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Phytohaemagglutinin as a modulator of DNA repair measured by chromosome aberration analysis in micronucleus assay in ionizing radiation biodosimetry

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Background. *There are some correlations between cell's ability to remove DNA damage and proliferative activity. The aim of this study was to examine the influence of phytohaemagglutinin (PHA) on DNA repair capacity in isolated human lymphocytes exposed to ionizing radiation.*

Methods. *Lymphocytes were isolated from the whole blood using a Ficoll centrifugation. As the source of γ-rays ^{60}Co source Alcon, CGR-MeV was used. To achieve the absorbed dose of 2 Gy a total exposure to radiation lasted for 1.24 minutes at room temperature. Possible differences in DNA repair efficiency were monitored by chromosomal aberration analysis and micronucleus assay, 48 and 72 h after the PHA stimulation, respectively.*

Results. *The number of dicentric chromosomes and acentric fragments were significantly increased in lymphocytes stimulated by phytohaemagglutinin immediately after the irradiation compared to the cultures where the activator was added after 1, 2 and 4 h. The micronucleus assay did not show any significant differences in the number and distribution of micronuclei regardless of the time when the mitogen activator was added.*

Conclusions. *The observed non-significant decreases in the total number of chromosomal aberration and micronuclei suggest that phytohaemagglutinin does not significantly contribute to the DNA repair.*

Key words: ionizing radiation; DNA damage; DNA repair; phytohaemagglutinins; chromosome aberrations; micronucleus test

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Introduction

During the last few decades ionizing radiation became unavoidably present in human lives. It is widely used in the variety of medical diagnostic procedures as electric energy source of nuclear power plants. It affects a human organism on daily basis in the form of cosmic radiation. Since air flights are becoming more common way of travelling, and it is

known that cosmic radiation is significantly higher in upper layers of the atmospheres, the total dose population is expected to increases. It is known that ionizing radiation (IR) deposits its energy in cellular structures through discrete ionization events that are essentially randomly distributed in space. Unlike chemical agents, whose damaging potential is strongly dependent on diffusion processes and thus may be affected by subcellular structures, IR is highly penetrating: the physics and subsequent chemistry associated with the photon absorption and the ionizing events that occur along fast electron track are complete within a few microseconds.[1] Ionizing radiation causes a wide spectrum of chemically different types of lesions in DNA of which the so-called locally multiply damaged sites (LMDS) are assumed to be biologically most important.[2,3] LMDS may consist of single-strand breaks (SSB) on opposite strands that, if located close to each other, may give rise to double strand breaks (DSB).[4] Thus, DSB induced by IR may arise as a direct consequence of one or more ionizing events or indirectly as a base or sugar damage on opposite strands. Ionizing radiation in physicochemical interaction with cellular DNA also produces a variety of primary lesions, alkali-labile sites, DNA-DNA and DNA- protein crosslinks, and damage to purine and pyrimidine bases.[5-8] DNA double strand breaks (DSB) are the most serious form of DNA damage. There are two pathways for the repair of DSB.[9] One is homologous recombination (HR) which occurs during late S and G2 phase of the cell cycle and the other pathway is non homologous DNA end joining (NHEJ). It is predominant during G0, G1 and S phase of the cell cycle.[10] If not repaired, DNA lesions could cause cell death. If misrepaired, DSBs contribute to chromosomal aberrations and genomic instability. Ionizing radiation produces chromosome aberrations (involving two chromatids) at the S phase and the chromatid aberrations at G2. To detect genetic alterations at the chromosome level using chromosome aberration and micronucleus assay the cells should be induced to enter the G1 phase and undergo division.[11-14]

Phytohaemagglutinin (PHA) selectively stimulates T lymphocytes to enter mitosis. The widespread popularity of peripheral blood culture as means of chromosome analysis has been largely dependent on that mitogen.[15-23] There is some correlation between cell's ability to remove the DNA damage and its proliferative activity. It is well established that unstable aberrations like dicentric chromosomes, chromosome breaks and acentric fragments can be eliminated during the cell division. It was suggested that the regulation of DNA repair is dependent on cell cycle. It involves the expression of DNA repair enzymes within the defined program of gene control during the cell cycle. Some authors have shown that immediately after the irradiation mitogen-stimulated cells have a higher frequency of chromosome aberrations than the cells resting in G_0 phase before the addition of mitogens.[15]

Our study aimed to examine the influence of PHA on DNA repair capacity of isolated human lymphocytes. Cell cultures were started and PHA was added 0, 1, 2 and 4 h after the irradiation. Possible differences in the DNA repair efficiency was monitored by chromosomal aberration analysis and micronucleus assay, 48 and 72 h after the PHA stimulation, respectively.

Methods

Isolation of lymphocytes

The whole blood sample was taken from the cubital vein of a healthy adult male volunteer using heparinized vacutainer (Becton Dickinson, USA). There is no record that prior to the study the volunteer was exposed to any physical or chemical agent that might in-

terfere with the results. Lymphocytes were isolated from the whole blood sample by a Ficoll centrifugation method.[24] One milliliter of the whole blood was resuspended in 8 ml of Ham's F-10 essential medium supplemented with L-glutamine, bovine serum (20%), penicillin (100 I.U./ml) and streptomycin (100 μg/ml).

