Immunocytochemical Detection of Interaction Products of cis-Diamminedichloroplatinum(II) and cis-Diammine-(1,1-cyclobutanedicarboxylato)platinum(II) with DNA in Rodent Tissue Sections

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ABSTRACT

Calf thymus DNA was modified in vitro by cis-diamminedichloroplatinum(II) (cisDDP), complexed with methylated bovine serum albumin and used to immunize rabbits. The anti-cisDDP-DNA antiserum obtained was applied in a double peroxidase-antiperoxidase staining procedure to localize cisDDP-DNA and cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA)-DNA interaction products in cryostat tissue sections of mice and rats. Rats received cisDDP (0–10 mg/kg) and were killed after 24 h. Mice received cisDDP (0–15 mg/kg) or CBDCA (200 mg/kg), and were killed after 2 h–162 days. For each time-dose combination two mice or one rat were used; agents were given i.p. Specific nuclear staining was observed in all tissues examined from cisDDP- or CBDCA-treated animals. No significant nuclear staining could be observed in tissue sections from control rats and mice. The extent of staining after cisDDP was dose and time dependent. The lowest dose of cisDDP after which specific nuclear staining could be detected varied from tissue to tissue [e.g., 0.1 mg/kg, pancreas (mouse); 0.5 mg/kg, liver, kidney (mouse, rat)]. The longest time interval after a single dose of 6 mg/kg cisDDP in which adducts could be visualized also depended on the tissue and varied between 9 days (spleen, testis) and 162 days (kidney). The staining intensity in liver and kidney, measured microdensitometrically, decreased relatively fast in the first days after treatment, but much slower thereafter. In the kidney, cisDDP-induced DNA modification showed regional variation: inner cortex > outer cortex > medulla (rat) and cortex > medulla (mouse). In the mouse kidney, a small subpopulation of tubular cells in close association with the renal corpuscles showed a remarkably high staining intensity after both cisDDP and CBDCA administration. Tissues that showed clear cisDDP-induced histological alterations (kidney, pancreas, testis, and duodenum) also showed moderate to high levels of cisDDP-DNA interaction products. A correlation between cell damage (measured histologically) and cisDDP-DNA binding within one tissue type was demonstrated in the rat inner renal cortex, the murine renal cortex, and in duodenal epithelial cells of both mice and rats.

INTRODUCTION

The potent antitumor drug cisDDP is routinely used in the treatment of testicular, ovarian, and bladder cancer, while the demonstrated activity against a wide range of other malignancies, such as carcinoma of the head, neck, and stomach, or brain tumors, needs further research to establish the drug’s usefulness (1). Side effects of cisDDP administration include myelosuppression, neuro-, and gastrointestinal toxicity, while nephropathy is regarded as the dose-limiting factor in humans (1, 2) and laboratory animals (3, 4). It is believed that both the cytostatic and the cytotoxic effects of cisDDP, in vitro as well as in vivo, are mediated by its interaction products with DNA (5–9). For the detailed investigation of the relationship between DNA interaction and the in situ cytotoxicity, studies at the level of the individual cell become necessary. Until recently, however, it has not been possible to study the binding of cisDDP to DNA at the level of the individual cell. We therefore prepared a rabbit antiserum against in vitro-modified cisDDP-DNA and used this anti-cisDDP-DNA antiserum to visualize cisDDP-induced DNA modifications in tissue sections of mice and rats. We also studied the cross-reactivity between our antiserum and DNA adducts induced by CBDCA. This second generation platinum antitumor drug was chosen because of the apparent similarity between cisDDP-DNA and CBDCA-DNA interaction products (10).

This report shows the remarkable sensitivity and specificity of the immunocytochemical method, originally developed for the visualization of carcinogen-DNA adducts in tissue sections (11–13). The immunocytochemical technique provides us with the opportunity to investigate the formation and persistence of cisDDP-DNA adducts and to correlate the newly obtained data with existing data on the cytotoxic and antitumor effects of both platinum compounds.

