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CIS-DIAMMINEDICHLOROPLATINUM(II) TOXICITY IN HUMAN MELANOMA CELLS AND LYMPHOCYTES AS RELATED TO CELLULAR PLATINUM ACCUMULATION, DNA CROSS-LINKING AND INHIBITION OF DNA SYNTHESIS

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Abstract

The cytotoxic effect of cis-diamminedichloroplatinum(II) (cis-DDP), as measured by a dye exclusion assay was much more pronounced in bone marrow cells and phytohemagglutinin (PHA)-stimulated lymphocytes than in a human melanoma cell line. DNA synthesis measured by incorporation of ^{3}H-thymidine was much more sensitive to cis-DDP in PHA-stimulated lymphocytes than in melanoma cells. These differences were not caused by a difference in drug accumulation since measurements of cellular platinum content gave similar results in both cell types. The total amount of DNA cross-links and DNA interstrand cross-links induced by cis-DDP was measured with alkaline elution of DNA. In both PHA-stimulated lymphocytes and melanoma cells low total levels of DNA cross-links and DNA interstrand cross-links were found immediately after drug exposure, followed by a protracted increase in DNA cross-linking for 6–12 hours during further incubation after removal of cis-DDP. The relationship between the concentration of cis-DDP and peak levels of total DNA cross-links as well as DNA interstrand cross-links was linear in both cell types. Cis-DDP was found to induce 5.6 times higher total levels of DNA cross-links and 6.1 times higher levels of DNA interstrand cross-links in PHA-stimulated lymphocytes than in melanoma cells. The incorporation of ^{3}H-thymidine was much more reduced in PHA-stimulated lymphocytes than in melanoma cells at similar levels of DNA cross-linking. Thus, both reduced DNA cross-linking and lower effect of DNA cross-links on the DNA synthesis may contribute to the greater resistance of melanoma cells to cis-DDP.

Key words: Cis-platin, DNA cross-linking, human melanoma cells, human lymphocytes.

Disseminated malignant melanoma is usually resistant to chemotherapeutic drugs (6). With cis-diamminedichloroplatinum(II) (cis-DDP) given as single agent less than 20% of patients obtain objective tumor regressions, which generally are only partial and of limited duration (6). The reason for the resistance of malignant melanoma to chemotherapy is largely unknown.

Clinical drug resistance is determined by the relative sensitivities of tumor and normal tissue to drug. Drug resistance occurs when the tumor is less drug sensitive than the normal tissues, which prohibits dose escalation to the levels required for tumor response. In drug resistant tumors the response rate may increase if the dose is increased above the usual level of normal tissue tolerance. In metastatic malignant melanoma this has recently been demonstrated with high dose cis-DDP therapy coupled with selective protection of normal tissues with the thiophosphate compound WR-2721, which gave a response rate of over 50% (13).

It is therefore relevant to compare the effect of cytostatic agents on resistant tumor cells and sensitive normal cells in order to characterize the mechanisms which make tumor cells tolerate drug levels that are toxic to normal cells. Cis-DDP toxicities in normal tissues include moderate myelosuppression (19) and inhibition of PHA-responsiveness of peripheral lymphocytes (21). In this study we have compared the cytotoxic effects of cis-DDP on a human melanoma cell line (RPMI 8322) to those on freshly obtained human bone marrow cells and PHA-stimulated lymphocytes.

There is considerable evidence of the cytotoxic effect of cis-DDP being due to reactions with DNA (37), but the nature of the lethal lesion(s) has not been determined. Cis-DDP causes 3 main types of DNA lesions: DNA-protein cross-links, DNA interstrand cross-links, and DNA in-

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trastrand cross-links (51). The amount of DNA interstrand cross-links induced by cis-DDP has been correlated to the cytotoxic effects in different cell types (8, 53). The less active isomer trans-diamminedichloroplatinum(II), while forming large numbers of DNA-protein cross-links (24), induces low levels of DNA interstrand cross-links (48, 49).

However, it has recently been demonstrated that the most frequent lesions induced by cis-DDP are DNA intrastrand cross-links (33). In vitro data indicate that this lesion may cause cytotoxicity by blocking DNA replication (34).

In the present study the cytotoxic effects of cis-DDP were estimated with the dye exclusion technique of Weisenthal et al. (46, 47). Following exposure to cis-DDP, the cellular concentration of platinum was measured with atomic absorption spectroscopy, and the total amounts of DNA cross-links and DNA interstrand cross-links by alkaline elution (22, 25). The results were related to the effects on DNA synthesis as measured by ^{3}H-thymidine incorporation.

Material and Methods

Drug and chemicals. Cis-DDP was obtained from Bristol-Myers Co., New York, USA, as a sterile powder. Immediately before use, 10 mg of cis-DDP was dissolved in 10 ml of sterile water, and diluted to the desired drug concentrations. Methyl-^{3}H-thymidine (5 Ci/mmol, 1 mCi/ml) was obtained from the Radiochemical Centre, Amersham, UK.

