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INFLUENCE OF NOVOBIOCIN ON γ-IRRADIATED G_0-LYMPHOCYTES AS ANALYZED BY CYTOGENETIC ENDPOINTS

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ABSTRACT

Experiments with novobiocin (NB) post-treatment were performed to verify its effect on the frequencies of micronuclei (MN) and chromosomal aberrations (CA) induced by γ-irradiation (0.75, 1.5 and 3.0 Gy) in human lymphocytes at G_0-phase. The frequencies of MN significantly decreased by 44 and 50%, for the treatment with NB 50 µg/ml (30-min pulse) after radiation doses of 1.5 and 3.0 Gy, respectively. However, CA frequencies were not significantly affected. No significant effect on CA was observed when lymphocyte cultures were exposed to a single dose of 2.0 Gy at the G_0-phase and posttreated with 25 µg/ml NB for three hours either immediately after irradiation (G_0-phase) or after 24 h (S-phase). The significant suppressive effect of NB on MN frequencies supports the hypothesis that NB interaction with chromatin increases access to DNA repair enzymes.

INTRODUCTION

The antibiotic novobiocin (NB), a nonspecific inhibitor of DNA topoisomerase II (DNA topo II) enzyme, can interfere with the action of many antitumoral agents, including topo II inhibitors, such as amsacrine (m-AMSA), mitoxantrone, adriamycin and ellipticine (Utsumi *et al.*, 1990; Stetina and Veselá; 1991; Lee *et al.*, 1992; Sakamoto-Hojo *et al.*, 1994). NB increases the radiation-induced aberrations at G_2-phase of the cell cycle in Chinese hamster V79 cells (Takahashi *et al.*, 1985), inhibits the repair of DNA single-strand breaks and causes partial DNA degradation in irradiated HeLa cells (Seregina *et al.*, 1996).

NB cannot be regarded as a specific inhibitor of DNA topo II in mammalian cells since it can disturb many biological processes, including eukaryotic DNA replication, transcription and repair (Mattern and Scudiero, 1981; Mattern *et al.*, 1982; Seregina *et al.*, 1996), as well as chromosome condensation (Aijiro and Nishimoto, 1985). Even though many studies have shown that treatment of cells with inhibitors of DNA synthesis may increase the frequency of chromatid-type aberrations in human lymphocyte cultures which were previously exposed to radiation or chemical mutagens (Kihlman and Natarajan, 1984; Kihlman and Anderson, 1985; Sakamoto-Hojo and Takahashi, 1991; Traganos *et al.*, 1991), there is evidence that NB suppresses the induction of chromosomal aberrations (CA) by camptothecin (Palitti *et al.*, 1994), mitoxantrone (Medeiros and Takahashi, 1994) and ellipticine (Sakamoto-Hojo *et al.*, 1994) under certain cell treatment protocols.

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Although the induction of CA by γ-irradiation is quite well known, the mechanisms underlying the repair of DNA lesions in cycling cells leading to chromosome damage still need to be clarified. As it is known that NB interferes with the activity of many enzymes, the objective of the present study was to determine the influence of NB posttreatment on the induction of micronuclei (MN) and CA in γ-irradiated G_0-lymphocytes.

MATERIAL AND METHODS

Cell culture and irradiation

Human peripheral blood lymphocytes from normal, healthy male and female donors aged 20-30 years were grown in RPMI 1640 medium supplemented with 20% fetal calf serum plus penicillin and streptomycin. Cells were stimulated with 4% phytohemagglutinin (PHA) prepared in our laboratory from *Phaseolus vulgaris*. In all experiments, cells were irradiated before PHA stimulation (at the G_0-phase of the cell cycle) using a 60-cobalt (60-Co) γ-radiation source (Department of Genetics, Faculty of Medicine of Ribeirão Preto-USP) at a dose rate of 0.1 Gy/min.

