Sensitive Detection of DNA Modifications induced by Cisplatin and Carboplatin in Vitro and in Vivo Using a Monoclonal Antibody

Michael J. Tilby, Caroline Johnson, Richard J. Knox, Jacqueline Cordell, John J. Roberts, and Christopher J. Dean

Experimental Unit, Section of Medicine [M. J. T., C. J., J. C., C. J. D.], and Molecular Pharmacology Unit, Section of Drug Development [R. J. K., J. J. R.], Institute of Cancer Research, Sutton, Surrey, England, SM2 5NG

ABSTRACT

An assay that is based upon a monoclonal antibody (ICR4) is described that enables the quantitation of cisplatin-induced adducts on DNA down to 3 nmol Pt/g DNA (i.e., 1 Pt adduct/10^10 bases), the level necessary to produce toxic effects in cells in vitro and in vivo, using just a few micrograms of DNA. Detection is possible below this level (although probably not necessary for in vitro studies) but the cross-reactivity of unmodified DNA sequences complicates absolute quantitation of adducts. Therefore, it will be possible to investigate the distribution of clinically useful platinum drugs in patients undergoing chemotherapy. Rats of strain F344 appeared to be the best, among several tested, for the production of antibodies to modified DNA, and they were used for the production of hybridomas which secreted antibodies that bound to DNA that was highly modified with cisplatin but not to normal DNA were obtained. One (ICR4) was chosen for further characterization because of its relatively strong binding to DNA modified to a moderate level with cisplatin. The characterization included the development of a sensitive competitive enzyme-linked immunoabsorbent assay and the use of DNA that had been reacted with cisplatin both in vitro and in vivo. The levels of platination of both types of DNA samples were determined by atomic absorbance spectroscopy. For DNA that had been exposed to cisplatin in vitro, 50% inhibition of antibody binding was caused by about 15 fmoles of total DNA-bound Pt/assay well. At moderate levels of platination, heating of the DNA solution at 100°C for 5 min increased its immunoreactivity such that 50% inhibition was caused by 2.5 fmoles Pt adducts/well. Pt adducts on DNA extracted from cells that had been treated with cisplatin were less immunoreactive than DNA treated with cisplatin in vitro, but after heating the immunoreactivity increased such that 50% inhibition in the assay was caused by 2 fmoles Pt adducts/well. This sensitivity was invariant over a wide range of levels of platinum adduct frequency. DNA adducts formed by the second generation anticancer drug carboplatin were recognized similarly to the adducts formed by cisplatin, but those formed by the clinically inactive trans-diaminedichloroplatinum(II) or chloro(diethylenetriamine)-platinum(II)-chloride were not significantly immunoreactive. Control DNA cross-reacted in the competitive assay but the immunoreactivity per mol base was 10^7 times lower than the immunoreactivity of cisplatin adducts.

INTRODUCTION

The widely used antitumor drugs cisplatin [cis-diaminedichloroplatinum(II)] and carboplatin [cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II)] appear to kill cells as a result of their interactions with genomic DNA (1). A number of important questions regarding the effect or lack of effectiveness of these drugs in the clinic could be answered directly if the extent of these DNA interactions could be reliably measured in small numbers of cells removed from patients. Drug doses in the biological range induce relatively low frequencies of DNA base modification [up to 30 nmol Pt/g DNA, (2)], so that large quantities of material (≥10^6 cells) are needed for reliable AAS determination of platinum adducts. This precludes many investigations on clinical material and at present immunological techniques appear to have the greatest potential for providing direct and sensitive detection methods.

A number of antisera and antibodies that specifically recognize DNA modified with cisplatin have been described previously. Two assays based upon antisera raised against cisplatin-modified polymeric DNA either were incapable of detecting (3) or showed a greatly reduced sensitivity (4, 5) for DNA modified with this drug in vivo, compared to DNA modified in vitro. For the assay described by Poirier et al. (4), this has been shown to be related to a reduced immunological recognition of platinum adducts at the overall frequency of base platination (Pt/base) was low, compared to the high levels of platination used to raise and characterize the antiserum (5). Assays with apparently analogous reduced sensitivities at low DNA modification levels have been described for other types of DNA modification (6–8). Another antiserum, which was prepared in essentially the same way as described in Refs. 3 and 4, provided good sensitivity for detection of DNA-cisplatin adducts in immunohistochemical procedures but appears not to have been characterized using other techniques (9). Monoclonal antibodies that recognize cisplatin-modified DNA have been described (10) but appeared to give assays of insufficient sensitivity for biological experiments. The problems associated with preferential recognition of highly platinated DNA are avoided in the work of Fichtinger-Schepe man et al. (11), in which the immunoassays were performed after hydrolysis of the DNA and chromatographic separation of the resulting nucleotides. As discussed below, sensitive immunological detection of platinum adducts on polymeric DNA could have certain advantages, especially if based upon monoclonal antibodies. Such a methodology would complement analyses of DNA hydrolysates.

