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Micronuclei induction by carboplatin in human lymphocyte subpopulations

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Micronuclei (MN) induction by carboplatin, cis-diammine-1,1-cyclobutane decarboxylate platinum (II) (CBDCA), in B and T lymphocytes was studied by the MAC (morphology/antibody/chromosome) method which allows the immunologic identification of different cell lineages. An increased frequency of MN in B and T lymphocytes in CBDCA-treated cultures compared with controls was observed ($p < 0.001$). CBD cells were found to be more sensitive to CBDCA damage. CBDCA-treated cultures showed a decrease, albeit statistically non-significant, in the proportion of CBD interphasic and mitotic cells. Furthermore, higher MN frequencies in isolated lymphocytes than in whole blood in both control and CBCDA-treated cultures were observed.

Key words: Carboplatin, MAC method, micronucleus, mutagenesis.

Introduction

Carboplatin, *cis*-diammine-1,1-cyclobutane decarboxylate platinum (II) (CBDCA), is a second generation cisplatin analog. It was selected on the basis of similar or increased therapeutic effects, compared with the parent compound, cisplatin, and reduced nephrotoxicity in experimental systems.[1] A phase II study of carboplatin has currently been carried out in a variety of cancers, demonstrating that carboplatin is very active in the treatment of small cell carcinoma of the lung, head and neck cancer, and in ovarian carcinoma, with little or no nephrotoxicity and less emesis than cisplatin, although thrombocytopenia was observed but proved reversible in most patients.[2,3]

The reaction of cisplatin and related platinum coordination complexes with DNA is considered the main mechanism of antitumor activity for this class of compounds.[4,5] It is known that O^6 or N^7 atoms of guanine are the primary binding sites of platinum compounds. This reaction with the formation of a closed-ring chelate results in interstrand and intrastrand cross-linking and DNA–protein cross-linkings.[6] It represents a similar mode of action to that observed with alkylating agents.[7] Furthermore, chromosomal damage on cultured human lymphocytes induced by CBDCA has also been demonstrated.[8,9]

Micronuclei (MN) are induced by agents damaging the chromosomes directly or the spindle apparatus. It is considered a sensitive method to evaluate genotoxic effects.[10] Recently, higher frequencies of MN in isolated lymphocytes than in whole blood cultures has been observed.[11]

There is evidence indicating that various lymphocyte subsets may differ in their sensitivity to genotoxic agents.[11–14] Different lymphocyte subpopulations may be distinguished by the MAC (morphology/antibody/chromosomes) method[15] that preserves the cell membrane and the cytoplasm, allowing the immunologic identification of different cell lineages. Thus the differential sensitivity of human lymphocyte subpopulations to different genotoxic agents can be studied.

In the present study the MAC methodology and the MN test were used to investigate the genotoxic effect of CBDCA in various human lymphocyte subpopulations. Furthermore, the MN frequencies in whole blood and isolated lymphocyte cultures were also evaluated.

Material and methods

Samples of peripheral blood from four healthy non-smokers male donors aged 27–45 years were analyzed.

Lymphocyte cultures

Nucleated cells were isolated from the samples by Histopaque (Sigma, St Louis, MO) density gradient

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centrifugation. These cells (2×10^6) were then cultured in 10 ml of RPMI 1640 medium containing penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (0.29 mg/ml), fetal calf serum (10%) and phytohemagglutinin (PHA) (0.3 μg/ml). The flasks were incubated for 72 h in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. CBDCA (1 μg/ml) (Sidus, Bernal, Buenos Aires, Argentina) was added 24 h after of incubation. Control cultures without CBDCA were similarly incubated at the same time.

For the detection of MN, no colcemid treatment was used, whereas for the determination of the relative proportion of mitotic cels, colcemid (0.1 μg/ml) treatment for 2 h was applied.

MAC methodology

After culturing, the cells were bathed at room temperature for 5 min in a solution composed of 1 part of a hypotonic solution (50 mM glycerol, 10 mM potassium chloride, 0.8 mM magnesium chloride and 10 mM sucrose in water) and 1 part of RPMI 1640 medium containing 20% fetal calf serum. Slide preparations of the cells were then made by means of a cytocentrifuge. The slides were air-dried for 18 h and then fixed for 1 min in a solution consisting of 50 ml of acetone and 40 μl of 30% formaldehyde.

