Formation of DNA Adducts by the Anticancer Drug Carboplatin: Different Nucleotide Sequence Preferences in Vitro and in Cells†

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ABSTRACT: We have studied the formation of adducts upon carboplatin treatment of isolated DNA and in cells. The major adduct formed in vitro, determined with atomic absorption spectroscopy and enzyme-linked immunosorbent assay, was the intrastrand cross-link *cis*-Pt$(NH_3)_2$d(pGpG)(Pt-GG) (58%). *cis*-Pt-$(NH_3)_2$d(pApG) (Pt-AG) (11%), *cis*-Pt$(NH_3)_2$d$(GMP)_2$ (G-Pt-G) (9%), and monofunctionally bound platinum (*cis*-Pt$(NH_3)_3$dGMP (Pt-G), 22%) were formed in smaller amounts. These relative occurrences of the adducts, average values found between 1 and 16 h of incubation, are comparable with those after incubation with cisplatin. The formation of carboplatin−DNA adducts was slow, and about 230-fold more carboplatin than cisplatin (molar dose) was required to obtain equal levels of platination after 4 h of incubation. However, less than 20 times more carboplatin was needed to obtain equal levels of cytotoxicity after 1 h of exposure of CHO cells. The percentages of the carboplatin−DNA adducts after 7−12 h postincubation of the cells (determined with ELISA), Pt-GG (30%), Pt-AG (16%), G-Pt-G (40%), and Pt-G (14%), were different from those of the in vitro data. After 12 h postincubation, the number of interstrand cross-links (determined by alkaline elution) amounted to about 10% of the G-Pt-G adducts and 3−4% of the total amount of adducts. The immunocytochemical detection (with antiserum NKI-A59) of the platinum−DNA modifications showed a pattern similar to that found for the various bifunctional adducts: the initially low levels slowly increased to maximum values within 7−12 h and then slowly decreased. In conclusion, carboplatin forms the same bifunctional adducts as cisplatin. However, in cells no preference for carboplatin binding on pGpG sequences was found.

Since its introduction, cisplatin[1] [*cis*-diamminedichloroplatinum(II)] has become an antitumor agent of major clinical importance [Carter, 1984; see references in Howell (1991)]. However, side effects such as nephrotoxicity, neurotoxicity, and severe nausea and vomiting have led to the development of second-generation platinum drugs. Carboplatin [*cis*-diammine(1,1-cyclobutyldicarboxylato)platinum(II)], which differs from cisplatin by the nature of the leaving group, cyclobutyldicarboxylate instead of the two chlorides, is one of the most promising second-generation platinum compounds. Like cisplatin, carboplatin exhibits a broad spectrum of antitumor activity, but with strong reduction of the side effects. For carboplatin the dose-limiting toxicity appears to be bone marrow toxicity (Calvert et al., 1982). Fortunately, this problem can be circumvented with good hematological support offered by hematopoietic growth factors, bone marrow transplantation (Shea et al., 1989), or modulating agents such as WR-2721 (Treskes & van der Vijgh, 1993). When applied in this way, carboplatin can serve as an alternative drug for patients who are highly sensitive to cisplatin toxicity, or it can be used in combination with cisplatin to expand the therapeutic armament (Gill et al., 1991). Recently, in the treatment of patients refractory to cisplatin chemotherapy, promising results were obtained with high doses of carboplatin in combination with other chemotherapeutic agents (Rodenhuis et al., 1992a) and autologous bone marrow or peripheral stem cell transfusion (Rodenhuis et al., 1992b).

The antitumor effect of platinum compounds is thought to be due to their interaction with DNA (Reedijk, 1992). The major reaction product of the bifunctional agent cisplatin with DNA (in vitro and in vivo) was found to be the cross-link between two adjacent guanines on the same strand of DNA (Pt-GG). The intrastrand cross-links Pt-AG, between adjacent adenine and guanine residues, G-Pt-G, in which cisplatin is bound to two guanines separated by another base as well as to two guanines in opposite strands (interstrand cross-links), and the monofunctionally bound platinum adduct Pt-G were found in smaller amounts (Eastman, 1986; Fichtinger-Schepman et al., 1985). The latter compound is the intermediate in the formation of bifunctional adducts. In cells small amounts of DNA−protein cross-links also are formed (Eastman, 1986; Plooy et al., 1984).

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); carboplatin, *cis*-diammine(1,1-cyclobutyldicarboxylato)platinum(II); Pt-GG, *cis*-Pt$(NH_3)_2$d(pGpG); Pt-AG, *cis*-Pt$(NH_3)_2$d(pApG); G-Pt-G, *cis*-Pt$(NH_3)_2$d$(GMP)_2$; Pt-G, *cis*-Pt$(NH_3)_3$dGMP; CHO, Chinese hamster ovary; AAS, atomic absorption spectroscopy; ELISA, enzyme-linked immunosorbent assay; R_b, number of platinum atoms bound per nucleotide.