In this paper we describe the production and initial characterization of a monoclonal antibody that provides a highly sensitive and specific assay for polymeric DNA modified with certain biologically active platinum compounds. We show that the sensitivity of the assay is essentially independent of the frequency of DNA modification and of whether the DNA is exposed to drug in vivo or in vitro and is sufficient to permit analysis of clinical samples containing just a few million cells.

MATERIALS AND METHODS

Solutions. Buffer A consisted of 50 mM NaCl, 50 mM sodium phosphate, and 0.2 mg/liter NaN3, pH 7.0. PBS was 10 mM Na/K phosphate, 140 mM NaCl, and 0.2 mg/liter NaN3, pH 7.5. PBS/Tween was PBS containing Tween 20 at 0.1% (v/v). Lysis buffer was 80 mM

Received 6/29/90; accepted 10/9/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1. This work was funded by the U.K. Cancer Research Campaign.

2. To whom requests for reprints should be addressed.


The abbreviations used are: AAS, atomic absorbance spectroscopy; ELISA, enzyme-linked immunoabsorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; trans-DDP, trans-diaminedichloroplatinum(II); dian-Pt, chloro(diethylenetriamine)-platinum(II)-chloride; K value, concentration of substance that causes 50% inhibition in the competitive ELISA.
Reaction of Drugs with DNA and RNA. DNA or RNA (2 mg/ml in H₂O) was reacted with various concentrations of cisplatin (50 nm to 1 mM) for 1 h at 37°C. Aqueous sodium acetate (30%, w/v) was then added to the reaction mixture to give a concentration of 1% and the DNA or RNA was precipitated with ethanol. This precipitate was washed four times with ethanol and then redissolved in H₂O. After a further 16 h incubation, the DNA or RNA was reprecipitated as above, washed with diethyl ether, and dried under vacuum.

Storage of Platinitated DNA. When highly platinitated DNA preparations were stored as frozen solutions at −20°C, they became insoluble, presumably as the result of delayed cross-linking reactions between DNA molecules that became concentrated in the center of the frozen sample. This was observed even though the DNA had been incubated extensively after the removal of unbound drug. Such an alteration was unacceptable for immunological experiments. Storage at −135°C or in liquid nitrogen prevented this problem.

Immunization Procedure. The most successful immunizations were essentially according to the method of Plescia et al. (12), using native DNA that had been reacted with cisplatin (23 μmol Pt/g DNA, Pt/base = 0.008). The DNA-methylated BSA suspension, containing 150 μg DNA/ml, was emulsified with an equal volume of Freund’s adjuvant. Complete adjuvant was used for the first and incomplete adjuvant for subsequent immunizations. Rats (strain F344 from the National Institute for Medical Research, Mill Hill, England) were each given 1 ml (final volume) of immunogen/challenge, distributed evenly over four sites s.c. and one site i.p. The rats were immunized 3 times at 4-week intervals, followed by an i.v. administration of immunogen without adjuvant 3 days before spleen cell fusions were carried out. Other immunizations using F344 rats made use of DNA precipitated with Ca₃(PO₄)₂ (13). This was collected by centrifugation, resuspended in PBS at 200 μg DNA/ml, and emulsified with adjuvant. The types of DNA, the adjuvant, and the immunization protocol were as above.

Hybridoma Production. Rat × rat hybridomas were prepared, according to the protocol described previously (14), by fusing the myeloma Y3Ag.1.2.3 (15) with spleen cells taken from immune rats. Each fusion generated 100 to 300 hybridoma cultures. Culture supernatants were screened initially using a radioimmunoabsorbent assay (16) in which the assay wells were coated with native DNA that had been reacted with cisplatin (146 and 2 μmol Pt/g DNA; Pt/base = 0.05 and 0.0007, respectively) or with normal native DNA.