Immunoperoxidase staining

After fixation, the cells were air-dried for 10 min and then incubated with primary antibody for 1 h. Then the cells were washed in phosphate- buffered saline containing fetal calf serum and treated with secondary biotinylated antibody (1 : 250) and avidin–DH biotinylated horseradish peroxidase (1 : 160) (Vectastain ABC Kit; Vector, Burlingame, CA) for 30 min. The cells were washed and then bathed for 20 min in the presence of hydrogen peroxidase and 3-amino-9-ethylcarbazole (0.2 mg/ml). Morphologic classification was performed by staining the cells with 10% Giemsa (Merk, Buenos Aires, Argentina) in Sorensen's buffer (pH 6.8).

Lymphocytes were characterized by means of the following monoclonal antibodies: CD8, CD4, anti-K and anti-L (all from Dakopatts, Carpinteria, CA). CD8 is a marker for suppressor/cytotoxic T cells and CD4 for helper T cells, K-light chain plus L-light chain is a pan-B cell marker.

MN determination by the MAC method was on cells in which the cell membrane and cytoplasm had been preserved.[10] The criteria for scoring MN were those of Countryman and Heddle[16] with the slight modification introduced by Heddle.[17] Only cells with proper cell morphology were accepted for MN analysis.

The frequency of cells with MN was determined by scoring 1000 cells with each monoclonal antibody from each individual. A total of 100 cells were analyzed to study the frequency of mitotic cells and 1000 to study interphase cells on each monoclonal antibody. Student's *t*-test was used for statistical comparisons.

Peripheral blood lymphocyte cultures

Whole blood (0.8 ml) was added to 10 ml of F-10 medium supplemented with 15% fetal calf serum and 2% PHA. The cultures were incubated at 37°C during 72 h, and 0.1, 1.0 or 5.0 μg/ml of CBDCA was added after 24 h of incubation and was not removed before harvesting. Control cultures were grown in indentical conditions.

After incubation, the cultures were resuspended in a prewarmed hypotonic solution of 0.075 M KCl for 4 min to preserve the cytoplasm and then fixed in methanol:acetic acid (3:1). The slides were stained with Giemsa (4%) for 10 min. For each experiment 1000 interphase cells were scored for MN.[16]

Differences between the number of MN were analyzed by the χ^2 test. The dose–response relationship was determined by means of the regression coefficients.

Results

The frequencies of MN in B and T lymphocyte subsets from PHA-stimulated cultures treated with CBDCA (1 μg/ml) were studied. That dose was elected taking into account our experience on the clastogenic effect of CBCDA on peripheral blood lymphocytes.[9]

In CBCDA-treated cultures, an increased frequency of MN in T and B lymphocyte subpopulations was observed compared with control (untreated) cultures ($p < 0.001$) (Table 1 and Figure 1). CBDCA-treated CD8 cells had a higher frequency of MN than did B lymphocytes ($p < 0.05$). Simultaneously, CBDCA-treated CD8 lymphocytes also showed some increase, albeit statistically non-significant, in the number of MN compared with that of CD4 cells ($p > 0.05$). On the other hand, no statistical differences in the frequency of MN were seen among the different subpopulations of control cultures ($p > 0.05$).

Table 1. Frequency of MN in B cells (K + L) and T cells (CD4, CD8) subpopulations in CBDCA-treated and control cultures

Antigen	Number of MN/1000 cells ($X \pm SD$)	
	control	CBDCA treated
CD4	6.75 ± 0.96	19.5 ± 3.41[a]
CD8	7.00 ± 1.89	23.5 ± 3.31[a,b]
K+L	7.50 ± 0.81	18.0 ± 3.55[a]

[a] Significant differences with respect to controls ($p < 0.001$).
[b] Significant differences with respect to B (K+L) cells ($p < 0.05$).

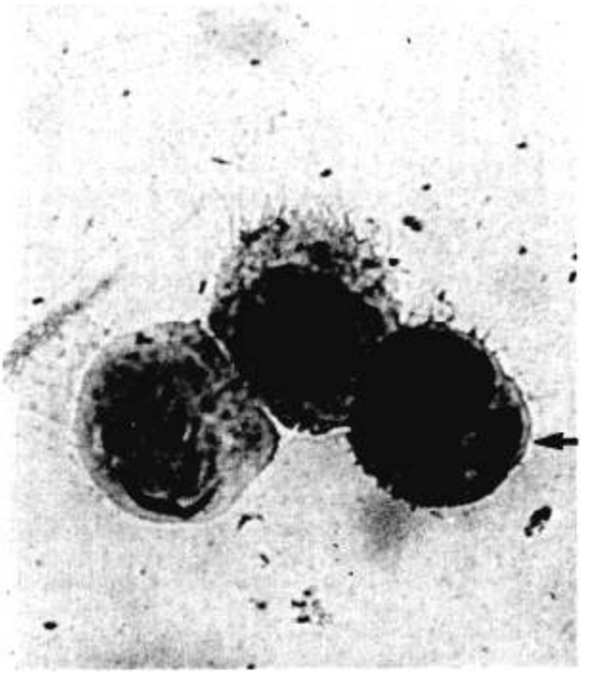

Figure 1. A CD8$^+$ lymphocyte with MN.

