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MICRONUCLEI FREQUENCIES IN PERIPHERAL BLOOD LYMPHOCYTES OF INDIVIDUALS EXPOSED TO DEPLETED URANIUM*

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One of the negative environmental impacts of the last armed conflict in Bosnia and Herzegovina was the use of radioactive ammunition containing depleted uranium. The United Nations Environment Programme measurements detected higher radioactivity at several examined sites in Bosnia and Herzegovina. One of those places is in the area of Hadžići, close to Sarajevo. This research included an evaluation of genetic load in human lymphocytes due to exposure to depleted uranium. The study included individuals who were located in the area of Hadžići and who were directly exposed to depleted uranium. The control blood samples were taken from individuals who lived in West Herzegovina which is considered environmentally uncontaminated. The results of the micronucleus cytochalasin-B test in peripheral blood lymphocytes showed increased micronuclei frequencies in the exposed group.

KEY WORDS: *Bosnia and Herzegovina, environmental genotoxicity, human lymphocytes, micronucleus*

War, as the most extreme form of violence, can result in exposure of humans to different chemical and physical genotoxic agents. During and for some time after the recent armed conflict in Bosnia and Herzegovina (1992-1995), the citizens of this country were exposed to various genotoxic agents and the impact on their genetic constitution has not yet been evaluated. Genotoxic agents which are present in the environment as a result of war activities and which may affect the genetic constitution of exposed persons include decomposed products of used ammunition, food additives, pharmaceuticals and food of suspicious quality and origin. Most mutagens and genotoxic carcinogens efficiently induce chromosomal changes associated with congenital abnormalities and neoplasia (1).

One of possible war-related genotoxicants is depleted uranium (DU). The origin of DU in the environment is connected with use of radioactive anti-tank ammunition containing DU in 1994 and 1995 in Bosnia and Herzegovina. Although DU has been used for the last 20 years in the manufacture of armour and ammunition (2), the first use of DU was in the Gulf War (1991) and it continued in the Balkans (Bosnia, Kosovo, and Serbia in mid 1990s). In the air strikes in Bosnia, NATO fired more than 10000 rounds of DU ammunitions (3.3 tonnes of DU). The United Nations Environment Programme experts confirmed higher radioactivity at several localities where DU was used. Higher radioactivity was also observed at the tank-repair facility and ammunition storage area in Hadžići near Sarajevo (3).

Depleted uranium is a waste product of the nuclear industry. It predominately contains radionuclide ^{238}U whose half-life is approximately $4470x10^6$ years (4). The main argument in favour of the frequent usage of

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DU is that it possesses only 60 % of natural uranium radioactivity, having been "depleted" of much of its most highly radioactive ^{234}U and ^{235}U isotopes. During decay, uranium isotopes emit alpha-particles, which possess high energy, but are poorly penetrating (5). When the ammunition containing DU hits a target, a fine aerosol is formed and particles are easily inhaled being especially dangerous if they enter deep into the lungs (4). Depleted uranium is an internal health hazard predominantly affecting skeletal tissue and kidneys (6). It has also been connected with the increased number of leukaemias and other cancers among the participants of the Gulf War and operations in the Balkans (4, 5).

A wide range of cytogenetic investigations has confirmed that different types of radiation cause various chromosomal damages. Human-lymphocyte micronucleus assay is a reliable and accurate method for assessing chromosome damage, including whole chromosome loss and chromosome breaks, as well as for testing genotoxicity. Micronucleus frequency in cultured human lymphocytes provides information about accumulated genetic damage occurring during the life span of circulating lymphocytes (7). Most peripheral blood lymphocytes live long (65 % to 85 %), especially T-lymphocytes. Estimations on the life span of human T-lymphocytes vary from 1.5 to 10 years (8). Although conducted several years after radiation exposure, numerous chromosomal aberration studies in the survivors of atomic bombing and in Chernobyl workers (9) have confirmed that human lymphocyte genome is a reliable biological model for this kind of investigation.

The aim of this study was to evaluate the genotoxicity of environmental DU using the micronucleus cytochalasin-B test and to determine human health risk. According to this aim and the possibilities of the applied method, we determined micronucleated binucleated cell frequencies and relative frequencies of micronuclei. We also compared the observed cytogenetic parameters between groups; and evaluated overall DU effects.