MATERIALS AND METHODS

Animals and Treatments. Inbred male Sprague-Dawley rats (250–270 g) from the SPF breeding colony of the Netherlands Cancer Institute, received a single cisDDP dose of 0, 0.5, 2, or 10 mg/kg body weight. For each dose one rat was used. Inbred male and female C3H/He/AfO mice (20–40 g) were obtained from the same breeding colony. Mice in group 1 received 15 mg/kg cisDDP and were killed after 2 h. Mice in group 2 received 0, 0.1, 0.5, 1, 2, 4, 6, or 8 mg/kg cisDDP and were killed after 6 h. Mice in group 3 received 6 mg/kg cisDDP and were killed after 6 h, or 1, 3, 9, 27, 81, or 162 days. Mice in group 4 received 200 mg/kg CBDCA and were killed after 4 h. For each dose-time combination two mice were used (one male and one female in group 1; two males in groups 2–4). cisDDP and CBDCA were dissolved in 0.14 M NaCl to give an injection volume of 1 ml/animal. Control animals were injected with 1 ml 0.14 M NaCl and were killed after 24 h (rats), or after 2 h, 6 h, 1, 3, 9, 27, 81, and 162 days (mice). One rat received an injection of 10 mg/kg AAF, dissolved in 1.2-propanediol, another rat 150 mg/kg benzo(a)pyrene, dissolved in olive oil, and both were killed after 24 h. Animals were kept under a 14-10 h light-dark cycle and fed standard laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. All agents were given by i.p. injection. Rats were killed by cervical dislocation, rats by decapitation.

Chemicals and Immunoreagents. cisDDP for injection into animals: a solution of cisDDP in 0.14 M NaCl, adjusted to pH 2–3 (Platinol;
Bristol-Myers, Weesp, The Netherlands); for the in vitro modification of DNA cisDDP from Ventrion (Karlsruhe, FRG) was purchased. CBDCA was purchased from Bristol-Myers (each vial contained 150 mg CBDCA and 150 mg mannitol). Other reagents used were MBSA, (Serva, Heidelberg, FRG); H2O2 (Merck-Schuchardt, München, FRG); CT-DNA, ovalbumin, imidazole, DAB, and RNase A from bovine pancreas (Sigma Chemical Co., St. Louis, MO); RNase T, from Aspergillus oryzae (Boehringer, Mannheim, FRG); NGS (The Netherlands Red Blood Cross Transfusion Service, Amsterdam, The Netherlands); GAR (against whole immunoglobulin G; Campino Benelux, Elst, The Netherlands); PAP (American Qualex, La Miranda, CA). Antisera were used in the following dilutions: GAR, 1:640; PAP, 1:3200.

Preparation of Antisera. The modification of CT-DNA by cisDDP [resulting in a drug/nucleotide ratio of 6.7 × 10^(-2), determined by AAS], the complex formation of cisDDP-DNA with MBSA and the immunization of rabbits with the cisDDP-DNA-MBSA complex were carried out as described by Poirier et al. (14), with the following exception: immunization of the rabbits with the MBSA complex of 330 μg cisDDP-DNA in Freund’s complete adjuvant at weeks 1, 2, 3, and 4 was followed by two booster injections in Freund’s incomplete adjuvant at weeks 8 and 14. Sera were tested for antibody activity in a direct ELISA, essentially as described by Van der Laken et al. (15), and used without purification. Preimmune NRS was collected from the rabbit, which was used for immunization with the cisDDP-DNA-MBSA complex. The preparation of antisera against synthetic cisDDP-nucleotide adducts have been described elsewhere (16, 17). Two of these antisera were applied in the immunocytochemical assay: the W101 antiserum, raised against the synthetic adduct cisPt(NH3)2d(ApG), and the 3/65 antiserum, raised against the synthetic adduct cisPt(NH3)2d(GpG). These antisera recognize the major cisDDP-DNA interaction products, i.e., the intrastrand cross-links of cisDDP on neighboring nucleobases (GG for W101 and AG for 3/65). The use of the antisera W101 and 3/65 was restricted to liver sections of cisDDP-treated rats.