Mitotic and interphase cells of different lymphocyte subpopulations were also studied in cultures with and without CBDCA (Table 2). The data showed a decrease, though statistically non-significant, in the proportion of CD8 interphasic and mitotic cells from CBDCA-treated cultures compared with controls ($p > 0.05$). On the other hand, no differences were found in the proportions of B and CD4 interphasic and mitotic cells between cultures with and without CBDCA.

Table 3. Frequency of MN from whole blood and isolated lymphocyte cultures with and without CBDCA treatment

Dose (μg/ml)	MN/1000 cells ($X \pm SD$)
Whole blood cultures	
0	4.00 ± 1.41
0.1	4.00 ± 1.83
1.0	5.00 ± 2.45
5.0	7.50 ± 4.50[a]
Isolated lymphocytes	
0	7.75 ± 1.25
1.0	14.50 ± 1.73[b]

[a] Significant differences with respect to controls ($p < 0.05$).
[b] Significant differences with respect to controls ($p < 0.001$).

Moreover, we studied the MN frequencies in whole blood cultures with different concentrations of CBCDA (Table 3). A significant increase in the frequency of MN compared with controls was only found for 5.0 μg/ml ($p < 0.05$) and a dose-dependent effect for the induction of MN by different doses of CBDCA was observed ($p < 0.02$). The results were compared with those observed by analyzing isolated lymphocyte cultures, and the latter showed a significant increase of MN frequencies in both controls ($p < 0.001$) and CBDCA-treated cultures ($p < 0.02$).

Discussion

Our results show that, compared with controls, CBDCA-treated cultures have significantly higher frequencies of MN and that different lymphocyte subpopulations are differently prone to MN formation.

MN are formed during the cell division from acentric fragments (clastogenic effect) or lagging

Table 2. Proportions (%) of different T and B cell subpopulations in interphase and mitotic cells from CBDCA-treated and control cultures

Lymphocyte subset	Interphase cells ($X \pm SD$)		Mitotic cells ($X \pm SD$)	
	control	CBDCA treated	control	CBDCA treated
CD4	45.00 ± 6.21	45.25 ± 3.59	38.66 ± 7.20	39.25 ± 4.27
CD8	40.75 ± 6.50	36.00 ± 4.54	44.66 ± 5.03	39.50 ± 5.74
K + L	10.25 ± 1.25	11.75 ± 1.25	13.33 ± 1.15	14.00 ± 1.63

chromosomes (aneugenic effect) that are easily detected in interphase cells as free intracytoplasmatic bodies. It is known that DNA is the cellular target for the platinum complexes, especially during S phase.[18] This suggest that the increased MN frequencies induced by CBDCA would be directly related to the clastogenic effect of this drug.

Our results on whole blood cultures showed a slight increased frequency of MN after CBDCA treatment (5 μg/ml) with a dose–response relationship. By using the MAC technique we were able to demonstrate that, although the genetic damage affects all different lymphocyte subsets, CD8 cells are more sensitive to the clastogenic effect of this drug.

Moreover, our findings on interphase and mitotic cells did not show any differences between CBDCA-treated and control cultures in the proportions of the different lymphocyte subpopulations. However, a decrease, albeit statistically non-significant, in CBDCA-treated CD8 cells was observed. This would reflect the susceptibility of CD8 cells to this drug and may be related to the inhibition of DNA synthesis exhibited by platinum complexes.[18]

Previous experiences have shown different sensitivities of each human lymphocyte subpopulation to different genotoxic agents.[11–14] Among them, the CD8 subpopulation was observed especially affected in smokers.[13]

On the other hand, the comparison of MN frequencies in both whole blood and isolated lymphocyte cultures showed a significant increase in the latter. The mutagenicity of Histopaque isolation[19] may explain the increased frequencies observed in isolated lymphocyte control cultures. In CBDCA-treated cultures, these findings suggest that purified lymphocytes in culture have the ability to metabolize this drug and/or that the binding of CBDCA to red cells may prevent the genetic damage. Similar results have been observed by us in AZT-treated human lymphocyte cultures.[11] Chromosome aberrations[20] and sister chromatid exchange[21] studies with different drugs have also found more damage in isolated lymphocytes than in whole blood cultures.

In conclusion, our results showed increased frequencies of MN induced by CBDCA in all human lymphocyte subpopulations with particular sensitivity of the CD8 subset and a significant increase in isolated lymphocytes compared with whole blood cultures.

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