cis-Diamminedichloroplatinum(II)-induced DNA Adducts in Peripheral Leukocytes from Seven Cancer Patients: Quantitative Immunochemical Detection of the Adduct Induction and Removal after a Single Dose of cis-Diamminedichloroplatinum(II)1

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ABSTRACT

The same four platinum-containing products identified in nucleolytic digests of DNA treated with cisplatin (cisDDP) in vitro now have been shown to be present in digested DNA originating from human cells after in vivo exposure. The immunochemical detection of these products at the fmol level became possible by the application of an existing and two newly raised rabbit antisera with specificities towards the various cisDDP-DNA derived products.

In DNA isolated from white blood cells of a number of cancer patients treated with the drug for the first time, intrasstrand cross-links on pGpG base sequences appeared to be the main adduct, followed by the intrasstrand cross-links on pApG sequences, interstrand cross-links, and/or intrasstrand cross-links on two guanines separated by one or more bases and a very low amount of monofunctionally bound cisDDP to guanine; typical proportions were 65, 22, 13, and <1%, respectively.

The induction and removal of the main adduct, the intrasstrand cross-link on pGpG sequences, have been studied in DNA from blood samples of six male patients after their first cisDDP treatment. The results indicate that the susceptibility of blood cells to cisDDP-DNA adduct formation can show strong individually determined differences. From the data it is also clear that a substantial part of the adducts is removed within the first 24 h after the cisDDP-infusion.

INTRODUCTION

Cisplatin, i.e., cisDDP,4 is successfully applied in the chemotherapy of several types of cancer, such as testicular (1, 2) and ovarian (3, 4) cancer. The antineoplastic activity is generally believed to result from the interaction of the drug with the DNA in the tumor cells. With isolated DNA, this interaction leads to the formation of different types of adducts through the reaction of the bifunctional platinum compound with N7 atoms of the nucleobases guanine and adenine. The major adducts are intrasstrand cross-links formed by the binding of cisDDP on two neighboring guanines (pGpG), on adenine and guanine in the sequence pApG (but not in pGpA), and on two guanines separated by one or more nucleobases [pG(pX)pG]. Other types of adducts formed are the interstrand cross-link on two guanines and monofunctionally bound cisDDP on guanine (5, 6). When the reaction proceeds inside cells, in addition to these products, cisDDP can form DNA-protein cross-links (7). It is uncertain which lesion is predominantly responsible for the antitumor effect. The DNA interstrand cross-link is often regarded to be a potential candidate for the cytotoxic action of cisDDP (7). Recently, however, Pinto and Lippard (8) provided evidence for the involvement of the intrasstrand cross-link on pGpG as an inhibitor of DNA replication. It is generally assumed that a cytotoxic DNA-adduct has to be a fairly persistent one, but few data are available on the persistency of cisDDP-DNA adducts in (tumor) cells.

Investigations into the induction and repair of the various cisDDP-DNA adducts in tumor cells of cisDDP-treated patients may give more precise information on the relevance of DNA-adducts in the antitumor action of cisDDP, and may result in identification of the possible active antitumor adducts. Because of the large variation in response of different tumors to chemotherapy with cisDDP or other platinum compounds, such studies should not be restricted to one type of tumor. A further aspect is the observation that in the treatment of apparently identical tumors pronounced interindividual differences can be encountered in the patient's response to the drug. A possible explanation could be that platinum uptake, adduct formation, and, particularly, adduct repair show large individual variability. In this situation, the individual characteristics of induction and repair of the platinum lesions are not necessarily restricted to the tumor cell; they might be a specific property of all cells of the individual. Because a comparative study on biopsies of tumors and of normal tissues from cisDDP-treated patients at various intervals after the drug's administration is not feasible, a different approach has to be followed to investigate this possibility. The most readily obtainable nucleated cells of these patients are their WBC, which can be analyzed for the presence of cisDDP-DNA adducts. If a correlation could be established between the induction and/or removal (repair) of the adducts in these cells on the one hand and the clinical efficacy of the therapy on the other, analysis of cisDDP-DNA adducts in WBC might become of great practical value; then, results obtained with blood samples taken after the first treatment could be predictive for the therapeutic effect and could be used to help to decide on continuation of the chemotherapy. Evidence favoring the existence of such a correlation has recently been reported by Poirier et al. (9) and Reed et al. (10, 11).