Preparation of Tissue Sections. Liver, kidney, heart, muscle (from the femur quadriceps), duodenum, spleen, testis, pancreas, brain, and thymus (the last three tissues were only collected from mice) were quickly removed, frozen on dry ice, and stored at −80°C. Cryostat sections (10 μm) were cut and mounted on ovalbumin-coated slides. In some experiments fresh tissues were fixed in 4% formalin (in 42 mM NaH2PO4 and 36.5 mM Na2HPO4, pH 7), embedded in paraffin, sectioned at 4-6 μm and stained with H&E.

Immunocytochemical Assay. The protocol of Heyting et al. (11) was used with some modifications, which are given below. The general outline of the staining procedure was as follows: cryostat sections were treated with methanol-H2O2 (to inactivate endogeneous peroxidases), RNases A and T, (to remove possible cisDDP-RNA adducts) and ethanol-NaOH (to denature the DNA and/or to increase the accessibility to antibodies). After incubation with NGS (to prevent nonspecific binding of the first antiserum (anti-cisDDP-DNA), the presence of bound antibodies was visualized by “double PAP staining,” i.e., by subsequent incubation of the sections in GAR, PAP, GAR, and PAP. The peroxidase substrate DAB was then added, together with imidazole and H2O2. The resulting product of the conversion of DAB by the peroxidase was a stable brown precipitate. Parallel cryostat sections were stained with H&E. Modifications of the method described by Heyting et al. (11) were: treatment during 10 min with 0.07 M NaOH in 30% ethanol instead of 3 min with 0.07 M NaOH in demineralized water; incubation with 10% NGS for 1 h at room temperature prior to incubation with anti-cisDDP-DNA antisem; CT-DNA (0.2 μg/μl) was added to the anti-cisDDP-DNA antisem just before incubation to diminish nonspecific nuclear staining; after each incubation with GAR and PAP, sections were washed in PBS, wash buffer and PBS (1 min each); final staining included incubation in 10 μm imidazole, 50 μM Tris-HCl (pH 7.5), 0.5 mg/ml DAB, and 0.01% H2O2 for 10 min in the dark. The anti-cisDDP-DNA antisem and the NRS were applied in the immunocytochemical assay in a dilution of 1:1800. The W101 and 3/65 antisera were used in a dilution of, respectively, 1:1600 and 1:100. In some experiments, 1:1800 diluted anti-cisDDP-DNA antisem was preincubated in wells coated with either cisDDP-modified or unmodified DNA (0.2 μg/μl) for 3 × 24 h at 4°C. Microdensitometry. Staining intensity was determined with a Knott (München, FRG) light-measuring device, coupled to a Leitz Orthoplan microscope. From 20 randomly selected cells in each investigated tissue section, the nuclear and cytoplasmic light transmission were measured (beam diameter, ± 5 μm). Specific nuclear staining was obtained by correcting the measured nuclear transmission for the background, measured in the cytoplasm of the same cells.

Histopathological Evaluation. H&E-stained cryostat and formalin-fixed sections were screened for histological changes. Nuclear enlargement, defined as the percentage of increase in mean nuclear diameter when compared with untreated animals, was measured with an ocular micrometer (amplification, ×1250). Determination of nuclear enlargement was restricted to liver, kidney [6 h (mice) or 24 h (rats) after cisDDP administration] and pancreas [6 h after cisDDP administration (mice)].

Statistics. Levels of significance were calculated using Student’s t test; P < 0.05 was considered indicative of a significant difference between groups.