Cells. A human melanoma cell line, RPMI 8322 (26), was used. Cells were grown in Eagle MEM medium with Earle's salts (Flow Laboratories, UK), supplemented with 2 mM L-glutamine, 10% foetal calf serum (FCS), 125 I.U. benzyl-penicillin and 125 μg streptomycin per ml. Human lymphocytes were obtained from heparinized fresh human blood from healthy donors by differential centrifugation in Ficoll-Isopaque (4). The lymphocytes ($0.5–1\times10^6$/ml) were stimulated for 48 h with phytohemagglutinin M (PHA) (Difco Laboratories, Detroit) in Parker 199 medium supplemented with 10% FCS, 125 I.U. benzyl-penicillin and 125 μg streptomycin per ml of medium. Human bone marrow cells were freshly obtained from healthy donors by biopsy of the iliac crest. Red cells were separated by centrifugation on a Lymphoprep (Nyegaard & Co. AS, Oslo, Norway) gradient at 1600 rpm for 20 min. Thereupon the cells were washed twice in phosphate buffered saline (PBS).

Dye exclusion assay. Toxicity was determined with a dye exclusion technique (46, 47). In summary, 0.5×10^6 cells were exposed to cis-DDP for 30 min in serum-free medium. The incubation was then continued for 48 h in fresh medium supplemented with 10% FCS. Parallel controls without drug were run. The cells were then concentrated in 0.2 ml medium, after which duck erythrocytes were added, followed by the addition of 0.2 ml of 2% fast green-nigrosine dye in 0.15 M NaCl. The suspension was then shaken on a whirl mixer and after 10 min sedimented onto microscope slides, using a SCA Shandon cytocentrifuge (500 rpm, 10 min). The cells were then counterstained by a modified hematoxyline-eosine technique. 'Living' cells stained pink and 'dead' cells stained green. The ratio of 'living' cells over duck erythrocytes was determined on each slide and expressed as the percentage of the untreated control.

Cis-DDP-induced inhibition of ^{3}H-thymidine incorporation. Human lymphocytes (0.8×10^6/ml) were stimulated with PHA for 48 h. RPMI 8322 melanoma cells were seeded into 35 mm Petri dishes with Eagle MEM medium supplemented with 10% FCS at a concentration of 0.8×10^6 cells/2 ml, and allowed to attach to the bottom overnight. The cells were exposed to different concentrations of cis-DDP for 30 min at 37°C in serum-free medium. After removal of the drug the incubation of the cells was continued in drug-free medium supplemented with 10% FCS. At 6, 24 or 48 h respectively after drug treatment, a 2-h pulse of ^{3}H-thymidine (10 μCi/ml) was given. The labelling was terminated by putting the cells on ice, removing the medium containing ^{3}H-thymidine, and washing the cells once in PBS. The RPMI 8322 cells were harvested by gentle scraping with a rubber policeman and transferred to test tubes. The cells were spun down, precipitated with 10% trichloroacetic acid (TCA), washed 3 times in 5% TCA and once in 70% ethanol, and plated onto 2.5 cm glass fibre filters (Type GF/B, Whatman Ltd, UK). The ^{3}H-activity on the filters was released with Soluene (Packard Instrument Company, Downers Grove, IL, USA) and measured by liquid scintillation counting.

Cellular accumulation of platinum. Human lymphocytes (34×10^6/34 ml) were stimulated with PHA for 48 h in large test tubes. RPMI 8322 cells (22×10^6/44 ml) were grown overnight in large test tubes. Following the removal of 4 ml of each cell suspension for the analysis of cellular protein content according to Lowry et al. (27), the cells were exposed to cis-DDP for 30 min. The drug exposure was terminated by putting the cells on ice, removing the drug and washing the cells once in drug-free medium. The cells were thereafter spun down and the pellets allowed to dry. The cell pellets as well as samples of the drug dilutions were then analysed for platinum content with flameless atomic absorption spectroscopy with a Pye Unicam atomic absorption spectrophotometer PU 9000 at Analytica AB, Täby, Sweden.

Cis-DDP induced DNA cross-linking. The alkaline elution technique (22, 25) was used with minor modifications as previously described (16, 18). PHA-stimulated lymphocytes and melanoma cells, labelled with ^{3}H-methylthymidine, were incubated for 30 min with cis-DDP in serum-free medium, followed by incubation in drug-free medium with 10% FCS for 0–12 h. The cells were then resuspended in ice-cold medium with 2% FCS, irradiated with