Immunosassay Plates and Plate Coating. The plates and the methods for coating with DNA were as described previously (16), except that the DNA used had been reacted with platinum drugs. For screening of hybridoma supernatants, the plates were coated by drying DNA onto the plastic surface. In order to ensure the uniformity of coating necessary for accurate ELISA, the surface of the plastic was saturated with BSA and then the DNA was cross-linked to the protein with UV light (16). For competitive ELISAs, the DNA (175 μmol Pt/g DNA; Pt/base = 0.06) was applied at 1 ng/well.

ELISA. This was essentially as described previously (16), except that antibody dilutions were in PBS/Tween containing BSA (1 μg/ml). In brief, the procedure entailed the transfer of aliquots of antibody solution (50 μl) to DNA-coated 96-well plates. These were incubated for 60 min and then washed 5 times. A β-galactosidase conjugate of F(ab')2 sheep/rat immunoglobulin was added to the wells, and after a further 90-min incubation and 10 washes the solution of the enzyme substrate (4-methylumbelliferyl-β-D-galactoside) was added. After incubation for 30 min to 2 h, the fluorescence in each well due to 4-methylumbelliferone was measured using a plate reader (Microfluor; Dynatech). Assays using the rat sera were read using a spectrofluorimeter (16).

Competitive ELISA. This was as described previously (16), with the following alterations. The antibody was ICR4. This was added as hybridoma culture supernatant at a final overall dilution of 1:40,000. Incubation with enzyme substrate was overnight at 37°C.

Treatment of Cells with Drugs. Cells (500 ml at 4 × 10⁵/ml) of the Walker 256 tumor cell line, grown as previously described (2), were treated with various concentrations of cisplatin (2 μM to 1 mM) in standard Walker growth medium at 37°C. After 2 or 4 h the cells were pelleted by centrifugation and washed 4 times in ice-cold PBS, an aliquot (2 × 10⁷ cells) was removed, and both the bulk pellet and the aliquot were rapidly frozen at −80°C.

Isolation of Cellular DNA Using Hydroxyapatite. This method, which reliably gives good recoveries of DNA from small numbers of cells, was developed from previous protocols (17, 18). When a potassium phosphate concentration of 80 mM was used, recovery of RNA from the hydroxyapatite column was undetectable (i.e., <5% of a similar applied amount of DNA), whereas DNA recovery was essentially complete. The potassium phosphate concentration, which is lower than that previously reported (17, 18), has been found to be invariant between several batches of Biogel-HTTP, despite interbatch differences in the concentrations of phosphate determined by the manufacturer to be necessary for elution of DNA in the absence of other buffer components.

Cells (up to 2 × 10⁸) were lysed in 2 ml of lysis buffer, sonicated (using Sonics and Materials VC-600 ultrasonic processor fitted with a cup-horn, at maximum power for 2 min), and then incubated (37°C, 15 min) with 10 μg of heat-treated (19) RNase A. The resulting solution plus an additional 3 ml of lysis buffer was mixed with 5 ml of phenol reagent (20) at room temperature for 15 min. After centrifugation (800 × g, 15 min) the aqueous phase was removed and the organic layer was washed with an additional 0.5 ml of lysis buffer. First 25 ml of 6 M urea/0.08 M potassium phosphate (pH 6.8) and then 0.5 g hydroxyapatite (DNA-grade Bioel-HTTP from Bio-Rad) were added to the DNA solution, which was mixed in an end-over-end mixer for 15 min at room temperature. The suspension was transferred into and centrifuged in a spun column device, constructed from a 10-ml plastic syringe barrel in which had been placed a glass fiber filter (Whatman GF/A) supported on a disc of stainless steel mesh. All centrifugations were at 47 × g for 5 min. This caused all the liquid to drain through the column without drawing air into the gel. The eluates were discarded. When all the suspension had been transferred to the column, the gel was washed by centrifugation successively with 5 ml and 2 × 10 ml of 5 mM urea/0.08 M potassium phosphate (pH 6.8). Finally, the DNA was eluted by washing the column with 0.2 ml and then 2 ml of 0.5 M potassium phosphate (pH 6.8). The 2-ml eluate was collected and then reduced in volume, using an ultrafiltration device (Centricon, M, 10,000 cut-off; Amicon), to about 50 μl. Buffer A (1 ml) was added and the volume was reduced again to 50 μl. Finally, 400 μl of buffer A were added and the device was centrifuged in the inverted orientation to collect the concentrated and desalted DNA solution. RNA and protein contamination was undetectable (i.e., <5%, w/w, for each). RNA was determined by chromatographic analysis of DNA samples which had been enzymatically hydrolyzed to nucleosides (using RNase, DNase I, P1 nuclease, and alkaline phosphatase). Protein was determined with BCA (bicinchoninic acid) reagent (Pierce). Ratios of A₂₆₀/A₂₈₀ for the resulting DNA preparations were in the range of 1.75–1.95.