Recently, we have started to analyze DNA from WBC collected from patients at various times after the beginning of the cisDDP-containing combination chemotherapy. In these studies we applied newly developed immunochemical methods for the quantitative detection of the individual DNA adducts. Preliminary results indicated that the amount of cisDDP adducts is strongly influenced by the interval between the onset of the cisDDP treatment and the moment of sampling (12). In this report the results are presented of a study on the induction and

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4 The abbreviations used are: cisDDP, cis-diamminedichloroplatinum(II); cisDDP-DNA adducts, cis-diamminedichloroplatinum(II)-containing deoxy- (oligo)-nucleotides; ELISA, enzyme-linked immunosorbent assay; ID50, amount of inhibitor giving 50% inhibition in the competitive enzyme-linked immunosorbent assay; IA50, amount of inhibitor giving 50% inhibition in the competitive enzyme-linked immunosorbent assay.
removal of cisDDP adducts in WBC of patients during the first 24 h after the initial cisDDP-treatment.

MATERIALS AND METHODS

Collection of Blood Samples. Blood samples were obtained from seven patients who received their first chemotherapeutic treatment with cisDDP. For patients A, B, C, and D, the treatment consisted of an infusion of 20 mg cisDDP/m², preceded by an injection with either etoposide (VP16-213) or vinblastin (patient D). In addition, patient A received the antibiotic bleomycin. Patients E and F were treated with vinblastin, the antimetabolite methotrexate, and 100 mg cisDDP/m². Patient G received cisDDP as single drug in a dose of 30 mg cisDDP/m². All patients received cisDDP by infusion over a 3-h period. Blood was taken by venipuncture shortly before the administration and at regular intervals after the onset of the infusion. Two 10-ml portions were collected in plastic tubes, each containing heparin sodium (TromboliquinR; 0.1 ml/tube), which were stored at -70°C until further handling.

DNA Isolation from WBC. To obtain the WBC, 3 volumes of cold 0.9% NH₄Cl solution were added to the thawed blood samples to lyse preferentially the erythrocytes. After about 5 min at 0°C, the WBC were collected by centrifugation for 15 min at 215 × g. The pellets were washed once with 0.9% NaCl solution and resuspended in 2.5 ml 10 mM Tris-HCl/1 mM EDTA, pH 7.8, to which NH₄HCO₃ was added just before use (final concentration, 0.1 M), for the inactivation of residual proteolytic activity of this rather heat-stable enzyme, the digestion mixture was incubated for 2 h at 37°C. The solutions were extracted for 15 min at room temperature with an equal volume of phenol (saturated with 10 mM Tris-HCl/1 mM EDTA/0.1 M NaCl, pH 7.8), and DNA was precipitated from the aqueous phase with 2 volumes of cold (−20°C) absolute ethanol after prior addition of 0.1 volume of 3 M sodium acetate/1 mM EDTA, pH 5.5. The DNA was collected on glass rods, rinsed in 80% ethanol, and dissolved in 0.5 to 1.5 ml of the Tris/EDTA buffer (volume depending on the yield of DNA). Subsequently, coprecipitated RNA was digested in a 2-h incubation at 37°C with RNase A (Sigma; 75 μg/ml, heated at 80°C for 5 min to destroy any RNase activity) and RNase T1 (Boehringer; 75 units/ml), followed by extraction with an equal volume of chloroform/isooamyl alcohol (24/1) and alcohol precipitation (see above). After dissolution in 0.5 to 1.5 ml of the Tris/EDTA buffer, the DNAs were precipitated once more with alcohol.

Enzymatic Digestion of DNA. The DNA samples were digested at a concentration of, at most, 1 μg/ml with the enzymes deoxyribonuclease I (EC 3.1.4.1) and DNA nucleases P1 (EC 3.1.4.1; Boehringer) as described before (6), except that NaCl was omitted. After the overnight digestion, the amount of digested DNA in the samples were determined from the absorbance at 260 nm (5). The samples were incubated for 2 h at 37°C with proteinase K (0.5 to 2 mg/ml, depending on the batch used), followed by heat inactivation of the enzyme (5 min at 100°C). Before chromatography, the samples were adjusted to pH 8.8 with 1 M Tris and centrifuged for 10 min in an Eppendorf centrifuge.