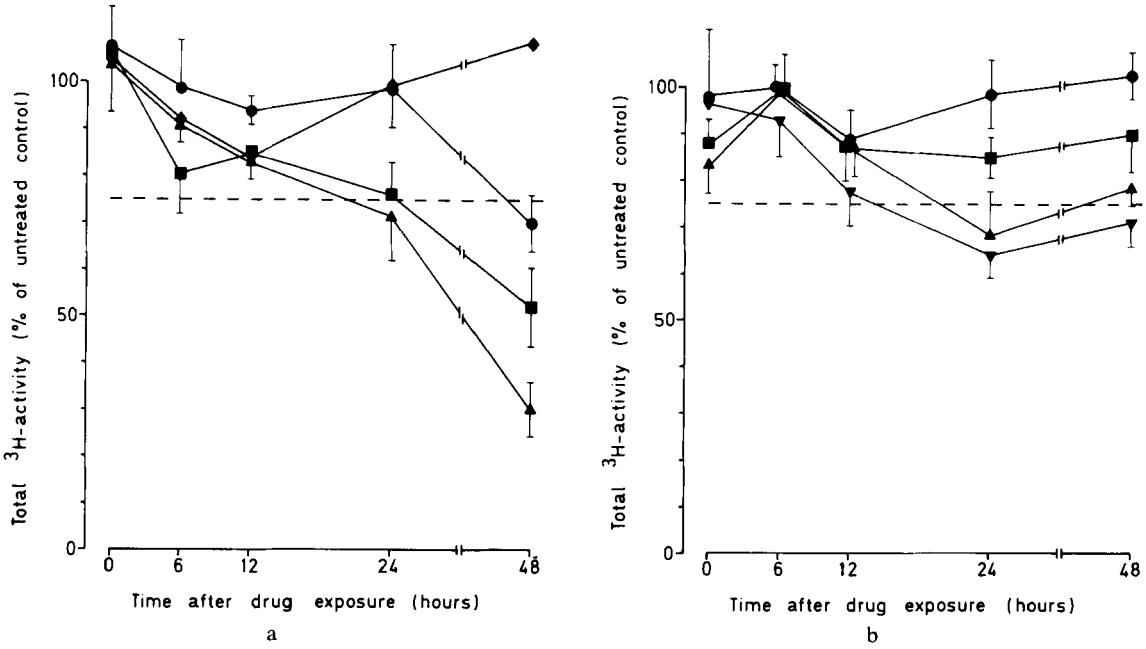

Fig. 1. Total ^{3}H-activity (on filters and in eluate) obtained at alkaline elution analyses 0–48 h after exposure to cis-DDP (0.5 (◆), 1 (●), 2 (■), 4 (▲), 10 (▼) $\times 10^{-5}$M), expressed in percentages of the ^{3}H-activity obtained from alkaline elution experiments on untreated control cells (mean±SEM). a) PHA-stimulated lymphocytes. b) RPMI 8322 melanoma cells.

600 R (with a Siemens Stabilopan x-ray apparatus operating a 140 kV, 20 mA, with a 4 mm aluminium filter), collected on polyvinyl chloride filters (pore size 2 μm, diameter 25 mm, Millipore Corp., Bedford, Mass., USA), washed with 10 ml ice-cold PBS and lysed with 5 ml sarkosyl-EDTA solution (sarkosyl 20 g/l, 0.02 M EDTA 7.44 g/l, 5 M NaOH added to pH 9.5). For the anaysis of DNA interstrand cross-links, another 2 ml of lysis solution containing 0.5 mg/ml of proteinase K was added and allowed to remain in contact with the filters for 1 h (22).

The filters were washed with 5 ml 0.02 M EDTA (acid form), pH 9.5. The DNA was then eluted from the filters during 10 h with a tetraethylammoniumhydroxide-EDTA solution (0.02 M EDTA (acid form) 5845 g/l, tetraethylammoniumhydroxide added to pH 12.1—for the analysis of DNA interstrand cross-links 0.1% sarkosyl was added) at a flow rate of 0.035 ml/min using a Minipuls 2 peristaltic pump (Gilford Medical Electronics, Villiers–Le Bel, France) and collected in 5 fractions. DNA remaining on the filters at the end of elution was removed by hydrolysis in 1 M HCl at 60°C for 1 h, followed by treatment with 2.5 ml 0.4 M NaOH at room temperature for 1 h and vigorous mechanical agitation. The ^{3}H-activity in each sample was analysed by liquid scintillation counting after adding 1.4 volumes of Instagel (Packard Instrument Company, Downers Grove, IL, USA).

Extensive descriptions of the measurement of DNA cross-links by alkaline elution have been published (22, 25). In brief, the presence of DNA cross-links (DNA interstrand and DNA-protein cross-links) results in a relative decrease in the rate of elution of DNA from x-irradiated cells. If the elution is carried out without previous proteinase K treatment the combined effect of DNA-protein cross-links and DNA interstrand cross-links is measured. Treatment with proteinase K prior to elution eliminates the effect of DNA-protein cross-links, allowing for the measurement of DNA interstrand cross-links. The amount of DNA cross-links is calculated from the following formula (25):

$$CLF = \sqrt{\frac{1-r_0}{1-r}} - 1$$

CLF = cross-linking factor, r_0 = fraction of DNA remaining on filter at the end of elution in irradiated control cells, r = fraction of DNA remaining on filter in cis-DDP-exposed, irradiated cells. By multiplying CLF by the radiation dose (600 R), the frequency of cross-links is expressed as R-equivalents.

One possible pitfall in studies of DNA cross-links induced by bifunctional DNA reactive drugs relates to loss of DNA due to cytotoxicity of the drug, which may cause underestimate of DNA cross-linking due to preferential loss of cross-linked DNA. This problem becomes especially important when the levels of DNA cross-links are followed for a prolonged period after drug exposure. Thus, a decrease in DNA cross-linking during prolonged observation may be due both to removal of cross-links by cellular repair mechanisms and to drug-induced killing of cells containing high levels of DNA cross-links. In order

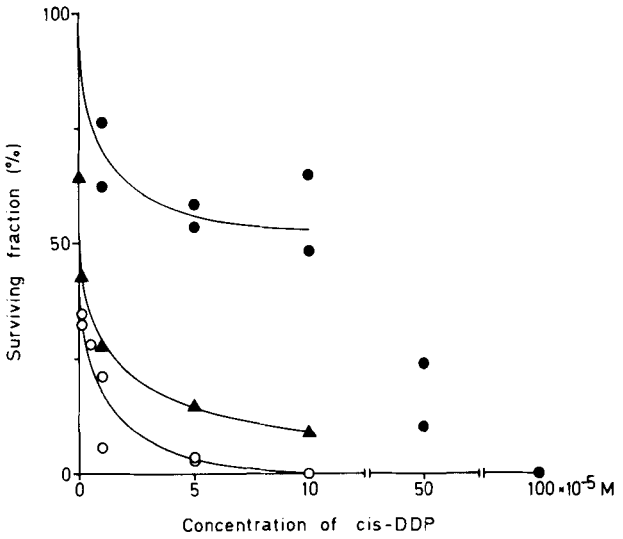

Fig. 2. Cell survival as measured by the dye exclusion technique of Weisenthal 48 h after exposure of bone marrow cells (▲), PHA-stimulated lymphocytes (○) and RPMI 8322 melanoma cells (●) to cis-DDP. Symbols indicate results of separate experiments.