Extraction of DNA from Cells Using Ethanol Precipitation. Frozen cell pellets (up to 2 × 10⁶ cells) were thawed directly into a lysing solution containing sodium p-aminosalicylate, and the DNA was extracted as previously described (2).

Determination of DNA Platinum Level by AAS. The specific binding of platinum to DNA was determined by atomic absorption spectrophotometry, as previously described (21, 22).

RESULTS

Production of Antibodies. Our initial attempts to obtain antibodies against cisplatin-modified DNA used hooded CBH/Cbi rats, as used previously (16). Although performed in essentially the same way as later successful experiments, these immunizations failed in terms of both serum responses and yield of appropriate hybridomas (seven fusion experiments). It was subsequently noticed that, even before immunization, rats of...
this strain contained significant levels of antibodies recognizing control DNA. Similarly high levels of this type of antibody were found in the sera of several other strains of rats tested, but particularly low levels were found in nonimmunized rats of strain F344 and these animals were chosen for subsequent immunizations.

Three F344 rats were immunized with DNA complexed with methylated BSA, and all rapidly developed good serum responses. Three fusions were carried out using spleen cells from two of the immune rats and these yielded five, five, and two hybridomas secreting antibodies that bound to cisplatin-modified DNA but not to normal DNA. We also immunized three more F344 rats with DNA precipitated with Ca$_3$(PO$_4$)$_2$. These all developed weaker but nevertheless positive serum responses specific for cisplatin-modified DNA. Spleen cells from two of these animals yielded a total of three hybridomas which secreted antibodies that bound to cisplatin-modified DNA but not to normal DNA.

Hybridoma supernatants were screened on assay wells coated with DNA that was modified with cisplatin at either high or low levels, in order to identify, at an early stage, antibodies that would be most suitable for analysis of biological samples. Of the antibodies which gave a high degree of binding to the highly modified DNA, the immunoassay signal for the DNA platinated at the lower level varied between <1% and 17% of the signal for the highly platinated DNA. One antibody, now designated ICR4 (clone CP9/19), was selected for further experimentation because of its class (IgG), its high binding to DNA platinated at the low as well as the high levels, and its lack of binding to control DNA.

Noncompetitive ELISA. ICR4 culture supernatant was serially diluted and applied to wells coated with varying amounts of DNA carrying various levels of cisplatin modification (Fig. 1). There was no detectable binding of antibody to native control DNA (applied at 100 ng/well). Two different drug-treated native DNA preparations were used, having a 75-fold difference in their levels of platination (187 and 2.5 $\mu$mol Pt/g DNA; preparations 1 and 2, respectively, of Table 1). The amounts of antibody which bound to the wells, as indicated by the fluorescence intensity, were dependent upon the amount of platinum adducts applied to the wells and not the quantity of actual DNA (Fig. 1A). After extended incubation with substrate, binding of antibody was clearly detectable when the supernatant had been diluted nearly 10$^5$-fold (Fig. 1B). When plates were coated with heat-denatured control DNA, a significant amount of antibody binding was observed. This was dependent upon the amount of DNA applied to the wells, but the titration curves showed no plateau, even at low levels of DNA coating, and antibody binding became undetectable at moderate dilutions of the supernatant. These observations indicate that this binding was due to a low affinity and/or a nonspecific interaction of the antibody molecules with denatured DNA. It was not dependent upon UV irradiation of the DNA during the plate-coating procedure, since DNA coated directly onto the plastic surface without irradiation gave similar results (data not shown). This type of noncompetitive assay is of limited application (16) and, therefore, all further studies to be described used competitive assays.