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On the basis of the chemical structure of carboplatin, it could be expected that the same bifunctional DNA adducts will be formed by carboplatin compared with cisplatin. However, the replacement of the two chloride ligands in cisplatin by the didentate ligand cyclobutyldicarboxylate in carboplatin makes carboplatin more resistant to aquation and therefore less reactive toward DNA than cisplatin. The binding of the second arm of carboplatin to DNA (i.e., the chelation) in vitro is reported to be 7−18 times faster than binding of the first arm (Knox et al., 1986; Frey et al., 1993), probably because of the higher lability of the leaving group once carboplatin has been monoaquated (Knox et al., 1986) or monofunctionally bound (Frey et al., 1993). The latter authors also reported that, instead of hydrolysis, a direct attack of nucleotides on carboplatin is an important first step in the interaction of carboplatin with DNA. Because carboplatin adducts are formed more slowly, larger doses and/or longer incubation periods are required to obtain DNA platination levels equal to those obtained with cisplatin. With the alkaline elution technique, it was established that the maximum level of interstrand cross-links is reached 6−12 h later in L1210 cells treated with carboplatin than in cells treated with cisplatin (Micetich et al., 1985). Immunocytochemically, with the platinum−DNA modification-recognizing antiserum NKI-A59, equally slow adduct formation has been observed in carboplatin-treated cultured cells and in white blood cells from patients after a carboplatin infusion (Terheggen et al., 1991a; den Engelse et al., 1991). The differences between dose levels of cisplatin and carboplatin required to obtain equal levels of DNA platination upon in vitro incubations appeared to be much larger (>100-fold) than those resulting in equitoxicity in cultured cells (20−40-fold; Los et al., 1991; Knox et al., 1986; Terheggen et al., 1991a). Also in patients an only 4−20 times higher dose of carboplatin than cisplatin is sufficient to reach equal levels of DNA platination (Blommaert et al., 1993; Gill et al., 1991). To explain these quantitative differences, it has been suggested that the relatively strong effect of carboplatin in cells may be due to, for example, preferential enzymatic cleavage (Cleare et al., 1978), different uptake, or membrane transport. Also, the presence of, for instance, NaCl, glutathione, and/or free oxygen radicals in the cellular and nuclear environments may play a role (Knox et al., 1986; Tonetti et al., 1993), while in patients different pharmacokinetics compared with cisplatin (Elferink et al., 1987) may have an additional influence.

Although the same bifunctional adducts are expected to be formed by both agents, at the onset of the study only interstrand cross-links had been identified for carboplatin (Knox et al., 1986). The presence of equal amounts of other bifunctional adducts was suggested on the basis of the equal abilities of cisplatin and carboplatin to unwind supercoiled DNA (Knox et al., 1986). However, it was still uncertain whether all of the other adducts were formed, and whether they were formed in the same relative amounts as after cisplatin treatment. Both factors are of importance with regard to the therapeutic effects, since there is still much uncertainty concerning the cytotoxic potential of the various individual adducts. In the present study, we have investigated the formation of the various platinum−DNA adducts after carboplatin treatment of DNA in vitro and in cultured Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

In Vitro Reactions of Carboplatin and Cisplatin with DNA. DNA solutions (salmon sperm DNA, Sigma Ltd., Poole, U.K., 0.5 mg/mL in 10 mM phosphate buffer, pH 7.2) were reacted with freshly prepared carboplatin solutions (Paraplatin, Bristol-Meyers, Weesp, The Netherlands) at 37 °C. After treatment, DNA was precipitated with 2 vol of absolute ethanol (−20 °C) after prior addition of 0.1 vol of 3 M sodium acetate/1 mM EDTA (pH 5.5) and washed twice with 80% ethanol. The R_b values were determined after enzymatic digestion. The relative occurrences of the various carboplatin−DNA adducts were investigated by incubating DNA samples with 1.35 μM carboplatin for 4 h, 1.35 mM for 4 and 16 h, or 3.38 mM for 1, 2, and 4 h. For comparison, DNA was incubated with 5 μM cisplatin (Platinol, Bristol-Meyers, Weesp, The Netherlands) for 4 and 16 h at 37 °C. At the end of the incubations, DNA was precipitated and washed (see earlier) and then dissolved in and dialyzed overnight against 0.5 M NH_4HCO_3 at 37 °C (to inactivate the monofunctionally bound Pt compound). Finally, after dialysis against water, the samples were enzymatically digested and chromatographed, and column fractions were analyzed.

Enzymatic Digestion and Chromatography. DNA samples were digested with nuclease P1 and DNase I to the unmodified mononucleotides and platinum-containing (di)-nucleotides. The digests were chromatographed on an anion exchange Mono-Q column (Fichtinger-Schepman et al., 1985, 1987).

Determinations of Platinum Content. Platinum concentrations in digested DNA samples and column fractions were determined with atomic absorption spectroscopy (AAS) by using a Perkin-Elmer Model 4000 atomic absorption spectrometer equipped with an HGA-500 graphite furnace and an AS-400 autosampling system. The concentrations of nucleotides were calculated from UV absorbances. The DNA platination levels were expressed as R_b values (number of platinum atoms bound per nucleotide).