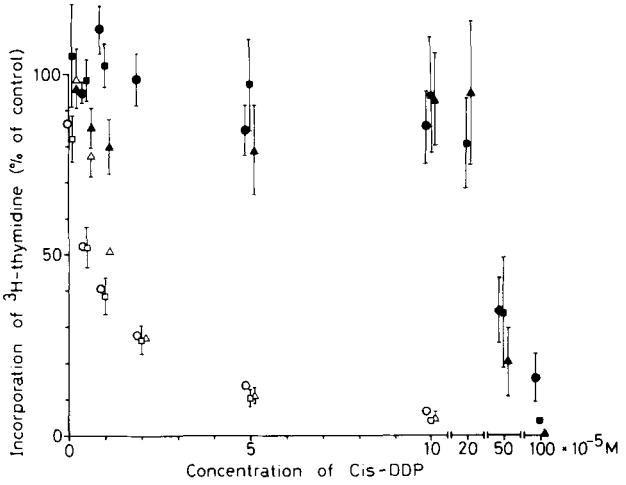

Fig. 3. Cis-DDP-induced decrease in ^{3}H-thymidine incorporation in PHA-stimulated lymphocytes and RPMI 8322 melanoma cells measured 6, 24 and 48 h following drug exposure. Symbols indicate mean values of 2–7 separate experiments, bars=SEM. PHA-stimulated lymphocytes 6 h (○), 24 h (□) and 48 h (△) after drug exposure. RPMI 8322 cells 6 h (●), 24 h (■) and 48 h (▲) after drug exposure.

to estimate cell killing the total ^{3}H-activity collected during each elution (i.e. the total activity in the eluted fluid as well as that remaining on filter at the end of elution) was calculated and compared in drug treated and control cell samples. There was a decrease in the total ^{3}H-activities in drug treated samples 24 and 48 h after drug exposure (Fig. 1). This decrease was most conspicuous for the PHA-stimulated lymphocytes. To limit the influence of drug induced cell death on the results, cross-linking data were used only when total ^{3}H-activity in drug treated samples was at least 75% (broken line, Fig. 1) of that of control cells.

Results

Cell survival. The cytotoxic effect of cis-DDP on PHA-stimulated lymphocytes was much higher than on RPMI 8322 cells (Fig. 2). In contrast, results obtained with fresh bone marrow cells and PHA-stimulated lymphocytes were similar, suggesting that PHA-stimulated lymphocytes may be used as a model for bone marrow cytotoxicity induced by cis-DDP. The further comparative studies were limited to PHA-stimulated lymphocytes and RPMI 8322 melanoma cells.

Cis-DDP induced inhibition of ^{3}H-thymidine incorporation. The inhibitory effect of cis-DDP on ^{3}H-thymidine incorporation in PHA-stimulated lymphocytes and RPMI 8322 cells was measured 6, 24 and 48 h respectively after drug exposure (Fig. 3). PHA-stimulated lymphocytes exhibited a high sensitivity to cis-DDP with marked decrease in ^{3}H-thymidine incorporation following low doses of cis-DDP, while RPMI 8322 cells exhibited a much lower drug sensitivity. Comparison with the data in Fig. 1 shows that this effect in PHA-stimulated lymphocytes was due to a reduced incorporation of ^{3}H-thymidine into DNA, rather than a loss of DNA due to cell death, since there was a marked reduction in ^{3}H-thymidine incorporation at drug concentrations which caused little loss of DNA.

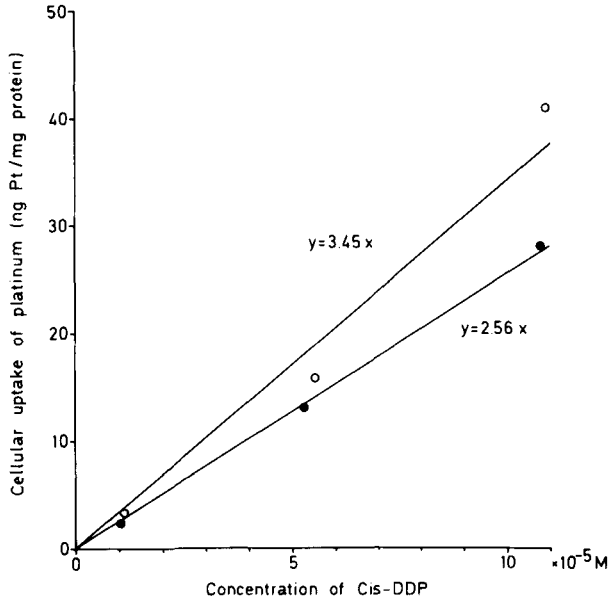

Fig. 4. Cellular accumulation of platinum (ng platinum/mg protein) in PHA-stimulated lymphocytes (○) and RPMI 8322 cells (●) following exposure to different concentrations of cis-DDP, measured by atomic absorption spectoscopy. Symbols indicate mean values of 2 separate experiments. Solid lines indicate results of regression analysis with zero intercept according to the equation y=ax (y=cellular platinum content (ng Pt/mg protein), x=concentration of cis-DDP (multiples of 10^{-5} M)).