Chemicals

Novobiocin (Sigma) was freshly prepared in deionized sterile distilled water and used at concentrations of 25 or 50 µg/ml of culture medium.

NB treatment at the G_0-phase of the cell cycle

Unstimulated lymphocytes from three donors (a female and two males) were irradiated with 60-Co γ-rays (0.75, 1.5 and 3.0 Gy) and posttreated with 50 µg/ml NB for 30 min in serum and in phytohemagglutinin-free medium. After treatment with NB, the cells were washed twice

with RPMI medium and incubated at 37ºC. For CA analysis, lymphocyte cultures were fixed 52 h after culture initiation and exposed to 0.4 µg/ml colchicine (Sigma) during the last 90 min of incubation. For MN analysis, the cytokinesis-block (CB) method (Fenech and Morley, 1985) was applied; 3 µg/ml cytochalasin B (Cyt-B, Sigma) was added to the cultures after 44 h of incubation, and cells were fixed after 72 h. MN were scored in 2000 binucleated CB cells per treatment per individual, using the criteria of Fenech and Morley (1985). The results were analyzed statistically by the Fisher exact test.

NB treatment at S-phase

Lymphocytes from three donors (all female) were γ-irradiated with 2.0 Gy at the G_0-phase of the cell cycle and posttreated with 25 µg/ml NB for 3 h, immediately after irradiation (G_0-phase) and 24 h after culture initiation (when most cells were in the S-phase). Lymphocytes were washed twice in serum-free medium, and reincubated for 52 h in complete medium at 37ºC. The Student *t*-test was applied to the results.

Chromosome preparation and analysis

Metaphase preparations for CA analysis were obtained by the conventional technique of Moorhead *et al.* (1960). For mitotic index (MI) calculation, mitotic cells were scored in 2000 cells per treatment per individual. CA were analyzed in a blind test and classified according to Savage (1975) and IAEA (1986). One hundred metaphases per treatment were analyzed in all experiments.

RESULTS

Effect of NB posttreatment in irradiated G_0-lymphocytes

a) Micronucleus analysis

The effects of NB posttreatment (30-min pulse) on γ-irradiated unstimulated lymphocytes were analyzed in three experiments with different donors (Table I). As expected, MN frequencies increased proportionally to the radiation doses (0.75 to 3.0 Gy). The induction of chromosome damage was significant ($P < 0.01$) and dose dependent, as can be seen in the distribution of cells with increased numbers of MN. There was a clear interaction effect between the radiation treatment and 50 µg/ml NB posttreatment, since significant reductions of 44 and 50% in the frequencies of MN ($P < 0.05$) were observed for the doses of 1.5 and 3.0 Gy, respectively (Figure 1), despite a considerable degree of variability among individuals.

b) Chromosomal aberrations

Similarly to MN analysis, three experiments were carried out in parallel to verify the effects of NB posttreatment on the induction of CA in γ-irradiated unstimulated-lymphocytes. A significant ($P < 0.01$) induction of CA was obtained for all lymphocyte cultures treated with radiation at all doses (Table II). There was a lack of interaction effects between the radiation and NB treatments. Differences in mitotic indices were not significant for all treatments ($P > 0.05$). The aberrations detected were predominantly dicentrics and double fragments.