Irradiation of isolated lymphocyte

As the source of γ-rays ^{60}Co source Alcyon, CGR-MeV was used. Vacutainer containing isolated lymphocytes was mounted in an acrylic phantom (dimensions: 20x20x15 cm^3), in depth of 5.5 cm, transversally to the axis of the irradiation. The radiation field was 15 x 15 cm^2, and the distance between the surface of phantom and the source of radiation was 80 cm. At total exposure to radiation lasted for 1.24 min at room temperature, thus the absorbed dose was 2 Gy.

Cultivation of lymphocytes

Phytohaemagglutinin (Murex Biotech Ltd.) (0,2ml) was added to lymphocyte cultures either immediately after the irradiation or after a certain recovery period (1, 2 or 4 h). Meanwhile cultures were held at 37°C.[13] Since the analysis was done in duplicate, for each stimulation time, 4 different cultures were started: for the chromosomal aberration analysis and for the micronucleus assay.

The analysis of structural chromosome aberrations

The structural chromosome aberration analysis test was performed according to current IAEA guidelines.[25] Simultaneously with cultures for the micronuclei assay, cultures for the chromosome aberration test were set up in the same manner. Duplicate cultures per sample were set up and incubated at 37°C for 48 h. After the PHA stimulation to arrest dividing lymphocytes in metaphase, colchicine (Sigma) (0.004%) was added 2 h prior to the harvest. Cultures were centrifuged at 1000 rpm for 10 min, the supernatant was carefully removed, and the cells were resuspended in a hypotonic solution (0.075M KCl) at 37°C for 20 min. After the second centrifugation, the cells were fixed with a freshly prepared fixative of ice-cold methanol/glacial acetic acid (v/v 3:1). Fixation and centrifugation were repeated several times until the supernatants were clear. The cell suspension was dropped onto microscope slides and left to air-dry. Slides were stained with 5% Giemsa solution (Merck). For each stimulation analysis was done in duplicate, a total number of 200 methaphases was scored. Structural chromosome aberrations were classified based on the number of sister chromatids and breakage events involved. Only metaphases containing 46 centromeres were analyzed. A total number of each type of aberrations, as well as the percentage of aberrant cells per subject were evaluated.

Micronucleus assay

The micronucleus assay was performed as described by Fenech and Morley with some modifications.[26] After the irradiation lymphocyte cultures were set up by adding 1 ml of isolated lymphocytes to 8 ml of F-10 medium (Sigma) supplemented with foetal calf serum (Sigma) and antibiotics penicillin (Pliva) and streptomycin (Krka). Following the stimulation with PHA, lymphocytes were incubated in vitro for 72 h at 37°C. Cytochalasin-B (Sigma) at the final concentration of 6 mg/ml was added to each culture at 44 h, and the cells were harvested after a further incubation of 28 h. After the treatment with physiological saline, cells were fixed with cold fixative, a mixture of methanol: acetic acid (v/v 3:1). The fixation step was repeated twice and cells were resuspended in a small volume of fixative solution and dropped onto clean slides.

Finally, they were stained with 5% aqueous solution of Giemsa dye (Merck) for 10 minutes. For each stimulation the analysis was done in duplicate, thus a total of 500 binuclear lymphocytes were scored. The data are expressed as the number of micronuclei per 500 binucleated cells as well as the frequency of binucleated cells containing one or more micronuclei.

Statistical analysis

The statistical significance of the results obtained was evaluated using the $\chi 2$ - test. The level of statistical significance was set at 5%. Chi-Square test was used to compare the frequencies of chromosomal aberration and micronuclei.

Results

Chromosomal aberration analysis

The number of dicentric chromosomes and acentric fragments was found to be significantly increased in all irradiated lymphocytes regardless of the start point of the PHA stimulation compared to the control ($p<5\%$). In irradiation exposed sample there were 97 acentric fragments and 12 dicentrics observed in PHA stimulated lymphocytes whereas in the control no dicentrics and 4 acentic fragments were found. The number of aberrations between cultures stimulated 1, 2 and 4 hours after the irradiation did not differ significantly ($p< 5\%$).

In cultures stimulated with PHA 1, 2 and 4 hours after the irradiation the number of acentric chromosomes and dicentric chromosomes significantly decreased compared to the cultures where mitogen was added immediately after the irradiation. One hour after the irradiation the number of acentrics was 57, two hours after the irradiation the number of acentric chromosomes was 69 and four hours after the irradiation the number of acentrics was 48. The number of dicentric chromosomes decreased compared to the number of acentric chromosomes. Immediately after the irradiation the number of dicentric chromosomes was 12, one hour after the stimulation the number of dicentrics was 1. Still, the difference in the number of dicentrics was not found to be significant (Table 1).