Cellular accumulation of platinum. Cellular accumula-

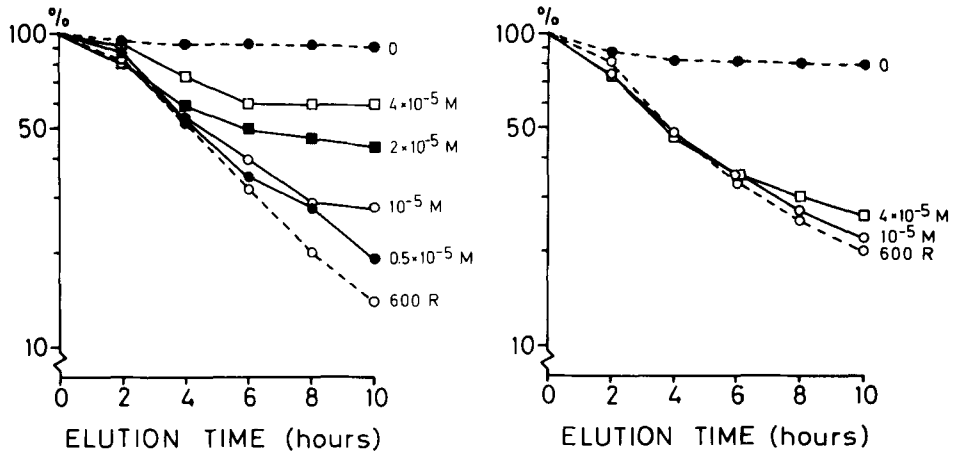

Fig. 5. Results of alkaline elution experiments measuring DNA interstrand cross-linking 6 h after exposure of cells to various concentrations of cis-DDP for 30 min. a) PHA-stimulated lymphocytes. b) RPMI 8322 melanoma cells.

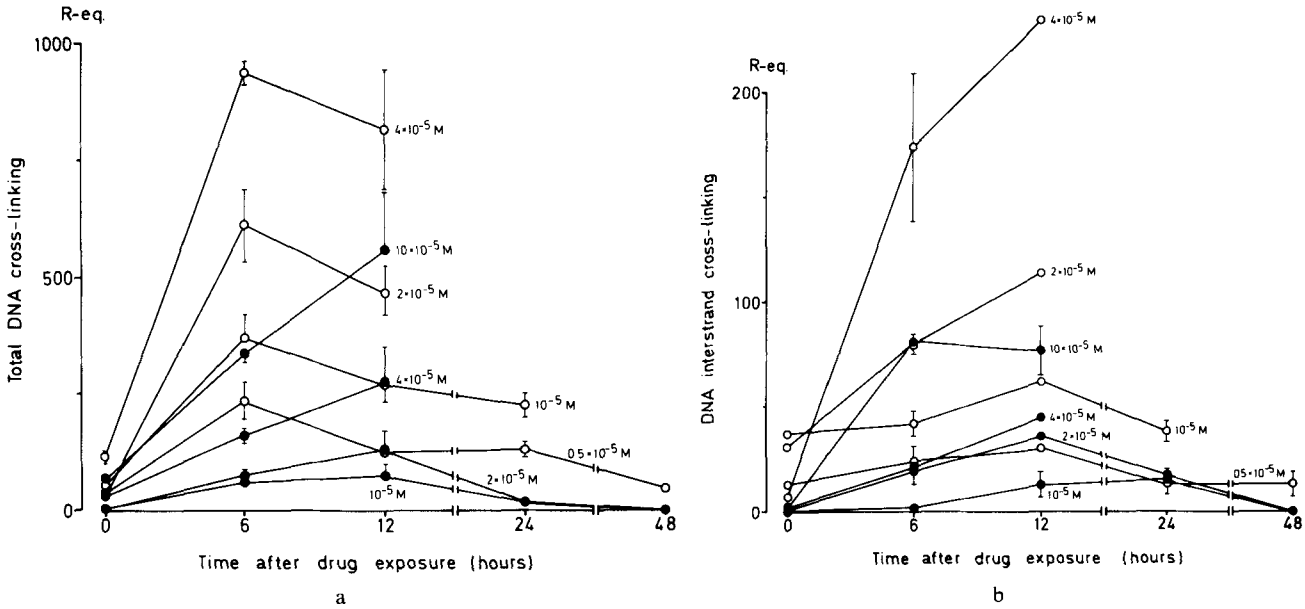

Fig. 6. DNA cross-linking in PHA-stimulated lymphocytes (○) and RPMI 8322 melanoma cells (●) measured with alkaline elution of DNA 0–48 h after exposure to cis-DDP for 30 min. a) Total DNA cross-linking. b) DNA interstrand cross-linking measured after treatment of the cell material with proteinase K. Symbols indicate mean values of 2–5 separate experiments, bars =SEM.

tion of cis-DDP as reflected by the cellular concentration of platinum following exposure to different concentrations of cis-DDP is illustrated in Fig. 4. In order to adjust for the differences in size between RPMI 8322 melanoma cells and PHA-stimulated lymphocytes, platinum content was related to the protein content of each sample. To correct for differences in drug dilution, the actual concentration of cis-DDP in the external medium at the beginning of incubation was calculated from the platinum content of each solution as measured by atomic absorption spectroscopy. Since both RPMI 8322 cells and PHA-stimulated lymphocytes exhibited an approximately linear relationship between concentration of cis-DDP and cellular accumulation of platinum, regression lines could be obtained for both cell types. The slopes of the regression lines were not significantly different (ratio: 1.35; $p=0.194$, Student's t-test), indicating a similar accumulation of platinum in both cell types.