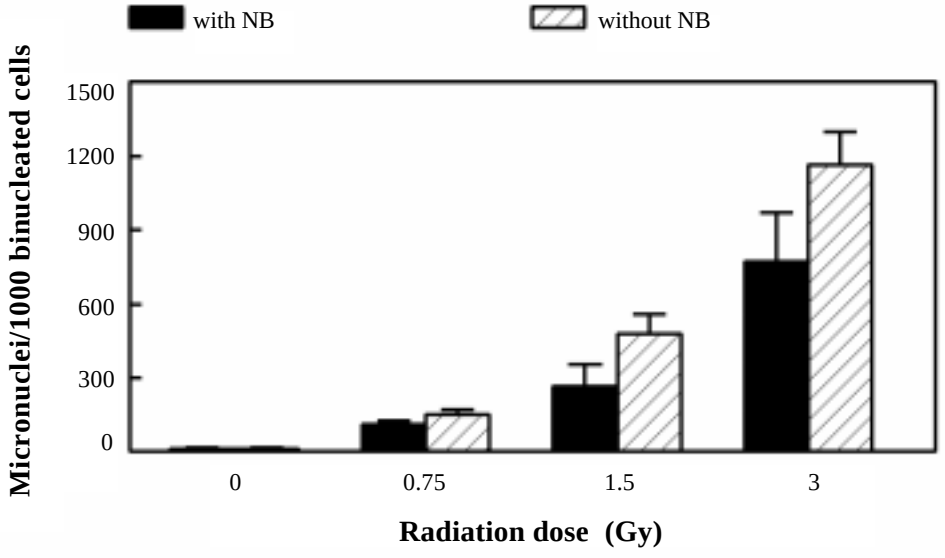

Figure 1 - Mean frequencies of micronuclei induced by gamma-irradiation in cytokinesis-blocked human lymphocytes posttreated with 50 µg/ml novobiocin (NB) (30-min pulse) at G_0-phase (pooled data for three experiments with different donors).

Table I - Frequencies of micronuclei (MN) in 0.75 to 3.0 Gy gamma-irradiated cytokinesis-blocked G_0-lymphocytes posttreated with 50 µg/ml novobiocin (NB) (30-min pulse). Three experiments (A, B, C) were performed with different donors and 2000 binucleated cells/treatment/donor were analyzed.

Treatment	Donor	Number of binucleated cells with *n* MN								MN/1000 cells
		0	1	2	3	4	5	6	Total	
Control	A	1994	5	1	0	0	0	0	6	3.5
	B	1982	17	1	0	0	0	0	18	9.5
	C	1988	10	2	0	0	0	0	12	7.0
NB	A	1997	2	1	0	0	0	0	3	2.0
	B	1992	7	1	0	0	0	0	8	4.5
	C	1979	19	1	1	0	0	0	21	12.0
0.75 Gy	A	1823	165	11	0	1	0	0	177	95.5
	B	1886	107	7	0	0	0	0	114	60.5
	C	1874	114	10	2	0	0	0	126	70.0
0.75 Gy + NB	A	1895	96	8	1	0	0	0	105	57.5
	B	1875	115	9	0	0	1	0	125	69.0
	C	1928	69	3	0	0	0	0	72	37.5
1.5 Gy	A	1643	303	50	2	1	1	0	357	209.0
	B	1488	402	98	9	2	1	0	512	319.0
	C	1681	271	42	6	0	0	0	319	186.5
1.5 Gy + NB*	A	1846	130	22	2	0	0	0	154	90.0
	B	1633	299	59	6	3	0	0	367	223.5
	C	1854	122	23	1	0	0	0	146	85.5
3.0 Gy	A	1183	533	215	56	7	4	2	817	595.5
	B	1084	552	279	68	14	3	0	916	692.5
	C	1364	421	165	41	7	2	0	636	456.0
3.0 Gy + NB*	A[a]	614	227	95	33	3	2	1	361	557.9
	B	1445	373	150	27	4	1	0	555	387.5
	C	1696	218	70	18	1	2	1	310	216.0

[a] 975 binucleated cells were analyzed. *Statistically significant, $P < 0.05$ (Fisher test).

NB posttreatment at different phases of the cell cycle in irradiated G_0-lymphocytes

The influence of NB treatment at different phases of the cell cycle was evaluated in G_0-lymphocytes irradiated with 2.0 Gy. There were no significant differences ($P > 0.05$) among treatments (Table III), indicating an absence of interaction effect between radiation and NB treatment at the G_0- and S-phase.