RESULTS

Controls of the Immunocytochemical Procedure

Nuclear staining in tissue sections from untreated rats and mice (Fig. 1, right) or in liver sections from AAF- or benzo(a)pyrene-treated rats was very weak and could not be discerned from the very low cytoplasmic background staining. This nuclear staining did depend on the antiserum dilution. The proper dilution (1:1800) was the lowest dilution that gave no significant nuclear staining in sections from untreated animals. Significant nuclear staining was also absent from liver sections of cisDDP-treated rats when the first antiserum (against cisDDP-modified DNA) was replaced by NRS or had been preincubated in wells coated with cisDDP-DNA. Cytoplasmic staining was not influenced by pretreatment with RNases. PAP-stained sections from untreated animals showed slightly more cytoplasmic and nuclear staining after the anti-cisDDP-DNA antisem than after NRS. Addition of CT-DNA to the first antiserum was used routinely since it led to a significant depression or total elimination of nuclear staining in tissues from solvent-treated animals.

Visualization of Adducts in Rats and Mice after Different cisDDP Doses (Groups 1 and 2)

Liver. Strong, specific nuclear staining and faint cytoplasmic background staining was observed in liver sections of cisDDP-
IMMUNOCYTOCHEMICAL DETECTION OF cisDDP-DNA ADDUCTS

Fig. 3. Immunocytochemical localization of nuclei with cisDDP-induced DNA modifications in murine kidney. Nuclei with highest staining intensity were closely associated with renal corpuscles. Kidney tissue was obtained from cisDDP-treated mice (group 1, 2 h after i.p. 15 mg/kg cisDDP). Bright-field optics; bar, 25 μm.

Fig. 4. Microdensitometrically measured nuclear staining intensities in different renal regions after immunocytochemical staining for cisDDP-induced DNA modifications. M: mice, 6 h after i.p. 8 mg/kg cisDDP; rat, 24 h after i.p. 4 mg/kg cisDDP. This distribution was independent of time after injection and dose of cisDDP. Points and bars, mean ± SD (20 measurements per section) from two animals (mice) or two kidney sections from one rat.

Table 1 cisDDP-DNA adducts in mouse and rat tissues

<table>
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<tr>
<th>Tissue</th>
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<tr>
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<td>0.1</td>
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<tr>
<td>Pancreas (M)</td>
<td>Liver (M+R)</td>
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<td>Heart (M)</td>
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Fig. 2. Relationship between the microdensitometrically measured nuclear staining intensity and the cisDDP dose (mice, group 2). Liver and kidney cryostat sections were stained for cisDDP-DNA adducts according to the standard method (see "Materials and Methods"). Points and bars, mean ± SD (20 measurements).

was applied in the PAP assay (Fig. 1, left). Nuclear staining intensity, measured microdensitometrically, correlated positively with dose, both in mice (group 2) (Fig. 2) and in rats (data not shown). cisDDP-induced DNA modifications could be visualized in nuclei of parenchymal cells but not in nuclei of blood vessel cells. It is as yet uncertain if nuclei of other nonparenchymal cells show cisDDP-induced DNA modification, because nonparenchymal cells (e.g., Kupffer cells) couldn’t be discriminated unequivocally from parenchymal cells in the immunocytochemically stained sections. The distribution of stained nuclei over the different lobular regions was homogeneous. The minimal dose of cisDDP after which modifications could be visualized was 0.5 mg/kg (Table 1). After all doses of cisDDP, a weak cytoplasmic staining was observed. This cytoplasmic background became lower when the dose of cisDDP increased, probably as a consequence of an increased competition for the anti-cisDDP-DNA antiserum by the nuclear cisDDP-DNA adducts. This type of cisDDP-related, weak cytoplasmic background staining was only seen in liver sections. Dose-dependent, specific nuclear staining was also seen in liver sections of cisDDP-treated rats when the 3/65 (after ≥0.5 mg/kg cisDDP) or the W101 (after ≥2 mg/kg cisDDP) antisera were used. The nuclear staining intensity, however, was much lower when compared to that obtained with the anti-cisDDP-DNA antiserum. In addition, the W101 antiserum gave rise to a considerable (cytoplasmic) background staining after cisDDP doses above 2 mg/kg.