DNA cross-linking determined by alkaline elution. The results of 2 representative experiments measuring DNA interstrand cross-linking 6 h after cis-DDP exposure are shown in Fig. 5. In PHA-stimulated lymphocytes cis-DDP caused a marked dose dependent decrease of the elution rate in irradiated cells, indicating the induction of increasing amounts of DNA interstrand cross-links. In RPMI 8322 melanoma cells this effect was much smaller, reflect-

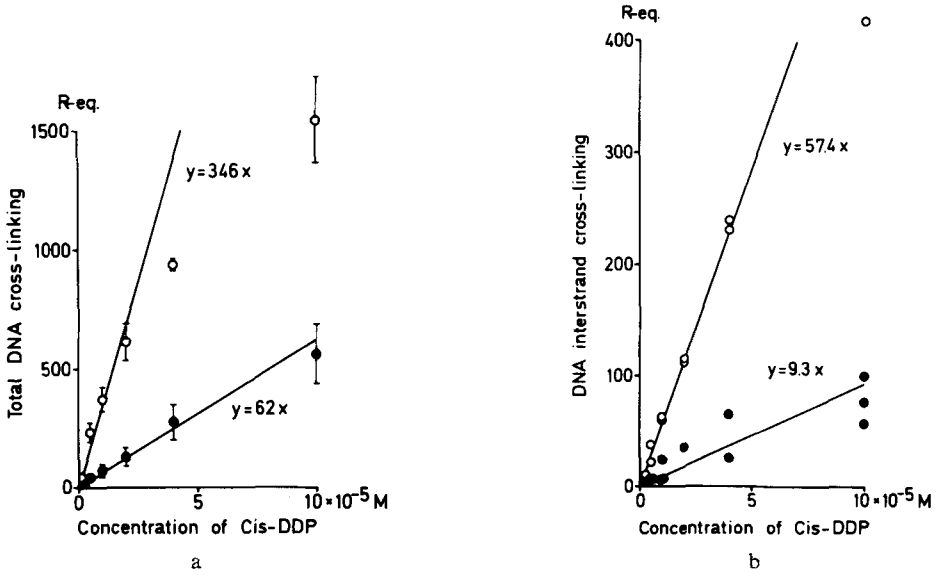

Fig. 7. Relationship between the concentration of cis-DDP during drug exposure and maximum levels of DNA cross-links in PHA-stimulated lymphocytes (○) and RPMI 8322 melanoma cells (●). Symbols indicate mean values of 2–5 separate experiments, bars =SEM. Solid lines indicate results of regression analyses with zero intercept according to the equation y=ax (y=DNA cross-links (R-equivalents), x=concentration of cis-DDP (multiples of 10^{-5} M)). a) total DNA cross-linking 6 h after drug exposure in PHA-stimulated lymphocytes and 12 h after drug exposure in RPMI 8322 cells. b) DNA interstrand cross-linking 12 h after drug exposure in PHA-stimulated lymphocytes and RPMI 8322 melanoma cells.

ing lower levels of DNA interstrand cross-links. A similar difference was obtained when total levels of drug induced DNA cross-links were measured with alkaline elution without prior treatment with proteinase K.

Fig. 6 illustrates the total amounts of DNA cross-links induced by different concentrations of cis-DDP in PHA-stimulated lymphocytes and RPMI 8322 cells when measured 0–48 h following drug exposure. In both cell types low levels of DNA cross-links were seen immediately after drug exposure, followed by a protracted increase in DNA cross-linking. In PHA-stimulated lymphocytes maximum levels of DNA cross-links were reached 6 h and in RPMI 8322 cells 12 h following drug exposure, after which time the amounts of DNA cross-links decreased.

There was an approximately linear relationship between the concentration of cis-DDP and maximum DNA cross-linking in RPMI 8322 cells at all dose levels, as well as for concentrations of cis-DDP of up to 2×10^{-5} M in PHA-stimulated lymphocytes (Fig. 7). In PHA-stimulated lymphocytes the highest concentration of cis-DDP gave a lower total yield of DNA cross-links than expected from the dose–response relationship established at lower concentrations, possibly indicating an underestimate of DNA cross-links with this method at very high levels of cross-linking. The ratio of the slopes of regression lines for the linear part of dose–response curves indicates an approximately 5.6-fold higher level of DNA cross-links in PHA-stimulated lymphocytes ($p<10^{-9}$, Student's t-test).

DNA interstrand cross-links were measured with alkaline elution after treatment of the cell material with proteinase K (Fig. 6). In similarity to the findings regarding total amounts of cross-links, DNA interstrand cross-links increased to maximum levels at 12 h after exposure, and then decreased in both cell types. There was an approximately linear relationship between maximum DNA interstrand cross-linking and concentration of cis-DDP at all dose levels in RPMI 8322 cells and for concentrations of cis-DDP up to 4×10^{-5} M in PHA-stimulated lymphocytes, with a 6.1-fold higher level of cross-links in PHA-stimulated lymphocytes ($p=0.00001$) (Fig. 7).