MATERIAL AND METHODS

Study groups

This study investigated over 60 blood samples of individuals from two groups. The exposed group included the employees of a tank-repair facility and ammunition storage in Hadžići who were environmentally exposed to DU. Blood samples were taken three times from this group: in November 2002, December 2002, and February 2003. The control group consisted of the inhabitants of West Herzegovina, which was spared war activities and is considered environmentally clean. Their blood samples were taken in March 2003. No volunteers from this group were professionally exposed to dangerous or toxic agents. The participants from both groups were volunteers not subjected to any medical treatment or radiation for diagnostic or therapeutic purposes. They completed a comprehensive questionnaire about demographic information such as age, lifestyle factors (tobacco usage), profession, medical history and exposure to chemical or radiotherapy. Fourteen subjects in the exposed group were smokers. Ten of them smoked less than 20 cigarettes a day for 15-20 years and the other four smoked over 20 cigarettes a day for over 20 years. In the control group, three subjects smoked less than 20 cigarettes a day for ten years and one subject smoked up to 40 cigarettes a day for ten years. Two subjects in this group smoked less than 15 cigarettes a day for 15 years and others in the same group smoked more than 30 cigarettes a day for over 20 years. In the exposed group 90 % of subjects were men, while in the control group, this ratio between sexes was more balanced (Table 1).

Table 1 *Demographic characteristics of analysed groups*

		Exposed group	Control group
Number of men		27	13
Number of women		3	17
Number of smokers		14	13
Age range / years		35-58	12-59
Average age / years	Men	46.07	37.23
	Women	46.3	41.88
	All	47.1	39.87

Method

Micronucleus cytochalasin-B test used in this study is based on the observation of small extranuclear formations (micronuclei) of genetic material in cytoplasm. The expression of micronuclei is the consequence of chromosome breaks or spindle disruption (7, 10, 11). Thus micronuclei originate from either acentric fragments or whole chromatids or chromosomes. Only dividing cells can express micronuclei. Cytochalasin B is used to stop dividing cells from performing cytokinesis and thus those

cells that have completed one nuclear division are recognized by their binucleated appearance.

Sample collection and cultivation

Fresh blood was collected by venepuncture and transferred into sterile, heparinized vacutainers. The lymphocytes were cultured in 15 mL sterile plastic tubes with conical bottom (NUNC) positioned obliquely and incubated at 37 °C. Blood (0.4 mL) was added to 5 mL of RPMI 1640 medium with L-glutamine, 20 % of foetal bovine serum, 10 μg mL^{-1} of phytohaemaglutinin and 200 IU mL^{-1} of penicillin-streptomycin solution (GIBCO BRL). On the 44th hour of phytohaemaglutinin stimulation, cytochalasin B (SIGMA) was added to the cultures to the final concentration of 4.5 μg mL^{-1}. Cells were harvested on the 72nd hour of cultivation. After centrifugation, cultures were briefly treated with a hypotonic solution (0.56 % KCl). In further processing, cold and fresh fixative (3:1, ethanol : acetic acid) was added to the cell suspensions. After the third fixation stage, cell pellet was gently resuspended once more in a few drops of fresh fixative and the suspension was dropped on clean, cold and dry microscope slides. Dry slides were stained with a 5 % Giemsa stain for 8 minutes and then washed in distilled water.

Microscopic analysis

Air-dried and coded slides were analysed using a light microscope at 400x magnification. Only cells with clearly visible cytoplasm and approximately similar-sized nuclei were analyzed. Micronuclei were scored in no less than 1000 binucleated cells per sample, according to the criteria given by *Surralles and Natarajan* (12). To minimize the impact of inter-scorer variability, all samples were scored and analyzed by two experienced researchers.

Statistical analysis

The obtained results were statistically processed and the significance was estimated by the Student's *t*-test, *t* % test and chi square test.

RESULTS AND DISCUSSION

Our cytogenetic analysis of peripheral blood lymphocytes showed that the number of binucleated cells, that is, cells with one or more micronuclei, ranged from 5 to 50 in the exposed, and from two to 43 in the control group. In both groups the most frequent micronucleated cells were the ones with one micronucleus. The absolute frequency of binucleated cells with one or more micronuclei as well as the relative frequency of micronuclei per 1000 binucleated cells (%) was determined for each subject (Table 2).

Paired Student's *t*-test did not show any statistical significance between the two groups in the average frequency of binucleated cells with one micronucleus ($t=1.92819$; $p=0.0637$), with two micronuclei ($t=0$; $p=1$), and with three micronuclei ($t=1.64886$; $p=0.11$). In addition, no statistical significance was found in the total number of micronucleated binucleated cells ($t=1.83738$; $p=0.0764$). The chi-square test showed no statistical significance in the distribution of binucleated cells with micronuclei in both analyzed groups ($0.10>p>0.05$).