Kidney. After administration of ≥0.5 mg/kg cisDDP to rats or mice, DNA modifications could be visualized in tubules of both the cortex and the medulla (Fig. 3). The staining intensity related positively to the cisDDP dose (Fig. 2). Nuclear staining was observed neither in blood vessel cells nor in the glomeruli, although in some cases nuclear staining of the parietal epithelium of Bowman’s capsules was observed. In the rat kidney, the staining intensity (measured microdensitometrically) was highest in the inner cortex, intermediate in the outer cortex, and lowest in the medulla (Fig. 4). This distribution was the same regardless of cisDDP dose or length of time after exposure. In the mouse, the differences between staining intensities of the inner and the outer cortex were much smaller, but again the medulla showed a relatively low level of DNA modification (Fig. 4). A striking aspect of the staining pattern of the mouse kidney was that nuclei from tubules, adjacent to renal corpuscles, showed a very high staining intensity. It is uncertain whether these stained nuclei belong to the convulated distal tubules or to the most proximal part of proximal tubules, although some observations suggest the latter (Fig. 5). Cytoplasmic and nuclear background staining in the kidney were invariably very low.

Pancreas. Mouse pancreatic acinar cells and islet cells showed significant nuclear staining after doses as low as 0.1 mg/kg cisDDP (Fig. 6). Nuclei of intra- and interlobular duct cells remained unstained. Islet cells exhibited a relatively high cytoplasmic background, which tended to be higher after higher cisDDP doses.

Muscle. In muscle tissue cisDDP-DNA adducts could be visualized after a minimal dose of 1 mg/kg (femur quadriceps)
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Persistence of Adducts in Mouse Tissues (Group 3)

After a single dose of 6 mg/kg cisDDP, DNA lesions were seen up to 9 days (testis, spleen), 27 days (pancreas, duodenum, and muscle), 81 days (liver, brain, and heart), or 162 days (kidney). In general, nuclear staining persisted longer in tissues with a relatively high initial level of nuclear staining (kidney, liver, heart, see Table 1). Two exceptions were observed: the brain, which had a low initial staining level, and the pancreas. In the latter organ cisDDP-induced DNA modifications could be visualized already after 0.1 mg/kg cisDDP 6 h after injection (see Table 1), but no cisDDP-specific staining could be detected longer than 9 days after administration of the much higher dose of 6 mg/kg (see above). This suggests that there is no consistent relationship between adduct persistence and initial nuclear staining intensity. Fig. 8 shows the time course of nuclear staining intensities of murine livers and kidneys; measurements in the kidney were restricted to the subpopulation of heavily stained nuclei from tubules, adjacent to renal corpuscles (see above). Adduct loss in both tissues was relatively fast in the first days after drug administration, but became much slower thereafter. Original nuclear staining intensity in the liver was lower than that in the kidney. Nuclear staining intensity was highest 6 h after treatment in the kidney and 1 day after treatment in the liver. These observations probably reflect differences in kinetics of adduct formation and loss between the liver and the kidney.

Visualization of Adducts in Mice after CBDCA Administration (Group 4)

In the kidneys of CBDCA-treated mice, DNA modifications could be visualized both in the cortex and the medulla, using the anti-cisDDP-DNA antiserum. The cortex showed higher adduct levels than the medulla, but within the cortex and the medulla staining intensity was distributed rather homogeneously. Tubular cells close to renal corpuscles showed most pronounced nuclear staining (Fig. 5), as was the case in kidneys or 0.5 mg/kg cisDDP (heart). Background staining in muscle tissue was very low.

Brain. The mouse brain showed nuclear staining in both the cerebellar and cerebral cortex after doses of ≥2 mg/kg cisDDP. Nuclei from the white matter were heavier stained than those from the gray matter.

Spleen. Nuclear staining was visible in the spleen of mice and rats after ≥2 mg/kg cisDDP, both in the white and the red pulp. Nuclear staining was faint, except in some scattered unidentified cells.

