Inductively Coupled Plasma Mass Spectroscopy Quantitation of Platinum-DNA Adducts in Peripheral Blood Leukocytes of Patients Receiving Cisplatin- or Carboplatin-based Chemotherapy

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ABSTRACT
Platinum-DNA adducts can be assayed in peripheral blood leukocytes by means of atomic absorption spectroscopy and ELISA, and high adduct levels have been correlated previously with favorable clinical response to platinum-based chemotherapy. Our purpose was to study adduct formation in peripheral blood leukocytes by means of a new method, inductively coupled plasma mass spectroscopy (ICP-MS), and to correlate adduct formation with clinical response and toxicity.

Platinum (Pt)-DNA adducts were measured by means of ICP-MS in leukocytes of 66 patients receiving a cisplatin- or carboplatin-based chemotherapy, collected either before the beginning of treatment and incubated in vitro with cisplatin or 1 and 24 h after the administration of drug to the patient.

The Pt-DNA adduct level in leukocytes from patients exposed to drug in vitro was 14.33 ± 14.71 fmol/μg DNA (mean ± SD), which was not significantly different from the value of 23.4 ± 19.53 fmol/μg DNA observed in leukocytes from nine healthy volunteers. In samples collected after the administration of chemotherapy, Pt-DNA adducts ranged from 1.91 ± 3.59 fmol/μg DNA (mean ± SD) at the 1-h time point to 2.61 ± 3.35 fmol/μg DNA at 24 h (P > 0.05). Adduct levels in leukocytes exposed in vitro did not correlate with adduct levels from patients treated with cisplatin-based chemotherapy (r = 0.085 and 0.011 at 1 and 24 h, respectively). At 24 h, adduct levels in patients receiving cisplatin (3.15 ± 3.64 fmol/μg DNA, mean ± SD) were significantly higher (P = 0.02) than those observed in patients treated with standard dose carboplatin (0.57 ± 0.73 fmol/μg DNA) and also higher than those in patients receiving high-dose carboplatin (1.18 ± 1.06 fmol/μg DNA), although the latter difference did not reach statistical significance (P = 0.071). No differences in adduct levels (mean ± SD) were evident between patients responsive (3.23 ± 3.51 fmol/μg DNA) and nonresponsive (2.34 ± 0.31 fmol/μg DNA) to chemotherapy.

INTRODUCTION
Cisplatin and the analogue carboplatin are antineoplastic agents with demonstrated activity in different types of cancer, particularly testicular, ovarian, lung, head and neck, and bladder cancers (1). However, for the majority of these cancers, with the notable exception of germ cell tumors, the proportion of responding patients is usually small; therefore, the identification of a marker that would predict the response to chemotherapy could be useful.

After intracellular acquisition, cisplatin and carboplatin form intra- and interstrand adducts in DNA (2). Pt-DNA adduct levels in normal tissues and in tumors can be determined by means of immunochemical methods (3-5) or by AAS (6).

Previous reports link the Pt-DNA adduct levels in peripheral blood leukocytes collected from patients receiving cisplatin- or carboplatin-based chemotherapy with the clinical activity of the two drugs, and the suggestion has been made that the level of Pt-DNA adduct formation in leukocytes could be a marker of chemosensitivity (3, 7-10). Furthermore, a correlation has been reported between adduct formation in leukocytes collected from treated patients and adduct formation in vitro cisplatin-treated leukocytes, thus suggesting the possibility that the rate of adduct formation in vitro could help in predicting the response to chemotherapy (11, 12). A similar conclusion was reached when

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3 The abbreviations used are: Pt, platinum; AAS, atomic absorption spectroscopy; ICP-MS, inductively coupled plasma mass spectroscopy; 5FU, 5-fluorouracil; DI, dose intensity; AUC, area under the curve of concentrations over time; CI, confidence interval.

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the DNA damage in leukocytes was determined by the PCR-stop assay (13).

In the present study, Pt-DNA adducts were measured in leukocytes from treated patients and in leukocytes collected before the administration of drugs, and incubated in vitro with cisplatin to confirm in a large cohort of patients the hypothesis that there is a correlation between leukocyte adduct formation in vivo and in vitro and between leukocyte adduct formation and response and toxicity.

A new method for the assay of Pt-DNA adducts was used: ICP-MS. ICP-MS is a powerful analytical technique for the determination of trace and ultra-trace amounts of elements in biological samples (14). The instrumentation is comprised of an inductively coupled plasma (normally an argon plasma) used as an ion source and a quadrupole mass spectrometer for ion detection and quantification. Because the inductively coupled plasma works at atmospheric pressure and the mass spectrometer requires a vacuum better than 10⁻⁵ millibars, an interface is placed between the two components. The ICP-MS method of measurement has several advantages for trace element determination: simultaneous multielement determinations; excellent detection limits, in particular for elements such as platinum; wide linear dynamic range; and high sample throughput.