For each examined individual, the relative frequency of micronuclei per 1000 binucleated cells was evidenced and basic statistical parameters were determined. The observed micronuclei frequencies were compared with the criteria for spontaneous micronuclei (4.4±2.6 per 500 binucleated cells) given by *Fenech and Morley* (13). In the exposed group, 18 individual samples (60 %) showed an increase in the micronucleus frequency while in the control group, the increased frequency of micronuclei was found in 11 blood samples (36.66 %). Seven subjects with the increased micronucleus frequency in the exposed group and five in the control group smoked tobacco. In other words, this study revealed that the increased number of micronuclei was more common in non-smokers. Moreover, the most extreme evidence of micronucleus formation was in a non-smoking women in the control group. Although men dominated in the exposed group, two of the three women from this study group showed increased micronucleus frequency. In the control group, eight of eleven subjects with increased micronucleus frequency were women. This suggests that sex is an important variable in studies that include cytokinesis-blocked micronucleus assay as a biomarker of chromosome damage, possibly connected with the loss of the X chromosome (14). We have also found an enormously high frequency of micronuclei in a control woman in comparison with other individuals from the same group. This could be connected with isolated exposure of this person to unidentified genotoxic agent.

The *t* % test confirmed statistically significant differences in the relative frequencies of micronuclei

Table 2 *Results of micronucleus cytochalasin-B test in the exposed and control group*

SAMPLE	Exposed group					Control group				
	Absolute frequency of micronucleated binucleated cells				Relative MN frequency / %	Absolute frequency of micronucleated binucleated cells				Relative MN frequency / %
	1 MN	2 MNs	3 MNs	Sum		1 MN	2 MNs	3 MNs	Sum	
1	28	-	-	28	2.8	9	2	-	11	1.3
2	8	-	-	8	0.8	5	-	-	5	0.5
3	11	2	-	13	1.5	19	1	-	20	2.1
4	10	1	-	11	1.2	10	-	-	10	1.0
5	15	2	-	17	1.9	11	-	-	11	1.1
6	20	1	-	21	2.2	10	-	-	10	1.0
7	11	-	-	11	1.1	3	-	-	3	0.3
8	11	1	-	12	1.3	18	1	-	19	2.0
9	14	1	-	15	1.6	9	1	-	10	1.1
10	30	2	-	32	3.4	4	-	-	4	0.4
11	16	-	-	16	1.6	39	4	-	43	4.7
12	46	4	-	50	5.4	9	-	-	9	0.9
13	8	1	-	9	1.0	3	-	-	3	0.3
14	13	3	2	18	2.5	8	-	-	8	0.8
15	14	-	-	14	1.4	20	3	-	23	2.6
16	14	1	-	15	1.6	21	3	-	24	2.7
17	6	-	1	7	0.9	16	1	-	17	1.8
18	13	-	2	15	1.9	6	1	-	7	0.8
19	12	-	-	12	1.2	9	1	-	10	1.1
20	5	-	-	5	0.5	8	-	-	8	0.8
21	18	1	2	21	2.6	13	2	-	15	1.7
22	9	-	-	9	0.9	12	3	-	15	1.8
23	15	1	-	16	1.7	13	2	1	16	2.0
24	13	3	-	16	1.9	3	2	-	5	0.7
25	21	1	-	22	2.3	2	-	-	2	0.2
26	21	1	-	22	2.3	3	-	-	3	0.3
27	18	1	-	19	2.0	3	-	-	3	0.3
28	19	2	-	21	2.3	22	3	-	25	2.8
29	6	1	-	7	0.8	17	1	-	18	1.9
30	18	1	-	19	2.0	10	-	-	10	1.0
Σ	463	31	7	501	54.6	335	31	1	367	40
x	15.4	1.03	0.23	16.7	1.82	11.11	1.03	0.03	1.23	1.33
s	8.26	1.03	0.62	8.81	0.95	7.91	1.21	0.18	8.85	0.98
sx	1.50	0.18	0.11	1.60	0.17	1.44	0.22	0.03	1.61	0.18
V	53.5	100	268	52.7	52.4	71.1	117	557	7.18	74.1

MN = micronucleus

between the exposed and control group (t %=2.1747; 0.05>p>0.02). We are fully aware that a more adequate control in this study would have been to compare this micronucleus frequency with results obtained from the same subject prior to exposure to DU, had these results been available.