Cell Cultures. CHO cells were cultured as monolayers in flasks (75 cm^2, Costar, Cambridge, MA) in Ham's F-10 medium, supplemented with 15% newborn calf serum, 1 mM L-glutamine (BDH, Poole, U.K.), penicillin (100 units/mL; Gist-Brocades NV, Delft, The Netherlands), and streptomycin (100 μg/mL; Gist-Brocades NV) at 37 °C in a 5% CO_2 incubator.

Cell Survival Experiments. Cells were trypsinized, counted, serially diluted, and subcultured in 6 cm Petri dishes (Greiner GmbH and Co KG, Nürtingen, Germany) at a density of 500−10000 cells per plate. At 4 h after the cells were seeded, the medium was replaced by medium containing 7.5% newborn calf serum, and freshly dissolved carboplatin was added at the desired concentration. After 1 h at 37 °C, the platinum-containing medium was removed, cells were washed twice with PBS (phosphate-buffered saline: 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, and 2.6 mM KCl, pH 7.4), and fresh medium with 15% serum was added. After 7 days of culturing, the cells were stained with 1% methylene blue, and the colony-forming ability of the treated cells was determined. For the survival curve, each time point was determined in at least two independent experiments with two dishes for each dose used.

Cell Preparations for Immuno(cyto)chemistry. CHO cells were incubated with 0.70 (ELISA) or 1.40 mM (immunocytochemistry) carboplatin for 1 h at 37 °C, washed twice with PBS, and postincubated in medium without carboplatin for 0, 1, 3, 7, 12, 15, 18, and 24 h. At these time points, the cells treated with 0.70 mM carboplatin were harvested and washed twice with cold PBS, and cell pellets were frozen at −80 °C until DNA isolation. Cytospin preparations were made of the 1.40 mM carboplatin-treated cells on ovalbumin-coated slides, fixed for 10 min in methanol (−20 °C) followed by 2 min in acetone (−20 °C), and then stored at −80 °C until immunocytochemical analysis. For the detection of cisplatin−DNA adducts, cells were treated with 40 μM cisplatin and postincubated in drug-free medium for 7 h.

Immunocytochemical Analysis of Platinum-Induced DNA Modifications. The immunoperoxidase staining procedure was carried out as described earlier (Terheggen et al., 1988), with omission of the RNase treatment since the latter did not influence the nuclear staining level (not shown). The general outline of the method is as follows: cytospin slides are treated with PBS/H_2O_2 (to inactivate endogenous peroxidases), followed by KCl, proteinase K, and ethanol/NaOH incubations (to denature the DNA and to increase the accessibility of the cisplatin−DNA adducts), 10% fetal calf serum (to reduce nonspecific antibody binding), and rabbit antiserum NKI-A59 raised against cisplatin-modified calf thymus DNA (Terheggen et al., 1991b). Then, the antibodies bound to the DNA adducts were visualized by triple PAP staining, i.e., sequential incubation of the cytospin preparations with goat anti-rabbit immunoglobulin (GaR), peroxidase−(rabbit)antiperoxidase complex (PAP), GaR, PAP, GaR and PAP and finally 3,3′-diaminobenzidine hydrochloride (DAB) as peroxidase substrate. Each sample was analyzed in duplicate in separate experiments on different days. A positive control, consisting of buccal cells of healthy persons incubated in vitro for 1 h at 37 °C with 5 μg/mL cisplatin, was included in each staining experiment to allow the comparison of results from separate stainings. The staining intensity of individual nuclei was measured as described previously (Terheggen et al., 1988). In each of two independently stained slides, the nuclear stain [defined as the sum of optical densities of the stained nuclear pixels (van Benthem et al., 1991)] of 20 randomly selected nuclei was measured and expressed in arbitrary units.

DNA Isolation. For the isolation of DNA from carboplatin-treated cells, we used a method described elsewhere (Fichtinger-Schepman et al., 1995). Briefly, thawed cell pellets (about 2×10^7 cells) were suspended in 5 mL of 10 mM Tris-HCl/1 mM EDTA (pH 7.8) (TE buffer), to which NH_4HCO_3 was added to a final concentration of 0.5 M just before use (TE/NH_3 buffer). After the addition of proteinase K (250 μg/mL), the cells were lysed by the addition of sodium dodecyl sulfate [final concentration 1% (w/v)] during a 10 min incubation at 37 °C, immediately followed by extraction with phenol for 10 min at 4 °C and precipitation of the DNA from the water layer. DNA was washed, dissolved in 2 mL of TE/NH_3 buffer, supplemented with 250 μg of proteinase K/mL, and dialyzed overnight at 37 °C against TE/NH_3 buffer followed by 2 × 1 h against TE buffer. Then, solutions were extracted with 2 mL of chloroform/isoamyl alcohol (24/1), DNA was precipitated, and after dissolution in 2 mL of TE buffer, RNA was digested with 75 μg of RNase A/mL and 75 units of RNase T1/mL (2 h, 37 °C). Finally, the DNA solutions once more were extracted with chloroform/isoamyl alcohol and the DNA was precipitated. After enzymatic digestion and column chromatography as indicated earlier, the adducts were quantitated immunochemically.