MATERIALS AND METHODS

Patient Selection and Treatment Schedule. Forty-nine men and seventeen women ranging in age from 22 to 72 years (median age, 54) gave informed consent to participate in this study between January 1, 1994, and December 31, 1995. Patient characteristics are listed in Table 1. Clinical staging was based on a complete history, physical examination, a routine biochemical profile, a complete blood cell count, and the results of imaging procedures for all patients before the beginning and after three cycles of chemotherapy. Response and toxicity were evaluated according to standard WHO criteria (15).

Entry criteria included a pathologically confirmed diagnosis of cancer; serum creatinine of <134 μmol, blood urea nitrogen <14 μmol, leukocyte count >3,000 cells/μL, platelet count >100,000/μL, hemoglobin >9 g/dL, and adequate hepatic function (total bilirubin <26 μmol; transaminases less than two times upper limit of normal); no evidence of cardiomegaly on chest X-ray; clinically normal hearing: life expectancy >3 months; and an Eastern Cooperative Oncology Group performance status ≤2. If patients had received cisplatin previously, they must never have suffered cisplatin-induced nephrotoxicity (peak creatinine >195 μmol) or disabling neurotoxicity.

Cisplatin was administered at the dose of 100 mg/m² as a single bolus (1-h infusion in 3% sodium chloride with pre- and posthydration) in combination with: 5FU 1000 mg/m²/day as a single dose in 5% dextrose in water over 1 h, either alone or with cyclophosphamide 600 mg/m² (ovarian cancer patients), or in combination with doxorubicin 50 mg/m² and etoposide 100 mg/m² on days 1–3 (cancer of unknown primary). High-dose carboplatin (300 mg/m² in 5% dextrose in water over 1 h on days 1–4) was given in combination with ifosfamide 12 g/m² (with mesna as auroprotector) and etoposide 1200 mg/m² over 4 consecutive days.

Planned and received DI were calculated by the method of Hryniuk and Bush (16); received DI was expressed as percentage of the planned DI. The effectively received carboplatin AUC was determined by dividing the administered carboplatin dose by the carboplatin clearance, calculated according to Chatelut’s (17) formula: 0.134 × weight × [218 × height × (1 – 0.00457 × age)]/creatinine expressed in micromolar concentration (with weight in kilograms, age in years, and sex = 0 if male and sex = 1 if female).

The analysis of toxicity and of received DI was performed for all the cisplatin-based cycles but will be reported here only for the patients treated with the cisplatin-5FU combination.

Blood Sample Collection. Thirty ml of blood were collected by venipuncture in syringes containing heparin sodium before the beginning of treatment and at 1 and 24 h after the end of infusion of cisplatin (or carboplatin), during the first cycle of chemotherapy. Immediately after the collection, blood was layered in three 50-ml tubes containing 10 ml of hystopaque-1119 and 10 ml of hystopaque-1077 (10 ml of blood in each tube), and then centrifuged at 700 × g for 30 min. Leukocytes were collected and washed twice in PBS solution (pH 7.4); the supernatant was removed, and the pellet was frozen at −20°C.
The lowest detection limit was 0.1 μg/liter for ICP-MS and 10 μg/liter for AAS.

Fig. 1 Standard curves for platinum determination by ICP-MS and by AAS. The lowest detection limit was 0.1 μg/liter for ICP-MS and 10 μg/liter for AAS.

Fig. 2 Pt-DNA adducts in leukocytes from patients (n = 66) treated with platinum-based chemotherapy. Dotted line represents the lowest limit of detection. Each data point ● represents a separate leukocyte sample (samples at 1 h = 41; samples at 24 h = 25).

In Vitro Cisplatin Treatment of Blood Samples. To study the \textit{in vitro} formation of Pt-DNA adducts, leukocytes collected from 45 patients before the beginning of chemotherapy were incubated \textit{in vitro} at 37°C for 2 h in RPMI 1640 containing cisplatin at a concentration of 15 μg/ml. After the incubation period, the cells were washed, and DNA was isolated, then treated following the steps described above. Pt-DNA adduct formation \textit{in vitro} was also evaluated in leukocytes collected from nine healthy volunteers.