Thymus. Staining of the mouse thymus was always weak and regarded nonspecific, since it was impossible to discriminate cytoplasmic from nuclear staining.

Duodenum. In duodenal cells, DNA modifications could be visualized after cisDDP doses of ≥2 mg/kg. Nuclear staining intensity in epithelial villus cells was much higher than in any other duodenal (e.g., crypt) cells. Weak cytoplasmic background staining was observed in both treated and untreated animals.

Testis. cisDDP-induced DNA modifications in testis tissue could be made visible after cisDDP doses of ≥2 mg/kg (rats, Fig. 7) or ≥4 mg/kg (mice). Nuclear staining intensity was relatively high in the interstitial cells and almost absent in cells of the germinative epithelium.

Background staining in muscle tissue was very low.
cells; unequivocal identification of other stained cell types of cisDDP-treated mice. In the liver, distribution of stained SD (20 measurements) from two mice.

**Histological Changes**

cisDDP-induced histological changes were observed in the kidney, the pancreas, the duodenum, and the testis of both mice and rats. Cell injury in the renal cortex of the rat was most pronounced in the inner cortex; this observation is in agreement with earlier reports (18-20). Tubular nuclei in the inner cortex of the rat kidney were enlarged by 35%, 24 h after a dose of 2 mg/kg cisDDP; this enlargement is statistically significant (P < 0.05). cisDDP did not induce enlargement of tubular cells of the outer cortex of the rat kidney under these conditions. The murine kidney showed statistically significant nuclear enlargement of the proximal tubules (17% at 6 h after 8 mg/kg cisDDP, P < 0.05), while nuclei from the distal tubes were not enlarged. Histological changes, like nuclear enlargement and nuclear disposition, were observed to the same extent in the inner and outer cortex of the mouse kidney. In the murine pancreas, cytoplasmic basophilia and edema were observed 6 h after ≥0.1 mg/kg cisDDP; nuclear enlargement was also observed (10% after 0.5 mg/kg, P < 0.05). Histological changes in the duodenum of mice and rats, observed after ≥0.5 mg/kg cisDDP, predominantly consisted of irregularities of the epithelial villus cells (vacuolation, edema). These changes in the duodenum, observed up to 27 days after 6 mg/kg cisDDP, were in agreement with literature reports (21). Minor changes were observed in crypt cells. In the testis of mice and rats both interstitial degeneration and a disturbance of the normal hierarchical organization in the seminiferous tubules were observed after ≥1 mg/kg (mice) or ≥2 mg/kg cisDDP (rats). Hypocellularity in the red pulp of the spleen, observed in both mice and rats already 24 h after ≥0.5 mg/kg cisDDP, was visible up to 27 days after 6 mg/kg cisDDP. Histological changes were absent from heart, muscle (femur quadriceps), thymus, brain, and liver of cisDDP-treated mice and rats.

**DISCUSSION**

This paper shows that interaction products of the antitumor drugs cisDDP and CBDCA with DNA in rodent tissues can be detected by an immunocytochemical peroxidase technique, the double PAP assay. This assay already proved to be useful for the localization of carcinogen-DNA adducts (O alkyldeoxyguanosine, deoxyguanosin-8-yl-(acetyl)aminofluorene, and deoxyguanosin-7-yl aflatoxin B1) in various rat tissues, such as liver, brain, pancreas, and oesophagus (11-13, 22, 23). The demonstrated recognition of both CBDCA- and cisDDP-induced DNA modifications by the anti-cisDDP-DNA antiserum suggests (at least partial) epitopic similarity of CBDCA and cisDDP interaction products with DNA. This suggestion is in line with recent results from Knox et al. (10), who reported that cisDDP and CBDCA induce highly similar if not identical lesions in DNA of cultured cells. The anti-cisDDP-DNA antiserum showed a very low affinity for unmodified DNA. However, nuclear staining was almost absent from tissue sections of untreated animals when CT-DNA was added to the first antisem during the peroxidase staining. Since the antiserum was raised in a similar way as that described by Poirier et al. (14, see also Ref. 24), it can be expected to show its highest affinity for the bidentate adduct of cisDDP in which two chloride ions have been replaced by two adjacent deoxyguanosines on the same DNA strand. Further characterization of the anti-cisDDP-DNA antiserum is the subject of present research.