Anion Exchange Chromatography. Separation of the digestion products of cisDDP-DNA adducts, DNA was isolated from WBC of treated patients and was enzymatically digested; after chromatographic fractionation of the digest, the adduct-derived products were assayed with the use of specific antisera. The rabbit antiserum W101 was used for the detection of the product cisPt(NH₃)₂d(GpGp)₃, derived from intranstrand cross-links on pGpG sequences, and serum 3/43 for the determination of Pt(NH₃)₂d(GpGp) from intrastrand cross-links on pGpG sequences, and serum 3/65 was used for the detection of Pt(NH₃)₂dGMP. Antiserum 3/65 was elicited against the hapten cisPt(NH₃)₂d(GpGp) (14), synthesized in the group of Prof. Dr. J. Reedijk, State University, Leiden, The Netherlands) coupled to bovine serum albumin (ICN Pharmaceuticals). The immunoglobulin preparation resulting after (NH₄)₂SO₄ precipitation was used at a 1:100,000 dilution for the measurement of the intrastrand adduct on pApG sequences.

RESULTS

For the quantitative determination of the different cisDDP-DNA adducts, DNA was isolated from WBC of treated patients and was enzymatically digested; after chromatographic fractionation of the digest, the adduct-derived products were assayed with the use of specific antisera. The rabbit antiserum W101 was used for the detection of the product cisPt(NH₃)₂d(pGpG), derived from intranstrand cross-links on pGpG sequences in DNA and for the determination of cisPt(NH₃)₂d(GMP)₂, originating from intranstrand cross-links on pG(pG)x,pG and from interstrand cross-links between two pGs (14). Two newly raised rabbit antisera were used for the detection of the other digestion products: serum 3/65 for the assay of cisPt(NH₃)₂d(pApG) from intranstrand cross-links on pApG sequences, and serum 3/43 for the determination of Pt(NH₃)₂dGMP, which results from cisDDP monofunctionally bound to DNA. For the immunochemical quantitation of these products in the appropriate fractions obtained after chromatography, standardized versions of the "competitive ELISA" were used.

A survey of the specificity and the sensitivity of the assays is given in Table 1, which shows for each of the three antisera the amount of the various products that is needed to reduce the response of the assay to 50% (IA₅₀). For the correct combination of antiserum and product, the IA₅₀ lies between 4 and 9 fmol, whereas for the unmodified nucleotide 10⁶ to 10⁸ times higher values are found. The detection limit of the adducts is about 1 to 2 fmol, obtained at 20% inhibition. With our method of separating the DNA digestion products, adducts in DNA can be assayed down to about 0.02 fmol/μg DNA, depending on the amount of DNA analyzed.
In the assay fixed amounts of the relevant antiserum were incubated with various amounts of "inhibitor"; subsequently the fraction of antibodies remaining was determined by ELISA. By interpolation, the IC50 was determined.

The amounts of the cisDDP adducts were calculated from the ID50 values of the fractions and the corresponding IC50 values found for the authentic reference compounds, determined in the same experiment. The frequency of the occurrence of the four adducts in the blood sample of patient A is given by the amount of adduct per μg of DNA, as is listed in Table 2; the data are also expressed as percentages of total adducts determined. It shows that the intrastrand cross-link on pGpG [resulting in the product cisPt(NH3)2d(pGpG)] is the main adduct (65% of the total of the four adducts). The next abundant adduct is cisPt(NH3)2d(pApG) (22%) followed by cisPt(NH3)2d(GMP) (13%) and the minor adduct Pt(NH3)3dGMP (<1%). As expected, in the t = 0 sample none of the cisDDP-DNA adducts could be detected.

We have been able to establish the presence of all four cisDDP adducts in blood samples of various cisDDP-treated patients, and in all cases cisPt(NH3)2d(pGpG) was by far the most abundant adduct. In general, the relative abundance of the four adducts followed the pattern found for patient A, although the exact percentages varied somewhat; moreover, in the WBC of some patients the adduct levels were rather low so that accurate determination was possible only for the major product. For this reason, in a comparative study with a number of patients on the induction and removal of cisDDP-DNA adducts in WBC of patients receiving their first cisDDP dosage, the quantitative assays were restricted to the main adduct. Fig 2 presents an overview of the amounts of cisPt(NH3)2d(pGpG) per μg of DNA that was isolated from blood samples of six male patients; the samples were collected at various intervals during the first 24 h after initiation of the treatment. The data show the absence of any adduct before treatment in all patients and a substantial number of adducts immediately after the 3-h infusion period, whereas the removal of the adducts appears to set in soon.