DISCUSSION

The influence of NB on radiation-induced chromosome damage in human lymphocytes was investigated. Posttreatment of cells with NB immediately after irradiation (G_0-phase) significantly reduced frequencies of MN. There was a decreasing trend in radiation-induced CA frequencies with NB, but it was not significant.

Combinations of NB with other genotoxic compounds have been studied by many authors. Lazutka and Rudaitiene (1992) reported that NB suppressed SCE induction by tumor necrosis factor (TNF-α) in human lymphocytes. Furthermore, Sakamoto-Hojo *et al.* (1994) showed that pretreatment of lymphocyte cultures with NB during the S/G_2 transition can reduce CA frequencies induced by the antitumoral drug ellipticine. Medeiros and Takahashi (1994) observed a similar result by combining NB with mitoxantrone during the G_2-phase of the cell cycle. However, the cell response to chemical and/or physical agents may be influenced by several factors, such as cell line or organism, drug concentration or dose, protocol of cell treatment, etc.

It is well known that the firm compactation of chromatin may impair the accessibility of DNA repair enzymes to the damaged sites, since variations in chromatin conformation and organization can affect the extent of DNA damage and repair (Wheeler and Wierowski, 1983; Yasui *et al.*, 1987). Chiu *et al.* (1986) showed that chromatin

Table II - Frequencies of chromosomal aberrations in gamma-irradiated G_0-lymphocytes posttreated with 50 µg/ml novobiocin (NB) (30-min pulse). Three experiments (A, B, C) were performed with different donors and 100 metaphases were analyzed per treatment for each donor.

Treatment	Donor	Mitotic index (%)	Chromosomal aberrations							Abnormal cells
			DIC	rings	DF	B'	B"	CR	Total	
Control	**A**	3.9	0	0	2	4	1	0	7	7
	B	7.5	0	0	4	4	0	0	8	8
	C	3.2	0	0	3	1	0	0	4	3
NB	**A**	1.0	1	0	3	3	0	0	7	7
	B	6.1	0	0	8	1	0	0	9	8
	C	3.1	0	0	1	2	0	0	3	3
0.75 Gy	**A**	1.7	7	0	9	2	0	0	18	16
	B	4.1	0	0	8	3	1	0	12	12
	C	1.8	2	0	9	0	0	0	11	9
0.75 Gy + NB	**A**	2.5	1	0	12	6	0	0	19	15
	B	4.6	3	0	17	0	1	0	21	18
	C	1.8	3	1	4	1	0	0	9	9
1.5 Gy	**A**	3.4	25	3	45	3	6	0	82	46
	B	3.1	5	0	17	0	0	0	22	18
	C	4.7	11	2	18	4	3	2	40	32
1.5 Gy + NB	**A**	2.2	25	4	37	11	6	1	84	55
	B	4.1	12	0	40	5	0	0	57	39
	C	4.1	15	3	23	3	3	1	48	38
3.0 Gy	**A**	3.2	51	5	86	2	5	0	149	71
	B	9.4	49	3	84	6	3	0	145	69
	C	2.8	56	1	58	3	2	2	122	66
3.0 Gy + NB	**A**	1.5	79	2	62	4	4	4	155	84
	B	4.2	34	2	63	2	0	2	103	62
	C	2.5	50	4	66	4	4	1	129	77

DIC: Dicentric chromosomes; DF: double fragments; B': chromatid breaks; B": isochromatid breaks; CR: complex rearrangements.

structure plays an important role in the formation and repair of DNA lesions in Chinese hamster cells exposed to γ-irradiation. Likewise, Udvardy and Schedl (1991) showed that chromatin structure, not sequence specificity, is the primary determinant in DNA topo II site selection, and that chromatin organization may provide a general mechanism for generating specificity in a wide range of DNA-protein interactions. Kapizewska and Lange (1991) observed that NB applied before and during irradiation prevents the induction of alterations in DNA supercoiling in two mouse leukemia cell lines differing in radiosensitivity, by modifying the DNA relaxation in the chromatin structure. Consequently, these alterations influence the X-ray-induced DNA damage and its repair.