**Heterogeneity of DNA Modification.** After a given dose of cisDDP, nuclear staining intensity varied from tissue to tissue, indicating different levels of cisDDP-induced DNA modifications. The apparent differences in cisDDP-induced DNA modification levels between different organs in the present immunocytochemical experiments parallel literature data on the total platinum content in tissues of mice (25), rats (3, 26), rabbits (27), and dogfish (28). In all these species relatively high levels of total platinum were found in kidney and liver; we observed the highest levels of cisDDP-induced DNA modification also in these organs. Heterogeneity of DNA modification within a tissue was observed in the kidney, the testis, and the duodenum. The relatively high level of DNA modification in the inner cortex of the rat (Fig. 4) is in line with AAS measurements by Choie et al. (19), who reported the highest total platinum concentration within the kidney in the corticomedullary junction. The correlation between total platinum levels and extent of DNA modification (nuclear staining intensity) is, however, not an absolute one. The spleen proved to be an exception: whereas high levels of total platinum after cisDDP treatment have been reported (3, 27), nuclear staining intensity was faint, even after doses of ≥4 mg/kg cisDDP (Table 1). The heterogeneous distribution of cisDDP-induced DNA modifications might reflect differences in blood supply or the existence of a certain blood-tissue barrier. The low modification levels in brain and testis might be explained in this way. However, even a close or continuous contact of cells with the (drug-containing) blood seems to be no guarantee for cisDDP-DNA adduct formation, in view of the low modification levels in the spleen and the apparent absence of DNA modification in endothelial cells (in all examined tissues) and renal glomerulius cells. So, other factors [e.g., drug uptake, pH, protein or Cl− content, (29, 30)] may well influence cisDDP-DNA adduct formation.

**Stability of DNA Modification.** Our results (Fig. 8) indicate a high rate of adduct loss in mouse tissues in the first days posttreatment and a very slow rate or no loss at all thereafter, indicating different levels of cisDDP-induced DNA modification.
especially in heart, brain, and liver (nuclear staining for up to 81 days after drug exposure), and kidney (nuclear staining for up to 162 days after drug exposure). Differences in adduct persistence between organs could originate from differences in cellular turnover rates and/or differences in DNA repair capacity (31). Adduct stability might differ between mice and rats, since Poirier et al. (32) reported little or no decrease in adduct levels (measured by ELISA) in total renal DNA from rats between 6 h and 13 days following a single i.v. injection of 6 mg/kg cisDDP. cisDDP-DNA adducts in cancer patients also seemed to be relatively stable: DNA modifications in white blood cells accumulated when cisDDP was given in 21- or 28-day cycles (7, 32). Fichtinger-Schepman et al. (17) observed a fast removal of cisPt(NH3)2(d(pGpG)) from white blood cells in cancer patients during a 24-h period immediately following treatment with cisDDP. Taken together, these data suggest that in human white blood cells an initial phase of rapid loss of cisDDP-DNA adducts occurs (17), but that complete adduct loss takes more than 1 month (7, 32). It is concluded that a substantial part of cisDDP-DNA interaction products is highly persistent, both in animals and humans. This persistence may have long-term toxicological or carcinogenic implications, e.g., late toxic effects or an increased risk of second cancer (3).