The DNA from cisplatin-treated cells was isolated as described previously (Fichtinger-Schepman et al., 1987).

Immunochemical Determination of Platinum−DNA Adducts. For the quantitation of the platinum products, present at identified positions in the column eluate, adduct-specific antisera were used (Fichtinger-Schepman et al., 1985, 1987). In this method, the dilutions of the fractions giving 50% inhibition of antibody binding in the competitive ELISA were determined and used to calculate the amounts of carboplatin− and cisplatin−DNA digestion products.

Alkaline Elution Method. This method was performed as described by Plooy et al. (1984). Briefly, CHO cells (10^5) were seeded in 6 cm Petri dishes, and after 6 h of incubation, [^{14}C]dThd was added to the control cells and [^{3}H]dThd to the cells to be treated. After 16 h of incubation, cells were washed twice with PBS; control cells were given fresh medium and the [^{3}H]dThd-labeled cells were treated with 0.70 mM carboplatin. After 1 h, cells were washed, given fresh medium, and postincubated for the desired time (0, 3, 7, 12, 15, 18, and 24 h). Subsequently, the cells were placed on ice, medium was removed, 1 mL of ice-cold PBS was added, and both control and carboplatin-treated cells were irradiated simultaneously with X-rays at 0 °C. Cells were harvested and mixed, and alkaline elution was performed. The number of DNA interstrand cross-links was calculated in femtomoles per microgram of DNA (cross-links/10^9 Da) (Plooy et al., 1984). The experiments were carried out in triplicate.

Statistical Analysis. The significance of the data was determined with the two-sided Student's *t*-test and correlation coefficients with linear regression analysis.

RESULTS

In Vitro Binding to DNA as a Function of Carboplatin Concentration and Incubation Time. Salmon sperm DNA was incubated for 4 h at 37 °C with various concentrations of carboplatin. As shown in Figure 1A, the number of platinum atoms bound per nucleotide (R_b value) increased linearly with the carboplatin dose (correlation coefficient, 0.997). To obtain a comparable level of platination with cisplatin during a 4 h incubation at 37 °C, an approximately 230-fold lower dose was needed (4.23 μM for cisplatin and 0.98 mM for carboplatin to obtain $R_b = 1.5 \times 10^{-3}$). To study the kinetics of platinum binding, DNA was incubated with 0.27 mM carboplatin for 1−120 h. The results in Figure 1B indicate that adduct formation increased linearly with incubation time (correlation coefficient, 0.994).

Identification and Quantitation of Carboplatin−DNA Adducts. The reaction products of carboplatin with DNA were determined as described for the identification and quantitation of cisplatin−DNA adducts (Fichtinger-Schepman et al., 1985). In this method, the platinated DNA is first treated with NH_4HCO_3 to prevent the conversion of monofunctional into bifunctional adducts during additional processing (however, 0.5 instead of 0.1 M NH_4HCO_3 was used) and then digested with nucleases followed by chro-

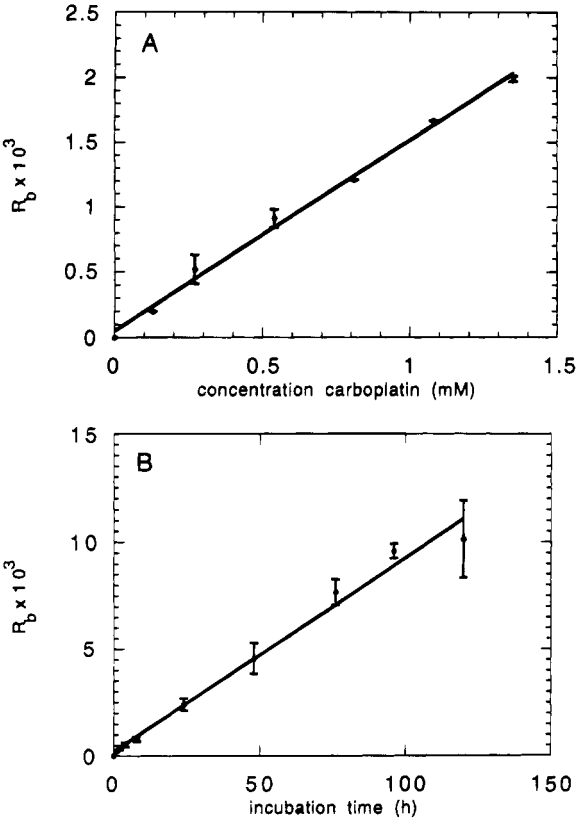

FIGURE 1: Number of platinum atoms bound per nucleotide (R_b) upon reaction of salmon sperm DNA with carboplatin: (A) R_b dependence on carboplatin concentration (exposure for 4 h at 37 °C, correlation coefficient of 0.997); (B) R_b as function of reaction time (exposure with 0.27 mM carboplatin at 37 °C, correlation coefficient of 0.994). Platinum contents were determined with AAS. Each point represents the mean (±SD) of at least two independent experiments.