**Determination of Pt-DNA Adducts.** DNA samples were analyzed for their Pt content by ICP-MS using an Elan 5000 Perkin-Elmer Sciex. Total Pt was determined by a quantitative method after dilution of the DNA samples in 2 ml of a 0.1% Triton solution in H2O (Sigma). Standard curves were generated by using aqueous serial dilutions of a stock of H2PtCl6H2O in 10% hydrochloric acid (SPEX Industries, Inc., Edison, NJ). Measurements were made as the integrated value of ion number in an analysis time of 3 min. Pt-DNA adduct levels were calculated as femtomoles of elemental Pt per microgram of cellular DNA (fmol Pt/μg DNA). The lowest limit of detection of the assay was 10 pg of elemental Pt; the coefficients of variation ranged from 1 to 3% (intraassay) and from 5 to 10% (interassay). Fig. 1 compares standard curves for ICP-MS and atomic absorption spectroscopy.

**Statistical Analysis.** Unless stated otherwise, the significance of the differences between experimental data was tested with Student's \( t \) test.

**RESULTS**

Two hundred-five cycles of chemotherapy were administered over the course of this study. Fifty patients received 156 cisplatin-based cycles; 10 patients received 40 carboplatin-based cycles, given at the standard target AUC of 3–6 mg-min/ml; the remaining six patients received nine high-dose regimens containing carboplatin given at the target AUC of 12–18 mg-min/ml.

Fig. 2 shows the individual data for Pt-DNA adduct levels determined in 101 samples (77% of the total number of planned samples): 60 collected at the 24-h time point and 41 collected at the 1-h time point after the first cycle of chemotherapy. Pt-DNA adducts were undetectable in 11 samples of the earlier time point (27%) and in 13 samples of the second time point (22%), giving a 24% overall incidence of samples below the detection limit of the method. The levels were undetectable at both time points in three patients treated with carboplatin and in three treated with cisplatin.

Leukocyte Pt-DNA adduct levels ranged from 1.91 ± 3.59 fmol/μg DNA (mean ± SD; 95% CI = 0.81–3.01) at the 1-h after chemotherapy time point to 2.61 ± 3.35 fmol/μg DNA (mean ± SD; 95% CI = 1.76–3.46) in samples collected at 24 h after chemotherapy (\( P > 0.05 \)). With both drugs, plasma levels were higher, although not statistically different, at 24 h.

Fig. 3 depicts individual Pt-DNA adduct levels in leukocytes treated \textit{in vitro} with cisplatin. No significant differences...
Quantitation of Platinum-DNA Adducts

Fig. 3 Pt-DNA adducts in leukocytes after in vitro incubation with cisplatin (15 μg/ml). Each data point ● represents a separate leukocyte sample (patients = 45; healthy volunteers = 9). ———— median.

Table 2 Association between adduct levels at 24 h and response to chemotherapy

<table>
<thead>
<tr>
<th>fmol Pt/μg DNA (mean ± SD)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.23 ± 3.51</td>
<td>Complete and partial (n = 20)</td>
</tr>
<tr>
<td>2.34 ± 3.01</td>
<td>Stabilization and progression (n = 28)</td>
</tr>
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CI = 2.1–4.2) was significantly higher (P = 0.02) than that observed in patients treated with standard dose carboplatin (mean ± SD = 0.57 ± 0.73 fmol/μg DNA; 95% CI = 0.22–1.24), and was also higher than the adduct levels in the high-dose carboplatin group (mean ± SD = 1.18 ± 1.06 fmol/μg DNA; 95% CI = 0.21–1.91), although this latter difference did not reach statistical significance (P = 0.071). Furthermore, the increase in Pt-DNA adduct levels in the patients receiving high-dose carboplatin was not proportional to the increased AUC.

The association between response to chemotherapy and adduct levels at 24 h was investigated in 48 patients. From the analysis were excluded the six patients treated with high-dose carboplatin, six with germ cell tumors, and six patients who were ineligible because the treatment was administered in an adjuvant setting (n = 2) or because of early loss to follow-up (n = 4). Overall, 20 responses were observed (42%; complete response, 5; partial response, 15). Although responsive patients showed a higher adduct level than patients showing disease stabilization or progression, the difference was not significant (Table 2).

Finally, the toxicity of treatment (hemoglobin decrease, peak creatinine) and the received DI were evaluated for the homogeneous group of patients treated with the combination cisplatin-5FU, the majority of whom (25 of 28; administered cycles, 76) had evaluable adduct levels at 24 h. In this subset of patients, the median adduct level at 24 h (1.97 fmol/μg DNA) was chosen as a cutoff to separate two groups with low and high adduct formation. As shown in Fig. 5, no differences were observed between the two groups in terms of received DI, posttreatment creatinine, and hemoglobin decrease.