**DISCUSSION**

The experiments described in this paper show for the first time that all four cisDDP-DNA adducts that were identified in in vitro-treated salmon sperm DNA and in DNA from cells exposed in vitro (12, 16) are also formed in human cells during in vivo exposure to cisDDP. This result was possible due to our new antisera against two of the adducts, which when combined with our earlier antisera allowed detection of all four adducts (Table 1). The proportion in which the four adducts are formed in vivo is very similar to the ratio observed after in vitro exposures; a strong preference is seen for the formation of the platinum compound was from the results obtained with the eluate fractions that were related to the corresponding values of the appropriate reference compound as measured on the same ELISA plate.

Table 1  IC50 values in the competitive ELISA

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Serum W101</th>
<th>Serum 3/43</th>
<th>Serum 3/65</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisPt(NH3)2d(pGpG)</td>
<td>4.0 ± 1.3</td>
<td>(1.3 ± 0.9)×10^2</td>
<td>(0.4 ± 0.2)×10^2</td>
</tr>
<tr>
<td>cisPt(NH3)2d(GMP)</td>
<td>9.2 ± 1.6</td>
<td>31.2 ± 7.8</td>
<td>(9.5 ± 1.4)×10^2</td>
</tr>
<tr>
<td>cisPt(NH3)2d(pApG)</td>
<td>(3.2 ± 1.8)×10^3</td>
<td>(0.7 ± 0.3)×10^3</td>
<td>6.5 ± 4.2</td>
</tr>
<tr>
<td>Pt(NH3)3dGMP</td>
<td>(2.4 ± 1.6)×10^4</td>
<td>4.4 ± 1.6</td>
<td>(0.3 ± 0.1)×10^4</td>
</tr>
<tr>
<td>dGMP</td>
<td>(5.8 ± 1.1)×10^5</td>
<td>(3.7 ± 1.1)×10^5</td>
<td>&gt;0.5 × 10^5</td>
</tr>
</tbody>
</table>

* Serum raised against cisPt(NH3)2d(pGpG).
* Serum raised against Pt(NH3)3dGMP.
* Serum raised against cisPt(NH3)2d(pApG).
* Mean ± SD of at least four different experiments.

Table 2  Analysis of DNA from WBC collected immediately after the cisDDP treatment of patient A

<table>
<thead>
<tr>
<th>cisDDP adduct</th>
<th>Amount of compound (fmol/μg DNA)*</th>
<th>% of total adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisPt(NH3)2d(pGpG)</td>
<td>5.6 ± 0.5</td>
<td>65</td>
</tr>
<tr>
<td>cisPt(NH3)2d(GMP)</td>
<td>1.1 ± 0.1</td>
<td>13</td>
</tr>
<tr>
<td>cisPt(NH3)2d(pApG)</td>
<td>1.9 ± 0.5</td>
<td>22</td>
</tr>
<tr>
<td>Pt(NH3)3dGMP</td>
<td>0.06 ± 0.04</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* The amount of DNA was based on the absorbance at 260 nm; the amount of the platinum compound was from the results obtained with the eluate fractions in the competitive ELISA (IC50, which were determined at least twice in different experiments) that were related to the corresponding values of the appropriate reference compound as measured on the same ELISA plate.
* Mean ± range.
Table 2. Patients B, C, and D formed about 15 times less cisDDP-pGpG adducts in WBC, as is illustrated by Fig. 2 and determined as cisPt(NH₃)₂d(pGpG) after nucleolytic degradation of the DNAs. The amount of adducts formed in WBC after in vivo administration (correlation coefficient, 0.91) has been established between the two patients. The regression, while intermediate adduct levels corresponded with an intermediate response (11). In addition, the analyses of WBC collected on several days during a cisDDP treatment and during several cycles indicated accumulation of cisDDP-DNA adducts as a function of the dose; Poirier et al. (9) and Reed et al. (10, 11) concluded that adduct removal is a rather slow process, taking 1 month at least.