matography. Figure 2A shows the elution pattern of a digest of DNA treated for 4 h with 1.35 mM carboplatin. The same four platinum-containing peaks could be observed as in cisplatin-treated DNA samples, i.e., Pt-G, Pt-AG, the main adduct Pt-GG, and G-Pt-G. Similar amounts of platinum (micromolar) in the collected column fractions were observed when determined with AAS or with the specific antibodies in competitive ELISA. To test whether the relative occurrence of the various adducts was dependent on drug concentration, a DNA sample was also treated with a 1000-fold lower concentration (1.35 μM) of carboplatin. In this case, however, the amounts of platinum in the column fractions were too low to be measured by AAS and therefore were detected with ELISA. The elution pattern and the relative occurrences of the various adducts were very similar to those of the 1.35 mM carboplatin-treated DNA sample (Table 1), and platination levels were exactly 1000-fold lower (not shown).

The relative occurrences of the various adducts formed under different in vitro incubation conditions are summarized in Table 1. During the first 16 h of incubation, only a minor shift from monofunctional to bifunctional adducts could be observed. In general, it can be concluded that the relative occurrences of platinum−DNA adducts after the in vitro treatments of DNA with carboplatin (15−30% Pt-G, 8−14% Pt-AG, 50−65% Pt-GG, and 4−17% G-Pt-G) are rather similar to those found after in vitro incubation of DNA with 5 μM cisplatin for 4 and 16 h at 37 °C.

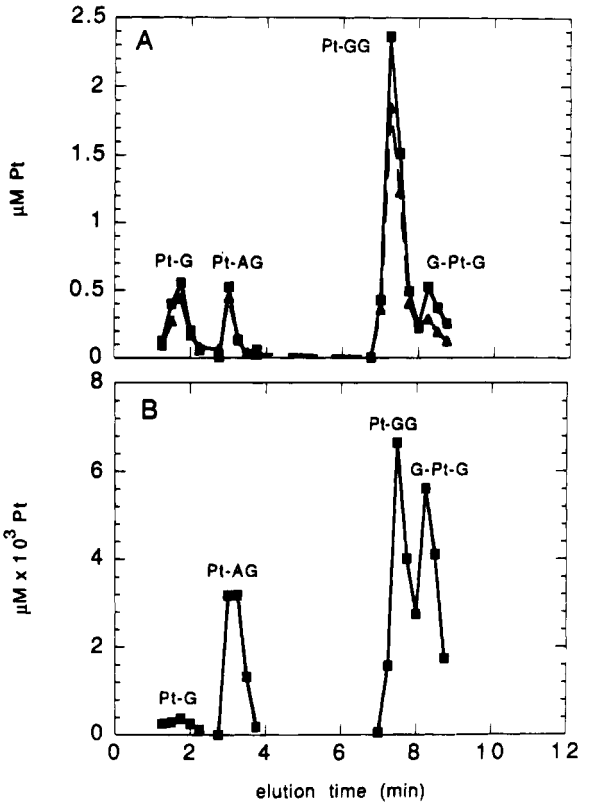

FIGURE 2: (A) Column chromatography of an enzymatically digested sample of salmon sperm DNA that had been reacted with 1.35 mM carboplatin for 4 h at 37 °C. The concentrations of platinum (micromolar) in the collected column fractions were detected with AAS (▲) as well as with ELISA (■). (B) ELISA data are given for a DNA sample from CHO cells treated with 0.70 mM carboplatin for 1 h at 37 °C and postincubated for 18 h in drug-free medium.

Survival of CHO Cells Treated with Carboplatin. To determine the sensitivity of CHO cells for carboplatin, cells were treated for 1 h with different concentrations of the drug, and their survival capacity was tested in a colony-forming assay. The results (Figure 3) indicate that the 10% survival concentration is about 0.70 mM carboplatin. A 17-fold lower molar concentration of cisplatin (40 μM) was required to reach an equal percentage of survival (data not shown).

Carboplatin−DNA Adducts in CHO Cells. Carboplatin−DNA adducts in cells were studied after incubating CHO cells for 1 h at 37 °C in the presence of 0.70 (ELISA and alkaline elution experiments) or 1.40 mM (for immunocytochemistry) carboplatin, followed by postincubation for 0−24 h in medium without carboplatin. To study the presence of the various platinum−DNA adducts, DNA was isolated from the cells, digested, chromatographed, and analyzed with the immunochemical assay (ELISA). As shown in Figure 4A, the bifunctional adduct levels were still low directly after treatment. The same holds for the monoadduct, Pt-G, which reached its maximum level after 3−7 h postincubation, followed by a decrease. The highest levels of the Pt-GG, Pt-AG, and G-Pt-G adducts were reached between 7 and 12 h postincubation, followed by a more gradual decrease. The total carboplatin−DNA adduct level, calculated from the sum of all four adducts present after 7 h postincubation of the cells, was about 45 fmol/μg DNA, which is equivalent to an R_b value of 1.5×10^{-5}. Similar to the in vitro carboplatin-treated DNA samples, the relative occurrences of the adducts in CHO cells did not vary