Therefore, many lines of evidence in the literature are showing that the repair mechanisms may be influenced by the state of chromatin relaxation, supporting the possibility that in the present study, NB may have acted by changing the DNA structure in irradiated G_0-lymphocytes, and consequently the DNA strands became more accessible to the enzymes responsible for DNA repair. Thus, NB interference in the processing of the DNA lesions, caused by ionizing radiation at the G_0-phase, may lead to a modulation of cell response by reducing the degree of chromosome damage.

No complete correlation between the CA and MN frequencies was verified when G_0-lymphocytes were irradiated and posttreated with NB. It is well known that radiation-induced MN arise not only from acentric chromosomes or fragments, but also from whole chromosomes which were not incorporated into the main nuclei during mitosis (Fenech and Morley, 1985); thus, the presence of MN indicates not only structural chromosome damage, but also an alteration in chromosome number (aneugenic effect). With the conventional micronucleus test it is not possible to distinguish among micronuclei arising from acentric fragments induced by clastogens and those arising from whole chromosomes induced by spindle poisons (aneugenic agents). Attempts to overcome this limitation of the micronucleus test have included measurement of micronucleus size and DNA

Table III - Frequencies of chromosomal aberrations in gamma-irradiated G_0-lymphocytes posttreated with 25 µg/ml novobiocin (NB) for three hours at G_0- and S-phases of the cell cycle. Three experiments (A, B, C) were performed with different donors and 100 metaphases were analyzed per treatment for each donor.

Treatment	Donor	Mitotic index (%)	Chromosomal aberrations							Abnormal cells
			DIC	rings	DF	B'	B"	CR	Total	
Control	**A**	4.7	0	0	0	0	0	0	0	0
	B	3.2	0	0	0	0	1	0	1	1
	C	5.5	0	0	1	0	2	0	3	3
2.0 Gy	**A**	2.5	25	0	32	4	3	2	66	44
	B	3.6	23	2	25	1	1	0	52	36
	C	4.3	22	4	51	0	1	0	78	54
NB (G_0)	**A**	5.5	0	0	0	0	2	0	2	2
	B	3.4	0	0	6	0	3	0	9	7
	C	5.3	0	0	2	0	1	0	3	3
2.0 Gy	**A**	2.7	22	4	33	2	1	0	62	45
+ NB (G_0)	**B**	2.9	19	2	29	1	6	0	57	43
	C	3.9	24	4	25	0	1	1	55	39
NB (S)	**A**	2.3	0	0	1	0	4	0	5	5
	B	4.9	0	0	4	0	0	0	4	4
	C	3.5	0	0	3	0	4	0	7	7
2.0 Gy	**A**	1.0	27	0	42	0	3	0	72	50
+ NB (S)	**B**	2.8	36	0	29	0	2	0	67	50
	C	2.1	19	1	36	0	0	1	57	42

Abbreviations defined in Table II.

content, C-banding and, more recently, *in situ* hybridization with DNA probes (Miller *et al.*, 1992).

Cornforth and Goodwin (1991) demonstrated that about 50% of all radiation-induced chromosome fragments in metaphase cells were found as MN in the subsequent interphase. Fragments which were unable to form MN could be incorporated into the daughter nuclei during division, however some fragments could coalesce and form micronuclei containing more than one acentric fragment (Savage, 1988; Kramer *et al.*, 1990; Cornforth and Goodwin, 1991). Miller *et al.* (1992) used simultaneous fluorescence *in situ* hybridization with telomere- and centromere-specific probes, to show that in nearly all MN containing a centromeric signal (spontaneous and radiation induced) the centromeric signal was accompanied by two to four telomeric signals in the same MN, indicating the presence of a whole chromosome. Similarly, the use of antibodies against centromeres has revealed that usually 30 to 50% of the total MN include whole chromosomes in untreated established cell lines and in biopsies from human rectal carcinomas (Weissenborn and Streffer, 1991).