Table

The proportions of DNA interstrand cross-links (ISCL) and DNA-protein cross-links (DPCL) in PHA-stimulated lymphocytes, 6 and 12 h following exposure to cis-DDP. The relative proportions of the 2 classes of DNA cross-links were calculated from the slopes of regression lines of the dose–response relationships

Time after exposure to cis-DDP	PHA-stimulated lymphocytes		RPMI 8322 melanoma cells	
	ISCL (%)	DPCL (%)	ISCL (%)	DPCL (%)
6 h	13	87	19	81
12 h	23	77	15	85

The Table shows a comparison of the proportions of the 2 types of DNA cross-links. The results at 6 and 12 h differed for PHA-stimulated lymphocytes, while the proportions of DNA interstrand cross-links and DNA-protein

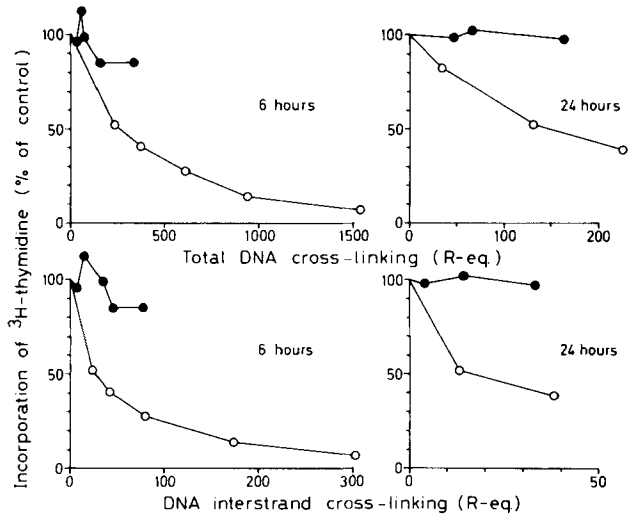

Fig. 8. Inhibition of ^{3}H-thymidine incorporation as a function of cis-DDP-induced DNA cross-linking in PHA-stimulated lymphocytes (○) and RPMI 8322 melanoma cells (●) as measured 6 h (left panels) and 24 h (right panels) after drug exposure. Upper panels: total DNA cross-linking. Lower panels: DNA interstrand cross-linking.

cross-links were relatively constant in RPMI 8322 cells. At the time of maximum total levels of DNA cross-links similar results were obtained in the 2 cell types, with DNA interstrand cross-links accounting for 13% of total DNA cross-links in PHA-stimulated lymphocytes and 15% in RPMI 8322 cells respectively.

Inhibition of ^{3}H-thymidine incorporation in relation to cis-DDP induced DNA cross-linking. The drug induced inhibition of ^{3}H-thymidine incorporation was related to the amounts of DNA cross-links induced in each cell type by different doses of cis-DDP (Fig. 8). At similar levels of DNA cross-linking the inhibition of ^{3}H-thymidine incorporation in RPMI 8322 melanoma cells was much smaller than that in PHA-stimulated lymphocytes. This difference between the cell types was seen when measured both at 6 h and at 24 h following drug exposure.

Discussion

Malignant melanoma cells are relatively resistant to cytostatic drugs (18, 29, 38, 43, 44). The present study indicated that a human melanoma cell line (RPMI 8322) is much more resistant to cis-DDP than bone marrow cells and PHA-stimulated lymphocytes (Figs 1–3). This could possibly be due to a differential intracellular accumulation of cis-DDP. It is not known whether cis-DDP enters cells by passive diffusion (12, 31) or by active transport across the cell membrane (5, 39, 41). In several investigations evidence for reduced drug accumulation in drug resistant cells has been obtained (1, 3, 11, 20, 36, 42, 45). However, the lower sensitivity of RPMI 8322 cells to cis-DDP is not due to reduced drug accumulation, as shown by measurements of cellular incorporation of platinum (Fig. 4).

In agreement with previous studies of other cell types, both the total amounts of DNA cross-links and the amounts of DNA interstrand cross-links were low immediately after exposure to cis-DDP (8, 30, 48–50, 52, 53). During the following drug-free incubation, both cell types exhibited protracted increase in total cross-linking and in DNA interstrand cross-linking (Fig. 6). The finding of maximum DNA cross-linking 6–12 h after cis-DDP exposure is in agreement with previous findings in animal cells (30, 35, 48–50, 52, 53), human tumor cell lines (7, 8), and fibroblasts (35).

Theoretically, if cis-DDP would induce a mixture of DNA cross-links and strand breaks, the subsequent resealing of these breaks could cause elution patterns mimicking increasing amounts of DNA cross-links. However, this is unlikely to be the case, since alkaline elution assays without previous x-irradiation of cells exposed to 10^{-4} M cis-DDP have failed to indicate any DNA strand breaks (data not shown). Thus, it is likely that the slow increase in cross-linking as measured by alkaline elution reflects a delayed build-up of DNA cross-links.

A 2-step model for the delayed formation of cross-links by cis-DDP has been proposed by Kohn (23). After the displacement of one of the chloride ligands, the cis-DDP molecule binds to a nucleophilic site on one DNA strand as a monoadduct, the N^7-atom of a guanine base being the most common binding site (51). Following this, after the displacement of the second chloride ligand, there is a delayed reaction with a second nucleophilic site on the complementary DNA strand. Alternatively, in the second reaction the cis-DDP molecule may bind to a site in chromatin protein, causing the formation of a DNA-protein cross-link. In our study DNA-protein cross-links, at the time of maximum total cross-linking, accounted for 87% of total cross-links in PHA-stimulated lymphocytes and 85% in RPMI 8322 cells (Table).