Table 1: Relative Occurrences of the Platinum−DNA Adducts[a]

treatment		Pt-G	Pt-AG	Pt-GG	G-Pt-G
	In Vitro[b]				
carboplatin					
1.35 mM, 4 h	(n^c = 1; AAS)	17	10	64	9
	(ELISA)	16	8	61	14
1.35 μM, 4 h	(n = 1; ELISA)	15	8	60	17
3.38 mM, 1 h	(n = 3; AAS)	30 ± 2	13 ± 1	50 ± 1	7 ± 2
2 h	(n = 1; AAS)	28	12	52	8
4 h	(n = 1; AAS)	23	12	56	9
1.35 mM; 4 h	(n=5; AAS)	26±6	12±1	54±6	8±1
16 h	(n = 1; AAS)	17	14	65	4
cisplatin					
5 μM, 4 h	(n = 2; AAS)	22 ± 0	17 ± 1	54	8 ± 0
16 h	(n = 1; AAS)	13	19	65	3
	In Cultured CHO Cells[d]				
carboplatin					
700 μM, 0 h	(n = 4; ELISA)	16 ± 15	13 ± 9	30 ± 7	40 ± 4
3 h	(n = 4; ELISA)	23 ± 15	13 ± 4	31 ± 10	33 ± 8
7 h	(n = 2; ELISA)	17 ± 10	16 ± 8	28 ± 8	38 ± 9
12 h	(n = 3; ELISA)	11 ± 7	15 ± 4	33 ± 7	41 ± 10
18 h	(n = 3; ELISA)	16 ± 11	20 ± 4	31 ± 10	33 ± 5
24 h	(n = 3; ELISA)	17 ± 11	18 ± 2	31 ± 8	35 ± 11
cisplatin					
40 μM, 7 h	(n = 3; ELISA)	9 ± 5	15 ± 7	57 ± 16	18 ± 8

[a] Relative occurrences ± SD (% of total adduct level) of the platinum−DNA adducts, measured by AAS and/or ELISA. Data are given for salmon sperm DNA treated in vitro with carboplatin or cisplatin at 37 °C under the conditions indicated and for CHO cells incubated with carboplatin or cisplatin for 1 h at 37 °C and postincubated in drug-free medium for the time periods indicated. [b] After in vitro carboplatin treatment the percentage of Pt-GG adducts was significantly higher than that of the G-Pt-G and Pt-AG adducts (p = 0.0001 for the 4 h samples). No significant differences between carboplatin-treated (at t = 4 h) and cisplatin-treated (at t = 4 and 16 h) DNA samples were found for the Pt-GG adducts (p = 0.6) and the G-Pt-G adducts (p = 0.09). [c] n is the number of independently performed experiments. [d] Compared with the DNA in carboplatin-treated cells, in in vitro carboplatin-treated DNA (at t = 4 h) the percentage of Pt-GG adducts was significantly higher than in carboplatin-treated cells (at t = 3 h, p = 0.0015, and at t = 12 h, p = 0.0022) and that of the G-Pt-G adducts was lower (at t = 3 h, p = 0.0001, and at t = 12 h, p = 0.001). The cisplatin-treated cells had a higher percentage Pt-GG than G-Pt-G adducts (p = 0.019). Compared with cisplatin-treated cells (at t = 7 h), a significantly higher percentage G-Pt-G adducts was found in the carboplatin-treated cells (at t = 12 h, p = 0.036), whereas the difference in Pt-GG adducts was not significant (p = 0.073). In all cases, no significant differences in the percentages of Pt-AG adducts were observed.

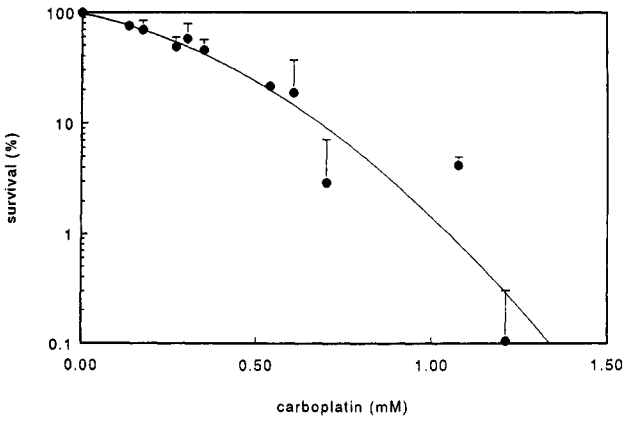

FIGURE 3: Survival curve of CHO cells treated for 1 h at 37 °C with different concentrations of carboplatin, as measured by their colony-forming ability. Each point represents the mean value ±SD from at least two independent assays.

considerably in the first 24 h of postincubation (Table 1). However, the percentages found for the Pt-GG (28−33%)