Therefore, the absence of correlation between CA and MN frequencies observed in our study is in accordance with the following evidence: some specific types of CA may form MN in preference to other aberrations (Wakata and Sasaki, 1987), multiple fragments may reside within a single MN (Jonhston *et al.*, 1997) and a considerable percentage of MN may have originated from whole chromosomes due to disturbance of the cell spindle (Walker *et al.*, 1996). According to Johnston *et al.* (1997), the interactions between the initial damage, repair processes, cell cycle-dependent variations in sensitivity and changes in cell kinetics can produce complex patterns of MN expression. In the case of CA analysis, the cell cycle kinetics of lymphocyte cultures should be considered as an important factor influencing the cell response when colchicine-arrested metaphases are scored for aberrations. Although cultures were fixed after 52 h, most of the aberrations were observed in 1st-cycle cells, since the chromosome-type ones were most frequent, resulting in a low percentage of 2nd-cycle cells.

Boei *et al.* (1996) showed that most chromosome breaks are repaired within a few hours after the induction of the damage in human lymphocytes, since the frequencies of radiation-induced aberrations decrease between the first and the second cycle, and are reduced drastically between the second and third cell cycle. Similarly, Darroudi *et al.* (1998) observed that in irradiated human lymphocytes (1, 2, 4 and 6 Gy) almost 50% of breaks were rejoined after 1 h, and about 20% of breaks remained after 18 h. In addition, these authors showed that at low doses of 1 and 2 Gy most of the exchanges were formed immediately, and, at higher doses, the frequency of exchanges increased at rates similar to those observed for the rejoining of breaks.

Thus, it seems that an efficient mechanism of repair process occurs immediately after cell irradiation, in-

dicating that in the present work, NB may have acted in a critical period after the induction of DNA damage, reducing the number of lesions which would be converted into chromosome damage. NB reduced the MN but not the CA frequencies induced by γ-irradiation during the G_0-phase of the cell cycle. Even though NB acts on different cell targets and by different pathways, our results support the evidence (Villeponteau *et al.*, 1984; Kapizewska and Lange, 1991) that NB interaction increases the accessibility of chromatin to DNA repair enzymes during the G_0-phase of the cell cycle by affecting its structure.

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RESUMO

Com o objetivo de estudar o mecanismo de indução do dano cromossômico, foi testada a influência do pós-tratamento com a novobiocina (NB) sobre as freqüências de micronúcleos (MN) e aberrações cromossômicas (CA) induzidas pela radiação gama (0,75, 1,5 e 3,0 Gy) em linfócitos humanos na fase G_0. Os experimentos realizados com 4 doadores demonstraram que as freqüências de MN sofreram um decréscimo estatisticamente significativo, 44 e 50%, para o tratamento com 50 µg/ml de NB (pulso de 30 min) após as doses de 1,5 e 3,0 Gy de radiação, respectivamente. Entretanto, o mesmo grau de interação não foi observado para as freqüências de AC e reduções não significativas foram obtidas para todos os tratamentos combinados em protocolos semelhantes. A ausência de um efeito de interação foi também observada quando os linfócitos em cultura foram expostos à dose única de 2,0 Gy na fase G_0 e pós-tratados com 25 µg/ml de NB por 3 horas logo após a irradiação (fase G_0) e após 24 horas (fase S) de incubação. O efeito supressivo da NB sobre as freqüências de MNs na fase G_0 do ciclo celular é discutido em termos de indução de modificações na estrutura da cromatina, aumentando o acesso às enzimas de reparo do DNA, e podendo, assim, ser observada uma modulação da resposta celular nos linfócitos irradiados em G_0 e pós-tratados com NB.

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