The report released by the United Nations Environment Programme in March 2003 confirms that DU from weapons in Bosnia and Herzegovina contaminated the local supplies of drinking water and was still found in the air. At the site of the Hadžići tank-repair facility, traces of DU were found in water from two wells: 0.38 μg L^{-1} (14 % DU in total U) and 0.55 μg L^{-1} (73.4 % DU in total U). For comparison, the current WHO provisional guideline for uranium in drinking water is 9 μg L^{-1}. Total uranium concentration found in the air ranged between 0.022 ng m^{-3} and 3.6 ng m^{-3}, with the DU content of 25 % to 99 %. The

expected range of natural uranium concentrations in the air is 0.008 ng m^{-3} to 0.8 ng m^{-3}. No DU was detected in the air samples taken outside the radius of 100 meters from the exposed sites. According to the United Nations Environment Programme (UNEP) experts, recorded contamination levels are very low and do not present immediate radioactive or toxic risks for the environment or human health (3). At this point however, we are not able to determine the dose-response relationship; one has to be aware of probable inhalation and long-term exposure to low concentrations and possible delayed effects of DU radioactivity.

Reports of leukaemias and other cancer cases among the servicemen in the Gulf War and the Balkans war may be associated with DU as one of the causative factors. The uranium oxide dust consists of a mixture of uranium oxides with different physical and biochemical properties. All of them are chemically toxic as well as radioactive.

The analysis of the slides revealed that, beside the number, the size of micronuclei also varied. This implies a different origin of the micronuclei. There are four recognised mechanisms of micronucleus formation: mitotic loss of acentric fragments, chromosome breaks and exchanges, mitotic loss of whole chromosomes, and apoptosis. Clastogenic agents usually induce the formation of smaller micronuclei while aneugenic agents are responsible for the formation of larger micronuclei (10, 15). The findings of both small and large micronuclei in this study are in accordance with our earlier findings of chromosomal and chromatid breaks and aneuploidy in exposed individuals (16). Examinations of sixteen UN Gulf War and Balkan War veterans, expected to be exposed to DU, showed a statistically significant increase in dicentric and centric ring chromosomes in comparison with controls (17).

Our analysis of micronuclei will be followed by an investigation of chromosomal aberrations and sister chromatid exchange in slides obtained from the same blood samples cultivated at the time of this study. These results will be presented separately.

CONCLUSION

As mutagenic substances are widely spread in the environment, defining a reliable control population in cytogenetic studies is an important issue. Despite this problem, our results show a statistically significant increase in micronucleus frequencies in the exposed group in comparison with the control group. These results suggest that DU leads to an increase in micronucleus frequencies and genetic load in exposed humans. The formation of small and large micronuclei indicates that DU acts both as a clastogenic and aneugenic agent.

A more detailed evaluation of the genotoxic effects of depleted uranium calls for additional in-depth cytogenetic and molecular investigations.

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Sažetak

FREKVENCIJA MIKRONUKLEUSA U LIMFOCITIMA PERIFERNE KRVI OSOBA IZLOŽENIH OSIROMAŠENOM URANU

Jedan od genotoksina, prisutnih u okolišu kao posljedica ratnih djelovanja u Bosni i Hercegovini jest osiromašeni uran. Njegovo porijeklo veže se za upotrebu radioaktivne antitenkovske municije s osiromašenim uranom. UNEP-ova mjerenja otkrila su povećanu radioaktivnost na nekoliko ispitanih lokaliteta od kojih je jedan na području Hadžića, u blizini Sarajeva. Naše istraživanje obuhvatilo je evaluaciju genetičkog opterećenja u humanim limfocitima periferne krvi osoba koje su bile nastanjene na području Hadžića te bile direktno izložene osiromašenom uranu. Kao kontrola u istraživanju, uzeta je krv od osoba nastanjenih na području zapadne Hercegovine, koja se smatra ekološki nekontaminiranom. Korištena je metoda mikronukleus-citokalazin B testa, a frekvencije mikronukleusa ispitanika iz obje populacije međusobno su komparirane. Rezultati istraživanja pokazuju povećanu frekvenciju mikronukleusa među ispitanicima eksponirane populacije.

KLJUČNE RIJEČI: *Bosna i Hercegovina, genotoksičnost okoliša, humani limfociti, mikronukleus*

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