Competitive ELISA. Serial dilutions of each sample of DNA were assayed and the results were fitted to the logistic equation, as described previously (16), from which the fitted parameters $K$ (i.e., concentration causing 50% inhibition) and slope were derived. Representative data and the fitted curves are shown in Fig. 2 and 3. In these results, the quantities of drug-DNA adducts are expressed as the amount of total platinum bound to DNA, as determined by AAS.

DNA Reacted with Cisplatin in Vitro. The first set of experiments utilized purified native DNA that had been reacted with cisplatin to give two levels of base modification. The $K$ values for the platinum adducts on these preparations were similar, with values of about 15 fmol/well (Table 1). It was discovered that, if these DNA solutions were heated (100°C, 5 min), the immunoreactivity of the DNA modified at the higher level (2.5 $\mu$mol Pt/g DNA; preparation 2) increased about 5-fold, whereas the immunoreactivity of the DNA modified at the lower level (187 $\mu$mol Pt/g DNA; preparation 1) was only slightly increased (Table 1, Fig. 2).

Single-stranded DNA. DNA was next heat denatured before being reacted with cisplatin (5.3 and 0.63 $\mu$mol Pt/g DNA; preparations 4 and 5, respectively). The immunoreactivities of the adducts on these preparations were similar to those of the heated preparation 2, which had been platinated in the native state to 2.5 $\mu$mol Pt/g DNA (Table 1). When solutions of these preparations were heated, as above, their immunoreactivities decreased slightly (Table 1).

RNA Reacted with Cisplatin in Vitro. RNA that had been reacted with cisplatin to give platination levels of 0.25 and 2.4 $\mu$mol/g caused no inhibition in the competitive ELISA, when present at the maximum concentrations tested (11 and 53 pmol Pt/assay well, respectively). RNA platinated to a level of 191
Fig. 2. Typical competitive ELISA data for DNA exposed to drug in vitro. Lines, logistic equations fitted to the data. The fitted parameters are indicated below. Concentration of competitor is expressed as mol bound platinum (□, ○, △, ▽) or mol DNA base (□, ○, △, ▽). Native DNA reacted with cisplatin (187 and 2.5 μmol Pt/g; preparations 1 and 2, respectively); △, preparation 1 (187 μmol Pt/g) assayed after heating (K = 12.8 fmol, slope = -1.3); ○, preparation 2 (2.5 μmol Pt/g) assayed without further treatment (K = 14.4 fmol/well, slope = -0.92); □, preparation 2 (2.5 μmol Pt/g) assayed after heating (K = 27, slope = -0.97); △, native DNA reacted with trans-DDP (3.6 μmol Pt/g) assayed after heating (K = 6.4 μg DNA and 22.8 pmol Pt/g/well, slope = -0.47). ○, native DNA reacted with cisplatin (7.5 μmol Pt/g) assayed after heating (K = 5.6 μg DNA and 41.3 pmol Pt/well, slope = -0.69). Control DNA (from Sigma): □, native; ○, after heating (K = 7.3 μg or 21 nmol DNA base/well, slope = -0.82). Points, mean readings from four wells; bars, SE. SE lie within symbols unless shown by bars.

Control DNA. Control DNA caused inhibition in the competitive assay but only at very high concentrations. Native DNA caused 44 ± 1% inhibition (mean ± SE) when present at 29 μg/well, the maximum concentration tested. After heating (100°C, 5 min), this DNA exhibited a K value of 7.0 ± 0.3 (mean ± SE) μg or 20 nmol/well (see Fig. 2).

DNA Reacted with Other Platinum Compounds. Native DNA was reacted in vitro with carboplatin to give preparation 3, which had a modification level of 0.95 μmol/g, similar to one of the cisplatin-modified DNA preparations (preparation 2) (2.5 μmol Pt/g). The immunoreactivities of these two preparations, per mol Pt, with and without heating, were very similar (Table 1).