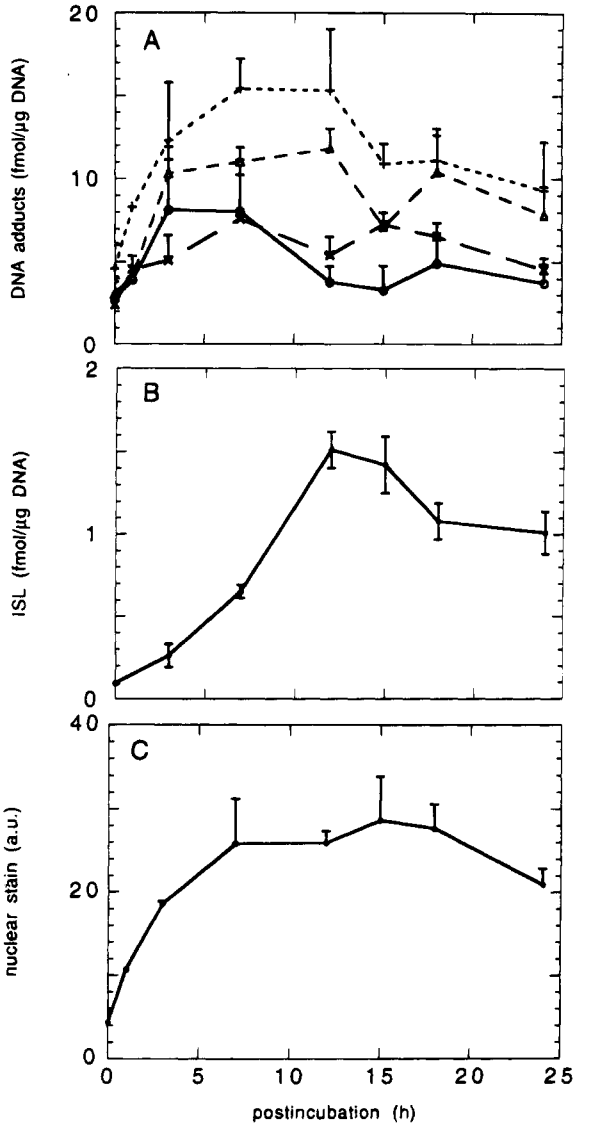

FIGURE 4: Formation of carboplatin−DNA adducts in CHO cells during postincubation in drug-free medium after treatment with 0.70 (A and B) or 1.40 mM (C) carboplatin for 1 h at 37 °C. Panel A shows the monofunctional adduct Pt-G (○) and the bifunctional adducts Pt-AG (×), Pt-GG (△), and G-Pt-G (+), as measured with competitive ELISA. Each point represents the mean value (±SEM) from at least two independent experiments. Panel B shows the interstrand cross-links (ISL) as measured with the alkaline elution technique. Each point represents the mean (±SEM) from three independent experiments. Panel C shows the results when the platinum−DNA damage was determined immunocytochemically (antiserum NKI-A59). Platinum−DNA damage is expressed in arbitrary units (au) of nuclear stain. Each point represents the mean value (±SEM) of three independent experiments.

and G-Pt-G (33−41%) adducts were significantly different from those found in the in vitro carboplatin-treated samples, as is illustrated in Figure 2A,B. The G-Pt-G percentage also significantly differed from the data obtained for cisplatin-treated DNA and cells (Table 1).

Because part of the G-Pt-G adducts are derived from interstrand cross-links, the presence of interstrand cross-links was determined separately with the alkaline elution method. As shown in Figure 4B, they were barely detectable immediately after the carboplatin treatment, but increased to a maximum level of 1.5 fmol/μg DNA after about 12−15 h, followed by a relatively slow decrease, as is also seen for the other bifunctional adducts (Figure 4A).

Figure 4C shows the formation and persistence of the platinum−DNA damage in CHO cells, as determined immunocytochemically with the NKI-A59 antiserum. Directly after the treatment, the nuclear stain slowly increased from the background value to a maximum between 7 and 18 h after the carboplatin treatment, followed by a gradual decrease.

DISCUSSION

We have studied the binding of carboplatin to DNA in vitro as well as in CHO cells. In vitro, a clearly linear increase in R_b value was observed when salmon sperm DNA was incubated with concentrations up to 1.35 mM (Figure 1A) and for time periods up to 120 h (Figure 1B). In earlier experiments, the formation of carboplatin−DNA adducts did not appear to be linear, but showed an initial fast step. This probably has to be ascribed to the presence of minor amounts of the aquated species in the drug sample used (Dijt, 1989). The observed slow rate of DNA platination is comparable with results reported by Knox, Terheggen, and Los (Knox et al., 1986; Terheggen et al., 1990; Los et al., 1991).

The formation of the same types of adducts after in vitro incubation of DNA with carboplatin and cisplatin (Table 1) is not very surprising since they both have the same amine ligands (only their leaving ligand is different). However, both compounds differ in their kinetics of hydrolysis and, consequently, in their reaction speed with nucleophiles such as DNA (Knox et al., 1986). Both compounds are believed to bind first in a monofunctional way; this step occurs much faster for cisplatin than for carboplatin. Even though the monofunctional adducts are different for cisplatin and carboplatin, both drugs seem to have a preference in vitro for reaction to guanines in a pGpG sequence, resulting in relatively high levels of the Pt-GG adduct. The preference for the formation of Pt-GG by cisplatin in cells as well as in vitro has been described before (Fichtinger-Schepman et al., 1985, 1987, 1989): the Pt-GG levels were substantially higher than the statistically expected 36.8% of total platination.