DISCUSSION

In this study, the quantitation of Pt-DNA adducts in peripheral blood leukocytes of patients receiving a cisplatin or carboplatin-based chemotherapy was performed by ICP-MS. This method gave a good reproducibility and was about 100 times more sensitive than standard AAS in detecting Pt-DNA adducts (Fig. 1). This is in agreement with two published papers focusing on the use of ICP-MS for the determination of Pt in biological samples such as blood (18), urine, and tissue specimens (19). The sensitivity of the ICP-MS allowed the detection of adducts in 76% of the samples. The percentage of samples below the detection limits of the different methods is rarely reported in the literature; we found this important information only in a paper by Reed et al. (3), who used a very sensitive ELISA method and found detectable Pt-DNA adduct levels in 37% only of the samples.

In previous studies, Pt-DNA adducts in peripheral blood leukocytes have been determined by ELISA (3, 5, 7), AAS...
Mean adduct values determined by AAS are usually in the range 5–500 fmol/μg DNA, about one to two orders of magnitude higher than in the present study. However, because the patients studied differ in terms of several variables, such as the type of administered chemotherapy, the doses of cisplatin, and the procedures followed for DNA isolation, it is impossible to make a direct comparison with the already published adduct values. Particularly, it has been demonstrated that the isolation of leukocytes done immediately after the blood collection (procedure followed in this study) leads to the determination of significantly lower adduct levels than the isolation of leukocytes done after thawing frozen whole-blood samples (21).

It is well known that the kinetic of reaction of carboplatin with DNA is slower than that of cisplatin (2, 24), and this observation can explain the lower level of adducts observed at 24 h in patients who received carboplatin compared with cisplatin-treated patients. In carboplatin-treated patients, the difference in adduct levels did not parallel the difference in the target AUC. Although this observation needs to be confirmed in a larger group of patients, it might suggest the utility of studying the formation of Pt-DNA adducts in the high-dose setting to help adjust the dose based on drug-DNA interactions rather than on plasma AUC.

A correlation between Pt-DNA adduct formation in leukocytes incubated in vitro with cisplatin and in leukocytes collected from treated patients, measured by an ELISA method, has been reported previously (11, 12), but is not confirmed in the present study, which involves a larger group of patients. This discrepancy could be due to several factors, such as the procedure of in vitro treatment (i.e., incubation of whole blood instead of isolated leukocytes), the different assay method, or the selection of the patients.

No differences in adduct levels were evident between patients responsive and nonresponsive to chemotherapy. The correlation between adduct levels in leukocytes and response to chemotherapy has been reported either as positive (3, 7–10), as lacking (22), or as limited only to patients with ovarian cancer who had adducts assayed by ELISA (23) by previous investigators (Table 3). These discrepancies could be due to the different types of tumors studied (which are likely to show different degrees of sensitivity to the platinum drugs with conceivably different thresholds of response), to the confounding factor of the other drugs used in the combinations, or to the fact that leukocyte adduct formation simply does not parallel adduct formation in tumors. Regarding this last aspect, data linking adduct formation in tumor samples and in normal tissues come only from autopsy studies (6, 25, 26), and it was these studies that raised the question of whether there were pharmacogenetic properties shared by tumors and normal tissues of a given
individual. However, this point of view is not generally accepted, and data from different clinical situations (e.g., the lack of correlation between the distribution of O\(^6\)-alkylguanine alkylation in tumor samples and in leukocytes) argue against this hypothesis (27). Furthermore, no data are available regarding the possible correlation between adduct formation in tumor samples from Pt-treated patients and the response to chemotherapy. Surprisingly, Pt–DNA adduct levels in blast leukocytes, collected from patients with acute nonlymphocytic leukemia and incubated \textit{in vitro} with cisplatin, were inversely correlated to the remission rate, regardless of whether the patients received carboplatin as a component of the treatment (28).

In patients treated with the cisplatin-5FU combination, no differences in renal toxicity, hemoglobin decrease, and received dose intensity were observed in the two groups of patients having either a high or low level of adduct formation. This fact might be due to the well-known differences in tissue distribution of cisplatin (29, 30), with leukocytes giving only a very limited representation of cisplatin action. In the present study, WBC nadirs were not regularly evaluated, and, therefore, it was not possible to assess differences in the degree of myelosuppression as a function of adduct formation. Furthermore, the adducts’ removal during the treatment, rather than the adduct levels measured on the first cycle, may be more closely linked to leukocyte toxicity (31).

In conclusion, in this paper we have shown that ICP-MS allows the determination of Pt-DNA adducts in leukocytes of patients treated according different doses and schedules of cisplatin and carboplatin combination chemotherapy. The lack of correlation of adduct levels with response and toxicity in this consistent group of patients supports the view that the handling of Pt drugs in leukocytes is very much different from that observed in other normal tissues and in tumors. Therefore, the determination of adduct levels in leukocytes does not enable one to predict the response and the toxicity to Pt-based chemotherapy.

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REFERENCES


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