Native DNA was reacted in vitro with trans-DDP or with dien-Pt to give levels of modification of 3.6 and 7.5 μmol Pt/g DNA, respectively. These preparations behaved similarly to control DNA in competitive assays. At the maximum concentrations tested, significant inhibition was observed only after these DNA preparations were heated (100°C, 5 min) (Fig. 3). The quantities of platinum adducts present when the DNA

\[ K = \frac{n}{N} \]

\[ n = \text{number of adducts} \]
\[ N = \text{number of DNA bases} \]

Emmerson et al. wrote about the effect of heating on DNA, stating that it caused a reduction in immunoreactivity of Melphalan adducts on RNA (16). It seemed of interest to investigate this in relation to the present assay, especially since sonication was to be used in the extraction of DNA from cells. Using DNA platinated at 2.5 μmol Pt/g (preparation 2), 2.7- and 2-fold reductions in immunoreactivity, respectively, were observed when the DNA was assayed with or without prior heating (Table 1).
Concentration was sufficient to cause 50% inhibition were about 23 and 41 pmol/well for trans-DDP and dien-Pt, respectively. Since these quantities had no significant effect on the assay, these adducts were recognized at least 10 times less effectively than the cisplatin or carboplatin adducts on heated DNA in preparations 2 and 3.

DNA Reacted with Cisplatin in Vivo. DNA was extracted from Walker cells that had been given a high dose of cisplatin (250 μM for 4 h). The level of DNA platination determined by AAS was 2.6 mol Pt/g. The platinum adducts on this DNA were about 10-fold less immunoreactive than the adducts on preparation 2 DNA (Figs. 2 and 3), which had been reacted with cisplatin in vitro to a similar level of modification (2.5 μmol Pt/g). After heating (100°C, 5 min), the immunoreactivity of the in vivo platinated DNA was increased about 100-fold (Fig. 3), to become similar to that of heated in vitro platinated preparation 2. All subsequent analyses of cellular DNA described here included the heating step immediately before the immunoassays. The duration of the heating was not critical, since the immunoreactivity of cellular or in vitro platinated DNA was constant when heated for between 2 and 15 min.5

In order to establish the utility of this immunoassay for biological samples, we determined the immunoreactivities of DNA preparations extracted from cells that had been treated with a wide range of doses of cisplatin. The levels of platination on the DNA samples were determined by AAS.

When DNA was extracted and concentrated using ethanol precipitation followed by drying, it sometimes proved difficult to redissolve the DNA to give a homogeneous solution that would pass through a 0.2-μm filter. Such preparations gave spurious immunoassay results, as might be expected if DNA conformation is critical for antibody recognition. Therefore, the hydroxyapatite method (see “Materials and Methods”) was developed and used for all the immunoassays described here. This hydroxyapatite method was designed for isolating small quantities of DNA; therefore, the use of AAS to determine platinum in these preparations was only possible with samples exposed to the higher cisplatin doses (at least 1 ng/5 pmol Pt was necessary for reliable AAS determination). Therefore, treated cultures were divided in two and extracted by both methods. Where the levels of platination could be determined by AAS on DNA extracted by both methods, the results were in agreement. Where the levels of platination were too low to be determined by AAS on the DNA extracted by the hydroxyapatite method, the values determined on the larger quantities of DNA prepared by ethanol precipitation was used. Full competitive ELISA curves similar to those shown in Fig. 3 were determined (in duplicate) for each DNA preparation. The mean fitted K values showed no significant dependency upon level of platination over the range studied (Fig. 4). Linear regression analysis gave a slope of −2.2 ± 1.5 × 10−4 (fmol/well)/(nmol/g), which is not significantly different from zero. The mean K value was 2.0 fmol Pt/well. In all of these assays the quantity of DNA present in the assay wells was less than 0.6 μg at 50% inhibition. This ensured that inhibition of antibody binding was caused only by the platinum adducts. Above this quantity, inhibition of antibody binding by the normal DNA sequences becomes significant. This restricted immunoassays to platination levels of ≥3 nmol Pt/g DNA (Pt/base = 10−6). It was clearly possible to detect lower levels of platination than this