For the correct determination of adducts present at the moment of sampling of the in vitro treated DNA, the excess of free carboplatin was removed by ethanol precipitation of the DNA, while the conversion of monofunctional to bifunctional adducts was blocked by the use of 0.5 M NH_4HCO_3. In experiments with carboplatin-treated cells, the cells were lysed quickly after thawing of the cell pellets, immediately followed by phenol extraction, ethanol precipitation of the DNA, and dialysis against 0.5 M NH_4HCO_3. This approach showed that, immediately after a 1 h exposure of CHO cells to carboplatin, very low levels of platinum−DNA adducts were present (Figure 4A). However, upon postincubation of the treated cells, these levels increased for many hours (Figure 4A); maximum bifunctional adduct levels were not reached until 7−12 h later. The number of monoadducts also increased with time but started to decrease after about 7 h. The increase in total platination level of the DNA during postincubation of the cells in drug-free medium indicates that there was still unbound carboplatin present in the cells after the washings, which reacted with cellular DNA during postincubation.

In the cellular DNA, the formation of the bifunctional interstrand cross-links was measured separately with the alkaline elution method (Figure 4B). Immediately after the carboplatin incubation, no interstrand cross-links were found. These adducts reached a maximum level after 12 h postincubation, which amounted to about 3−4% of the total platination and 10% of the G-Pt-G adduct level. After 24 h of postincubation, significant amounts of interstrand cross-links still could be detected with the alkaline elution technique, which indicates that at this time point hardly any degradation of the DNA had occurred by, for example, apoptosis. The slow formation of interstrand cross-links was in agreement with observations by Micetich in L1210 cells, in which maximum levels were found after 18 h (Micetich et al., 1985).

Slow adduct formation was also observed when the platinum−DNA damage was studied at the cellular level with antiserum NKI-A59 in fixed CHO cells (Figure 4C). Similar relatively slow formation of carboplatin−DNA damage measured with this antiserum has been reported for other cultured cells (Terheggen et al., 1990) and for buccal cells from carboplatin-treated patients (den Engelse et al., 1991). The immunostaining of the DNA damage appeared to correlate with cytotoxicity in a panel of cell lines treated with cisplatin (Terheggen et al., 1990), while staining in buccal cells from patients treated with carboplatin correlated with the tumor response (Blommaert et al., 1993). Recent results indicated that this antiserum preferentially recognizes bifunctional intrastrand cross-links (F.A. Blommaert et al., unpublished results).

Although the same types of platinum−DNA adducts were found after carboplatin treatment of isolated DNA and cultured cells, significant differences in the relative occurrences of the various adducts were detected. In the CHO cells a relatively large proportion of G-Pt-G adducts was found (33−41%), while in vitro only 4−17% of the total adducts consisted of G-Pt-G (Table 1); in contrast, the relative occurrence of Pt-GG adducts was lower in cells (28−33%) than in vitro (50−65%). These results suggest that the general preference for pGpG sequences in the adduct formation as found for cisplatin [Table 1; see also Fichtinger-Schepman et al., 1985, 1987, 1989; Eastman, 1986] does not exist for carboplatin in cells. This may be a more general phenomenon, since it has also been observed in white blood cells from patients treated with carboplatin (data not shown).

The difference in specificity between the adduct formation of carboplatin to DNA in vitro and in cells, which is not seen for cisplatin, is very intriguing, but not easy to explain. Possibly, the difference in structural complexity and protein environment between DNA in solution and in cellular chromatin plays an important role, for example, through impairment of the accessibility of the pGpG sequences by histones, which should then interfere selectively in the reaction with the carboplatin molecule and not with that of cisplatin.

Compared with cisplatin, carboplatin has low reactivity in vitro; much more (100−230-fold) carboplatin is required to obtain the same platination level (Knox et al., 1986; Terheggen et al., 1991a; this paper). However, to obtain similar cytotoxicity in cultured cells (Los et al., 1991; Knox et al., 1986; Terheggen et al., 1991a; this paper) and in patients (Blommaert et al., 1993; Gill et al., 1991), only a 4−20 times higher dose is needed. DNA platination levels after treatment of cells with carboplatin were in the same range as those after treatment with cisplatin (data not shown).

This relatively high effectivity of carboplatin in cells has been ascribed to enzymatic cleavage (Cleare et al., 1978) or to the presence of oxygen free radicals in the cell (Tonetti et al., 1993), which may activate the binding of carboplatin to DNA. Moreover, differences in pharmacokinetics between cisplatin and carboplatin can play an additional role in patients (Elferink et al., 1987). From this study it becomes obvious that, although carboplatin and cisplatin form the same bifunctional DNA adducts, carboplatin cannot be considered as just a slow-reacting substitute for cisplatin. Its activation may involve mechanisms other than those with cisplatin, leading to differences in DNA binding and cytotoxicity.

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