Sensitivity of the Immunocytochemical Assay. Determination of the lowest number of cisDDP-DNA adducts per nucleus, which can be visualized by our immunocytochemical technique, was hampered by the widely diverging literature data on the extent of cisDDP-DNA binding in experimental or clinical situations. According to Poirier et al. (32) 300 amol cisDDP-DNA adducts/µg DNA, as measured by ELISA, were present in bulk DNA of rat kidneys 6 h after i.v. injection of 6 mg/kg cisDDP. Poirier et al. (32) and Reed et al. (7) reported adduct levels of 50–200 amol/µg DNA for white blood cells from patients, 1 day after (cumulative) doses of 100–1500 mg/m2 cisDDP. In contrast, Fichtinger-Schepman et al. (9, 17, 33), who used antisera against specific adducts, reported much higher modification levels: the level of the cisDDP-DNA adduct cisPt(NH3)2(d(pGpG)) amounted to 0.5–9 fmol/µg DNA in white blood cells of patients 0–24 h after 3 h infusions of 20–100 mg cisDDP/m2 body area. cisPt(NH3)2(d(pGpG)) adducts in rats 1 h after 10 mg/kg cisDDP were as high as 10–400 fmol/µg DNA, depending on the tissue (34). The latter data were reported to be in good agreement with modification levels measured by AAS analysis in the same tissues. Until this issue is settled it is impossible to make a reliable estimation of the lowest number of adducts per genome which can be detected by our immunocytochemical assay. The detection limit using the double peroxidase technique can be as low as 4000 AAF-DNA adducts per diploid genome (12) or 1000–2000 aflatoxin B1-DNA adducts.

DNA Modification and Histological Alterations. We found that tissues, which showed clear, cisDDP-induced histological alterations (kidney, pancreas, testis, and duodenum), also showed moderate to high levels of cisDDP-DNA interaction products. In rat kidney, the highest total platinum content (as measured by AAS, 19), the highest level of cisDDP-induced nuclear staining (Fig. 4) and the most pronounced cisDDP-induced injury of predominantly proximal tubules (18–20, this report) are all localized in the inner cortex. The persistence of cisDDP-induced DNA modification in the murine kidney up to at least 162 days after drug exposure (this paper) correlates quite well with the persistence of functional kidney damage in C57/Hnu mice for up to 15 to 40 weeks after cisDDP exposure, depending on the dose (35). A similar situation is met in humans, where renal function (as judged from creatinine levels and 6Cr-EDTA clearance) continued to decrease during 12 months after initiation of cisDDP treatment (36). In the duodenum of mice and rats, the most severe cell injury and the highest levels of DNA modification were confined to epithelial villus cells. It is more difficult to relate adduct formation in pancreas (mice) or testis (mice, rats) with cytotoxicity, since histological changes in these organs were not restricted to a certain group of cells. In the liver and the heart (group 2; tissues collected 6 h after cisDDP administration), moderate or high levels of DNA modification were not accompanied by histological changes, although fine-structural studies are lacking. Morphological changes in these tissues were also absent at later times after drug exposure (group 3; tissues collected for up to 162 days after cisDDP administration). Hypocellularity in the spleen was not accompanied by high levels of DNA lesions. In this case, cell loss could also be the result of cisDDP-induced toxicity in other tissues, because the red pulp predominantly consists of recirculating lymphocytes. We conclude that the induction of morphological and/or functional tissue damage can be observed in general only in tissues with a relatively high level of initial and/or persistent cisDDP-induced DNA damage. The reverse is not always true: moderate or even high levels of DNA modification are not necessarily accompanied by histological changes.

In summary, we have been able to visualize cisDDP- and CBDA-induced DNA modifications in rodent tissue sections. The extent of modification varied not only between different tissues, but also between different cell types within one organ. We showed that a substantial part of cisDDP-DNA modifications are highly persistent. Future research will be concentrated on the role of cisDDP-DNA adducts in the radiosensitizing effect of cisDDP and the value of adduct measurement in tumors as a predictive tool in cisDDP chemotherapy. Preliminary results have already revealed a dose-dependent nuclear staining in murine solid tumors and cultured tumor cells after cisDDP exposure.

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Immunocytochemical Detection of Interaction Products of *cis*-Diamminedichloroplatinum(II) and *cis*-Diammine-(1,1-cyclobutanedicarboxylato)platinum(II) with DNA in Rodent Tissue Sections


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