Fig. 3. Typical competitive ELISA data for DNA extracted from Walker cells that had been exposed to cisplatin. Solid lines, logistic equations fitted to the data. The fitted parameters are indicated below. Concentrations of competitor is expressed as mol bound platinum (O, ■) or mol DNA base (○, □). DNA extracted from cells treated with 250 μM cisplatin for 4 h (2.6 μmol Pt/g DNA, determined by AAS): O—O, native (K = 142 fmol Pt/well or 55 ng DNA/well, slope = −1.22); ■—■, assayed after heating (K = 1.6 fmol Pt/well or 0.6 ng DNA/well, slope = −1.18). DNA extracted from Walker cells treated with cisplatin at 10 μM for 2 h, assayed after heating (K = 0.47 μg DNA/well, slope = −0.65). □—□, DNA extracted from cells not exposed to drug, assayed after heating. Broken lines, curves fitted to data for DNA exposed to cisplatin in vitro, as presented in Fig. 2. Preparation 2 DNA (2.5 μmol Pt/g): −−−−−−−−, native; ························, after heating. ————, control DNA after heating. Points, mean readings from four wells; bars, SE. SE lie within symbols unless shown by bars.

5 Unpublished observations.
The results of an experiment to determine the dose response for cisplatin adduct formation on DNA extracted from drug-treated Walker cells (Fig. 5) show the good agreement between AAS and immunological determinations. A $K$ value of 2 fmol Pt/well was used to calculate the immunoassay data.

DISCUSSION

The use of F344 rats for immunization appears to have been critical in obtaining a useful immune response against platinated DNA. It seems likely that initially high levels of antibodies that recognized normal sequences in the DNA immunogen were unfavorable for eliciting the desired antibodies and could explain our repeated failures when working with Hooded rats. This finding could be relevant to attempts to raise antibodies to other types of DNA modification.

Some of the monoclonal antibodies that we have generated exhibited extremely weak binding to plates coated with DNA that was modified to a low level with cisplatin or to denatured control DNA yet gave strong signals for DNA modified to a high level (data not presented). These could resemble the antibodies present in rabbit antisera raised against highly platinated DNA (3-5). It should prove interesting to characterize these antibodies further, compare their properties with those of ICR4, and determine whether they recognize structures of biological significance.

We observed binding of ICR4 to plates coated with denatured control DNA. Modification of DNA with platinum drugs causes conformational changes which result in the exposure of nucleosides (23); however, several observations indicate that binding of ICR4 to cisplatin-modified DNA does not simply depend upon recognition of single-stranded regions exposed as a result of such conformational changes. Firstly, in order to detect binding to assay plates coated with denatured DNA, the hybridoma supernatant must be at least $10^3$ times more concentrated than is necessary to detect platinum adducts (Fig. 1B). Presumably the quantity of single-stranded regions bound to wells coated with denatured control DNA is much greater than in wells coated with platinum-modified DNA. Despite the weak signals seen on the control DNA-control plates, there was no indication of a plateau in the assay signal at the higher antibody concentrations, unlike the situation with cisplatin-modified DNA. This indicates that antibody binding to the denatured control DNA was of very low affinity, possibly not involving the antigen recognition site. Secondly, the antibody distinguished with great specificity between platinum-modified and denatured control DNA in competitive assays. Thirdly, trans-DDP was more effective than cisplatin in causing local denaturation of DNA (23), yet trans-DDP adducts were not recognized significantly by ICR4.

The following observations are consistent with the possibility that ICR4 recognizes a conformation associated with intrastrand guanine-guanine cross-links. ICR4 recognized DNA adducts caused by trans-DDP or dien-Pt much less efficiently, if at all, compared with cisplatin adducts. After heat treatment, adducts formed by cisplatin on native DNA were recognized with similar efficiency as adducts formed on DNA that had been denatured before the drug treatment. Carboplatin adducts were recognized very similarly to cisplatin adducts, both before and after heat treatment. The bifunctional adducts formed by these two drugs are chemically identical (2). RNA adducts of cisplatin were not recognized to a significant degree, except possibly at very high levels of modification, and finally the immunoreactivity of DNA-cisplatin adducts was largely destroyed by nuclease digestion. Thus ICR4 could be, in this respect, similar to previously described antibodies and antisera raised against DNA modified with cisplatin (10, 24). Confirmation of this must await experiments using defined sequence oligonucleotides.

