in patients provides an opportunity to correlate adduct formation with a biological end point, which is in this case the response to therapy. From the computerized patient records it was possible to gather disease response data on 33 of the *cis*-DDP-exposed patients. Of these, 12 were given *cis*-DDP as a single agent and 21 received combination chemotherapy. The results are shown in Table 2 and suggest that among the individuals who did not form *cis*-DDP–DNA adducts there were fewer partial or complete remissions as compared to those who did form adducts ($p = 0.048$ by Fischer's Exact two-sided test). Since both the responding and nonresponding groups received essentially the same amount of *cis*-DDP, the formation of measurable quantities of adduct appears to correlate positively with a response to therapy. This type of analysis will become more definitive as data become available for all of the individuals under study.

Quantitation of *cis*-DDP–DNA Adduct Formation in Rat Tissues by ELISA

In an attempt to elucidate mechanisms underlying *cis*-DDP therapeutic efficacy in cancer patients, experiments were begun in animal models. Kidney was chosen as the tissue to monitor in these studies since it is a known target for *cis*-DDP toxicity. Although we do not yet have extensive animal data, the results available show certain distinct parallels within the human study. Table 3 shows data from two experiments in which we have investigated *cis*-DDP–DNA adduct formation with increasing *cis*-DDP dose, and persistence of the adduct after drug exposure, in kidney tissue of female rats. In experiment A (Table 3) the higher adduct levels were clearly a result of higher dose. One should note however, that 30 mg/kg is acutely toxic and animals do not survive for more than a few days. Therefore, when monitoring the time course of adduct removal (experiment B, Table 3) the lower dose of 6 mg/kg was chosen. Experiment B shows that little or no adduct was removed during 2 weeks subsequent to a single dose of *cis*-DDP. Therefore *cis*-DDP–DNA adducts persist in rat kidney for prolonged periods showing a distinct parallel to the results of the human studies.

Discussion

The ability to quantitate DNA adducts of *cis*-DDP in nucleated peripheral blood cells of cancer patients has been achieved through the use of a highly sensitive immunoassay and an antiserum with primary specificity directed towards the d(GpG)-N^7-diammineplatinum intrastrand adduct. The data show that many individuals given *cis*-DDP for the first time on 21- or 28-day cycles accumulated DNA adducts with cycle and dose. Accumulation is less clear in patients on 56-day cycles, suggesting that removal of this adduct or loss of adduct-containing cells may take place within a 2-month time period. These human results, however, have been obtained in circulating cells, and studies on solid tissues or tumors remain to be done. The data also show an apparent positive correlation between the formation of measurable adduct levels and disease response to *cis*-DDP treatment, although this relationship requires further investigation. Since there is a high probability that we are not measuring the only adduct formed, the possibility exists that other adducts may be contributing to the observed biological end point, that is disease response.

Table 2. Disease response in all patients grouped by treatment and by adduct status.

Treatment	ELISA status	Number of patients	Percent with partial or complete remission	Cumulative *cis*-DDP dose (mean ± SD), mg/m²
Single agent				
A		12		
	Never pos	4	0 (0/4)	756 ± 445
	Pos ≥ once	8	50% (4/8)	877 ± 395
Combination				
B		21		
	Never pos	6	50% (3/6)	543 ± 184
	Pos ≥ once	15	87% (13/15)	575 ± 219
A + B		33		
	Never pos	10	30% (3/10)	
	Pos ≥ once	23	74% (17/23)	

Table 3. *cis*-DDP–DNA adduct levels in kidneys of female rats exposed IV to *cis*-DDP.[a]

	cis-DDP dose, mg/kg	Time of sacrifice, hr	*cis*-DDP–DNA adducts, atmole/μg DNA
Expt. A	6	6 hr	130 ± 30
	30	6 hr	542 ± 28
Expt. B	6	6 hr	309 ± 35
	6	7 days	357 ± 31
	6	13 days	300 ± 14

[a] Animals were injected intravenously with either 6 or 30 mg/kg *cis*-DDP and either sacrificed 6 hr later or maintained for an additional 7 or 13 days. Values represent tissues from two or three rats assayed two or three times by ELISA (mean ± range).

Studies with animal models reported here show interesting parallels with the human data. Rat kidney was chosen as the most relevant tissue because of the significant kidney toxicity noted in patients and animal models. Attempts to measure adducts in rat blood were not successful because of the small yield of DNA. In performing the studies reported here we observed large interanimal variability in *cis*-DDP–DNA adduct formation among inbred animals in the same group. This variability may be related to feeding cycles since overnight fasting has reduced the variability in subsequent experiments (data not shown). It is clear, however, that *cis*-DDP–DNA adducts are formed in rat kidney in a dose-dependent fashion, and that these adducts are highly persistent, since little or no removal took place within the first 2 weeks after exposure. These studies are being extended to include rats bearing the Walker 256 sarcoma and nude mice carrying *cis*-DDP sensitive and resistent human ovarian tumors. In tumor-bearing animal models it may be possible to determine whether or not specific adduct levels are associated with antitumorigenic efficacy in both kidney and neoplasm, and if manipulation of drug administration may modulate adduct formation in tumor tissues.

Of the many possible sequelae *cis*-DDP may produce in humans, one of the most ominous is the generation of second malignancies, reports of which have been recently published (*14,15*). It is possible that the slow rate of intrastrand adduct removal contributes to the chemotherapeutic potency of *cis*-DDP and also that this adduct persistency may be related to the possible development of treatment-induced neoplasia. Adduct persistence is known to be associated with tumorigenesis in animal models. For example, in neonatal rats exposed to ethylnitrosourea, a neurooncogenic effect in brain tissue has been shown to correlate directly with the slow rate of repair of the O^6-ethyl-dG adduct formed by ethylnitrosourea (*16*). If reports of *cis*-DDP-related neoplasia become more frequent, a difficult therapeutic dilemma may ensue, and data generated in studies such as ours may be useful in determining if adduct accumulation is associated with risk of second malignancy. It is hoped that the ability to monitor *cis*-DDP–DNA adducts may allow the clinician to titrate *cis*-DDP for chemotherapeutic potency while minimizing immediate toxicity and the potential threat of second malignancy. Studies utilizing the ELISA to quantitate adducts in animal models may become a useful adjunct for designing drug treatment protocols and for elucidating mechanisms underlying drug efficacy.

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