The reason for the 5–6-fold lower formation of cis-DDP induced DNA cross-links in RPMI 8322 cells (Fig. 7), despite similar accumulation of drug in both cell types, is not understood at present. One possible mechanism might involve the higher content of glutathione (GSH) in RPMI 8322 cells as compared to PHA-stimulated lymphocytes (16), which might cause increased inactivation of cis-DDP with reduced binding to DNA, and/or increased quenching of cis-DDP-DNA mono-adducts resulting in reduced formation of DNA cross-links. Increased levels of GSH have been described in cis-DDP resistant L1210 cells (20, 36) and ovarian cancer cells (14).

RPMI 8322 cells also contain high levels of class Pi GSH-transferase (28), which might possibly catalyze a reaction between cis-DDP and GSH. However, when the GSH content of RPMI 8322 cells was reduced by buthionine sulfoximine (BSO) only minor potentiation of cis-DDP cytotoxicity and DNA cross-linking was observed (17). It has been shown that GSH can react with cis-dichloro(ethylenediamine)platinum(II) (an analog of cis-DDP) bound

as a monoadduct to DNA (9). If analogous quenching of cis-DDP-DNA monoadducts by GSH is of importance, then the intranuclear level of GSH may be more important than the overall cellular GSH concentration for resistance to cis-DDP. BSO is comparatively inefficient in reducing the intranuclear levels of GSH (10), which may explain the small potentiation of cis-DDP toxicity by this drug.

The lower induction of DNA cross-links in RPMI 8322 cells could alternatively be caused by increased capacity to remove cis-DDP bound as a monoadduct to DNA before cross-links have formed. It has been demonstrated that cis-DDP bound as mono- and diadducts to DNA can be removed by the UvrABC excinuclease enzyme complex of E. coli (2). Some recent reports indicate that cis-DDP resistant L1210 cells (40) and ovarian cancer cells (32) have increased capacity to repair cis-DDP induced DNA damage. However, it is not known whether enhanced repair of cis-DDP-DNA monoadducts contributes to reduced DNA cross-linking in cis-DDP resistant cells.

The rate of removal of established DNA cross-links could also affect cis-DDP cytotoxicity. Our results show that both RPMI 8322 cells and PHA-stimulated lymphocytes can remove DNA cross-links (Fig. 6). The loss of DNA due to drug induced cell death, made it possible to follow DNA cross-linking for prolonged times only after relatively low doses of cis-DDP. Thus the data does not allow comparison of the rate of repair of DNA cross-links between the 2 cell types. Moreover, the decrease in DNA cross-links as registered in alkaline elution analyses merely reflects the reversal of the drug induced covalent binding between the complementary DNA strands and between DNA and protein molecules. Those are likely to be early events which are followed by subsequent repair steps, and may therefore not be accurate measures of the rate at which DNA containing cis-DDP induced lesions is restored to normal.

Also the effects of DNA cross-links on DNA replication differ in the 2 cell types. At equal DNA cross-linking the incorporation of ^{3}H-thymidine was much less reduced in RPMI 8322 melanoma cells than in PHA-stimulated lymphocytes (Fig. 8). The melanoma cells thus have a higher capacity to maintain DNA synthesis in the presence of DNA cross-links. The mechanism responsible for the inhibition of DNA synthesis following exposure to cis-DDP is not well understood. DNA synthesis in PHA-stimulated lymphocytes was reduced to approximately 7% by a dose of cis-DDP which induced approximately 300 R-equivalents of DNA interstrand cross-links (Fig. 8). Assuming an induction of one single strand break per 10^9 bases per R of x-irradiation (22), this amounts to approximately one DNA interstrand cross-link per 3×10^6 bases. Since the size of the replication units in eucaryotic cells are in the magnitude of $50–300\times10^3$ bases (15), this would correspond to one interstrand cross-link per 10 replication units at the most. Thus, most replication units would contain no DNA interstrand cross-link and therefore the pronounced inhibition of DNA synthesis is unlikely to be caused by direct interference with DNA strand separation of these lesions. Similarly, it could be calculated that there was at the most one DNA-protein cross-link in every 2–3 replication units at the same level of inhibition of DNA synthesis.

However, the inhibition of DNA synthesis by cis-DDP may be due to direct obstruction of the DNA polymerase by the much more frequent DNA intrastrand cross-links, which have been shown to block DNA synthesis by the large fragment of E. coli DNA polymerase I (34). The lower inhibition of DNA replication in melanoma cells at equal levels of DNA interstrand cross-links might be caused by lower relative induction of DNA intrastrand cross-links and/or lower sensitivity of the DNA replicating enzymes of these cells to the presence of DNA intrastrand cross-links. Alternatively, the inhibition of DNA synthesis might not be due to direct interference with DNA as template for replication, but with drug induced inactivation of some gene(s) necessary for the passage of the cell through the S-phase. If this is the case, the finding that RPMI 8322 melanoma cells have 50% more DNA than normal diploid cells (B. Tribukait, personal communication), could indicate that such critical gene(s) might be present in additional copies. Thus, the depletion of the gene product(s) by drug induced gene inactivation would be less likely since this wold require the inactivation of an increased number of gene copies.

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