Our observations regarding the effects of heating on the immunoreactivity of DNA that has been modified with cisplatin or carboplatin are consistent with antibody recognition or access being increased by denaturation of the DNA. The small effect of heating on preparation 1 DNA, which had been highly modified with cisplatin (187 ± 0.95 µmol Pt/g DNA), compared to preparations 2 and 3 DNA, which had been modified to lower levels (2.5 and 0.95 µmol Pt/g DNA, cisplatin and carboplatin, respectively), could be due to rapid renaturation caused by the high frequency of interstrand cross-links in the more highly modified DNA. Conclusions similar to these, and a similar effect of heating DNA modified with cisplatin, were published by Fichtinger-Schepman et al. (11), using rabbit antisera raised against nucleosides that had been modified with cisplatin and conjugated to protein. A novel finding in the present work, however, is the very large effect of heating DNA that had been exposed to cisplatin in vivo. This could reflect the different conformational states of DNA in cells and DNA in solution at the time of the reaction with the drug (particularly since the in vitro treatment was in distilled water) or the effects of the purification procedures. An alternative explanation, which cannot be excluded at present, is that the heating promotes a delayed second arm reaction. This seems unlikely, because a 5-fold effect of heating was observed with the in vitro platinitated DNA, despite the fact that it had been incubated overnight in the absence of free drug, a step included in order to allow such second arm reactions to go to completion. In addition, similar effects of heating were observed for cisplatin and carboplatin adducts, despite a 3- to 4-fold difference in the rate of the second arm reactions of these drugs (2). It is possible that the
much lower molecular weight of purified DNA compared to DNA in cells could result in a greater proportion of cisplatin adducts being formed at or near strand ends, where the more immunoreactive DNA-adduct conformation might form spontaneously. We are at present investigating the nature of the heat-induced changes. It would be interesting to investigate the effects of biological repair processes on this property of DNA adducts.

The effects of sonication on the immunoreactivity indicate the importance of calibrating the assay using samples of known platination levels that have been prepared in the same manner as the samples that are to be analyzed. We have established that the sensitivity of the competitive ELISA for platinum adducts on DNA extracted from cells treated with cisplatin (and presumably carboplatin) is invariant over a large range of Pt/base values. Below the level of 3 nmol Pt/g DNA (i.e., Pt/base of about 10⁻¹⁶), the K values should decrease because of the additional immunoreactivity of the DNA, which must be present at sufficiently high concentrations to provide the equivalent of at least 2 fmol of Pt/well. It is easy to possible to detect and compare lower levels of platination than 3 nmol/g DNA (Fig. 3); however, accurate quantitation of the actual adduct frequencies will demand careful standardization.

The immunoassay described here does not permit the resolution of the various DNA adducts formed by cisplatin that was achieved using chromatographic analysis of nucleotides (11); however, the probable specificity of antibody ICR4 for a particular DNA conformation(s) and the marked effect of heating DNA extracted from cisplatin-treated cells that was revealed by this assay are of potential value. Furthermore, by using polymeric DNA, sample preparation is relatively simple and we have been able to determine clinically relevant levels of cisplatin-induced damage in the few micrograms of DNA that can be extracted from relatively small numbers (2 × 10⁶) of cells, both experimental and from patients. This compares very favorably with AAS determinations, where at least 100 times as many cells or 100 times as much DNA is needed for samples containing low levels of platination (2). The need for much smaller numbers of cells means that it is feasible to sort cells before analysis and to analyze a much wider range of clinical specimens. Furthermore, this antibody is proving valuable in the development of diagnostic tests for the detection of platinum adducts in DNA from tissues or body fluids. The immunoassay for platinum adducts on DNA has many potential uses in the clinical diagnosis of patients treated with platinum drugs. It is particularly valuable in the determination of the platinum drug content of whole blood, urine, exfoliated cells, and excised tumor tissues. The immunoassay for platinum adducts on DNA has considerable potential for the detection of the platinum drug content of excised tumor tissues. It is particularly valuable in the determination of the platinum drug content of whole blood, urine, exfoliated cells, and excised tumor tissues. The immunoassay for platinum adducts on DNA has considerable potential for the detection of the platinum drug content of whole blood, urine, exfoliated cells, and excised tumor tissues.

ACKNOWLEDGMENTS

We thank S. Pease and B. Stace for assistance with this work and the Johnson Matthey Research Centre for supplying the platinum compounds.

REFERENCES

Sensitive Detection of DNA Modifications induced by Cisplatin and Carboplatin \textit{in Vitro} and \textit{in Vivo} Using a Monoclonal Antibody