Micronucleus assay

Using micronucleus assay no significant differences in number of micronuclei were ob-

Table 1. Total number and distribution of chromosome aberrations in isolated human lymphocytes stimulated to proliferate after the indicated post-irradiation periods. Two hundred cells were analyzed per each PHA stimulation point.

Time after irradiation	Total number of abberations	Chromatid breaks	Chromosome breaks	Acentric fragments	Dicentric chromosomes	%of cells with abberations
Isolated lymphocytes irradiated with 2 Gy						
0 h	112[a]	3	/	97[a]	12a	38.0
1 h	58[a,b]	/	/	57[a,b]	1	21.5
2 h	76[a,b]	1	1	69[a,b]	5	29.0
4 h	53[a,b]	/	/	48[b]	5	23.0
Non-irradiated samples						
Lymphocytes	4	/	/	4	/	2.0
Whole blood	2	/	/	2	/	1.0

[a]statistically significant compared to the control $P < 0.05$

[b]statistically significant compared to the 0h PHA stimulation point $P < 0.05$

Table 2. Frequencies of micronuclei in binucleated human lymphocytes stimulated to proliferate after the indicated post-irradiation periods. Five hundred cells were analyzed per each PHA stimulation point.

Time after irradiation	Cells without MN	Cells with MN	S MN / 500 cells	Distribution of micronuclei				MN / Cell
				1 MN	2 MN	3 MN	4 MN	
Isolated lymphocytes irradiated with 2 Gy								
0 h	396	104	116	94	8	2	/	0.23
1 h	411	89	104	74	15	/	/	0.21
2 h	399	101	117	89	9	2	1	0.23
4 h	411	89	95	83	6	/	/	0.19
Non-irradiated samples								
Lymphocytes	499	1	1	1	/	/	/	0.002
Whole blood	498	2	3	1	1	/	/	0.006

MN = micronuclei

served, regardless of the time mitogen activator was added. In irradiated cultures the number of micronuclei per cell ranged from 0.19-1.23 compared to the control where it was 0.002.

Discussion

This study presented the possible influence of phytohaemagglutinin on DNA-repair scoring the number of chromosome aberrations and micronuclei. To initiate DNA damage lymphocytes were irradiated with 2 Gy [22,23] using a γ-ray ^{60}Co source. The number of acentric fragments was significantly increased in lymphocytes stimulated by phytohaemagglutinin immediately after the irradiation compared to the cultures where the activator after 1, 2 and 4 h was added (Table 1). That finding could indicate that DSB repair mechanisms are efficient in G_0 phase of the cycle and/or that the stimulation of lymphocytes to undergo division without having time to eliminate the majority of the DNA lesions in G_0 phase increases a misrepair rate resulting in the increased number of chromosome type aberrations. These results support the finding that the formation of unstable aberrations is cell cycle dependent and that most of double strand breaks can be fixed in first 24 h after the irradiation.[27]

The same result but with different approach was observed by Mayer *et al.* They showed that a higher level of the DNA repair events in stimulated cells does not necessarily reflect a higher DNA repair capacity. Additionally, they showed that all repair proteins needed for the repair of γ-irradiation induced DNA-damage are already present in G_0 cells at sufficient amounts and do not need to be induced once lymphocytes are stimulated to start cycling.[28] Only specific DNA repair genes were found to be up-regulated after the PHA stimulation of which most have an additional function in the DNA replication. The mitogen stimulation of lymphocytes may result in an increased removal of only specific types of DNA lesions as it was reported by other authors.[29,30] This observation might be explained by the cell cycle dependent regulation of specific DNA repair enzymes, that are more active in proliferating than in resting cells or by differences in the availability of deoxyribonucleotides which are necessary for the DNA excision repair which is not involved in DSB repair.[30,31] Mayer *et al.*[28] identified only 12 genes that responded with a more than 2-fold increase of transcripts to the mitogenic stimulus, with a maximum induction for each of the genes 72 h after the PHA treatment. A decrease in the number of chromosome type aberrations with the delay of PHA stimuli could indicate the gradual activation of addi-

tional repair capacities, but still the decrease was not to found to be significant. That observation is in the correlation with findings that more than 70% of all evaluated genes had constant expression levels within a twofold range compared to unstimulated.[28]

As shown in the Table 2. no significant differences were observed in the number of micronuclei, regardless of the time point when the mitogen activator was added. Nevertheless, the number of micronuclei for specific PHA stimulation point was significantly higher than the number of chromosomal type of aberrations. It indicates that all micronuclei formed do not originate from acentrics only but also from entire chromosomes.[32] Microtubules remain unsorted within the mitotic plane forming micronuclei.[11] Obtained results could indicate that the repair of those lesions is not dependent on the time passed between the irradiation and the mitogen stimulation. Our results show the baseline level of frequency of micronuclei after 3 cell cycles which is the same as Ramirez *et al.* observed.[32] The observed non-significant decreases in the total number of chromosomal aberration and micronuclei suggest that phytohaemagglutinin does not significantly contribute to the DNA repair. We could say that in order to maximize the sensitivity of the chromosomal aberration analysis phytohaemagglutinin has to be added immediately after the irradiation.

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