In contrast, our results (Fig. 2) demonstrated that the adducts induced during the first treatment are removed rapidly and therefore no simple accumulation of cisDDP-DNA adducts is occurring. However, our data also indicate an increase in adduct formation at repeated cisDDP infusions, which suggests a sensitization for cisDDP adduct formation by previous cisDDP treatments.

Fig. 2. Induction and removal of intrastrand cross-links of cisDDP with two neighboring guanines in DNAs isolated from WBC of six patients who received their first treatment with cisDDP (see text for dosages). The cross-links were determined as cisPt(NH₃)₂d(pGpG) after nucleolytic degradation of the DNAs. Bars, range of the data obtained from two different competitive ELISAs, each performed in four dilutions in duplicate wells. At the start of the 3-h cisDDP-infusion period no adducts could be detected in the samples (Y). O, patient B; □, patient C; A, patient D; ●, patient E; •, patient F; ×, patient G.

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The differences in the ability of individual patients to form adducts in their WBC is remarkable. It cannot be attributed to differences in treatment conditions, nor to the combination with other drugs, and could be concluded from another investigation (17). In that study on 12 patients, a good correlation (correlation coefficient, 0.91) has been established between the amount of adducts formed in WBC after in vivo administration of cisDDP in combination with various additional drugs and the induction of adducts during in vitro exposure to cisDDP as the single agent of whole blood obtained before the infusion given to these patients.

An important question is whether or not the amount of the cisDDP adducts formed in WBC after the first infusion has predictive value for the efficacy of the chemotherapy. The limited number of patients we screened does not permit an answer yet. Data suggesting a positive correlation between the amounts of cisDDP adducts induced in WBC and the tumor response have already been reported by Poirier et al. (9) and Reed et al. (10, 11). They determined the presence of adducts in DNA from WBC of patients by means of antibodies elicited against cisDDP-treated DNA. The blood samples were taken 12 to 18 h after the first as well as after subsequent cisDDP infusions. Repair between subsequent infusions or between treatment cycles was not investigated, however. The quantity of the adducts ranged from 0 fmol/µg DNA in samples of patients with a poor response to >0.2 fmol in at least one sample of the patients with a high rate of complete tumor regression, while intermediate adduct levels corresponded with an intermediate response (11). In addition, the analyses of WBC collected on several days during a cisDDP treatment and during several cycles indicated accumulation of cisDDP-DNA adducts as a function of the dose; Poirier et al. (9) and Reed et al. (10, 11) concluded that adduct removal is a rather slow process, taking 1 month at least.

In contrast, our results (Fig. 2) demonstrated that the adducts induced during the first treatment are removed rapidly and therefore no simple accumulation of cisDDP-DNA adducts is occurring. However, our data also indicate an increase in adduct formation at repeated cisDDP infusions, which suggests a sensitization for cisDDP adduct formation by previous cisDDP treatments.

The variation in the extent of adduct formation upon exposure in vivo or in vitro of blood cells from different individuals to cisDDP (9, 10, 11, 17, and this paper) is of importance for the interindividual differences in the response to chemotherapy with cisDDP. Genotoxic cytostatic drugs can pose a carcinogenic risk for patients treated with these compounds, as has been demonstrated for cyclophosphamide (18) and suggested for cisDDP (19, 20). Therefore, the findings described here for cisDDP have a more general bearing; since cisDDP does not need extensive bioactivation to react with DNA, the findings may be taken to indicate that the mutagenic/carcinogenic risk involved in exposure of humans to genotoxic agents could be individually determined already with regard to the first step, the formation of DNA adducts, also in case of exposure to direct-acting agents.

Further investigations on the formation and disappearance of the cisDDP-DNA adducts in WBC and other cell types are ongoing to gain insight into the mode of action of cisDDP as an antitumor agent and possibly as an inducer of secondary tumors; it is hoped that these and related studies will lead to a better scientific basis for the selection of chemotherapeutic drugs and for treatment regimens.

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8 Fichtinger-Schepman, unpublished data.

9 Fichtinger-Schepman, unpublished results.
QUANTITATION OF cisDDP-DNA ADDUCTS IN HUMAN WBC

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cis-Diamminedichloroplatinum(II)-induced DNA Adducts in Peripheral Leukocytes from Seven Cancer Patients: Quantitative Immunochemical Detection of the Adduct Induction and Removal after a Single Dose of cis-Diamminedichloroplatinum(II)
