INCIDENCE OF RADIATION-INDUCED MICRONUCLEI IN OCCUPATIONALLY EXPOSED SUBJECTS

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Abstract. The micronucleus test was applied in human circulating lymphocytes as an ex *in vivo* measure of chromosome breakage using the cytokinesis-block micronucleus assay (CBMN) in order to detect the genotoxic-induced action of ionizing radiation in occupationally exposed subjects. The analyzed lots comprised: 105 individuals professionally exposed (68 males and 37 females) and 40 controls (20 males and 20 females). The micronucleation was provided through the CBMN technique, by the micronuclei score in 1000 binucleated lymphocytes/individual. This paper presents: A. The structural and morphological effects on cell nucleus and B. The individual response to the physical noxa, depending on gender, age and length of work in the radioactive environment. When the control and the exposed groups were compared, the induced micronuclei show an increase with the age and with the time spent by the individual in the harmful environment in both genders; this effect is more pronounced in females. The two-tailed Student's "t"-test was applied for statistical assurance. As a conclusion, it is obvious that these studied individuals belong to a risk group; therefore, we recommend biological monitorization and work protection norms to be reinforced.

Key words: Micronucleus test, ionizing radiation, cytokinesis-block, human lymphocytes.

INTRODUCTION

Ionizing radiation and numerous chemical mutagens cause chromosomal aberrations, a proportion of these, usually referred as "asymmetrical events", give rise to chromosome fragments without spindle attachment organelles, (kinetochores, centromeres) which are named "acentric fragments" or micronuclei (MN) [2, 13, 16]. They can also derive from whole chromosomes**,** lagging behind in anaphase, finally showing both clastogenic and aneugenic effects [2, 15, 16]. Micronuclei are corpuscles covered by their own membrane, devised by the nucleus that can be observed in the cytoplasm during the interphase. Micronuclei are one of the best established biomarkers of DNA damage. They are used in the *in vitro* testing of chemical and radiation genotoxicity and as *in vivo* biomarker of

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exposure to genotoxins [14]. Some MN are also present in human lymphocytes as a result of a prior cell division *in vivo*, but most MN are expressed in lymphocytes after the cells are stimulated to divide *in vitro*. The micronucleated cells frequency analysis in human lymphocytes is accomplished by cytokinesis-block micronucleus assay (CBMN) relying on the observation that cells that have completed nuclear division and have their cytokinesis blocked with cytochalasin, express chromosome damage as MN in the resulting binucleated cells [7, 8, 10, 11, 18]. As a result of micronucleation in peripheral human blood in the occupationally exposed to ionizing radiations subjects, other nuclear events as "nuclear bud" (meaning the elimination of amplified DNA and possibly elimination of DNA-repair complexes), "nucleoplasmic bridges" between nuclei (originated from dicentric chromosomes whose centromeres are pulled apart to opposite poles of the cell during anaphase), apoptosis and necrosis could be distinguished [9, 11, 12, 17]. Besides the events induced in the structure and the morphology of the lymphocytary nucleus, considered to be the main target of this study, we also present data correlated with gender, age and the duration of the occupational exposure in the field of low frequency ionizing radiations, statistically appreciated by using two-tailed Student's test.

MATERIALS AND METHODS

After collecting the blood samples with lithium-heparin from the subjects in the vacutainer tube, 0.4 ml of aliquots of whole blood were added to 4.5 ml of culture medium (RPMI 1640, Sigma) supplemented with fetal calf serum 0.5 ml (Sigma), antibiotics 0.1 ml (Sigma) and 0.1 ml of PHA phytohaemagglutinin (Sigma). The blood was cultured in tissue culture vessels at 37 ºC, 5% CO_2 in a humidified atmosphere (Sanyo Incubator). In order to accumulate binucleated cells, 4.5 µg/sample of cytochalasine-B (Sigma) was added 44h post-PHA stimulation. Cytochalasine-B is difficult to dissolve in aqueous solution. The cytochalasine-B stock solution x 10 should be prepared in dimethylsulphoxide (DMSO, Sigma) and aliquoted in a small glass tube and stored until required at –20 ºC. The binucleated lymphocytes were harvested 28h after adding the cytochalasine-B.

The cells were centrifuged at 105×g for 8 min. and the supernatant culture medium was removed. In order to lyse the red blood cells, the sediment was hypotonically treated with 5 ml of cold 7.5 mM KCl (Sigma) and centrifuged immediately at 105×g for 4 min. The supernatant was removed and replaced with 5 ml of fixative consisting of methanol-acetic acid (3:1) (Merck), with 1% formaldehyde. The cells were then centrifuged at 65×g for 8 min. The cells were washed with two more changes of fixative, this time without formaldehyde and centrifuged in the same conditions. After the last centrifugation, the cells were resuspended gently in 1ml of fixative and the suspension was dropped on clean

glass slides and then fixed after air-drying. The cells were stained using a technique that can clearly identify nuclear and cytoplasmic boundaries. We used 5% Giemsa in potassium phosphate buffer (pH = 7.2) (Merck), the slides being examined in light microscopy (Nikon Eclipse E600, oc.x 10; ob.x 40).

The statistical analysis was performed by using the two-tailed Student's "t"-test.

RESULTS

The studied individuals were sub-divided according to age-group, gender and time-exposure groups. The analyzed groups included: 105 occupationally exposed subjects (68 males and 37 females) and 40 controls (20 males and 20 females).

It is known that the effective limit dose accepted for occupationally exposed subjects is 20 mSV/year (H.G. art. 20/2000).

The profile of micronuclei average (MN ± S.D.) as well as other values obtained by descriptive statistics, counted in 1000 binucleated lymphocytes/ individual in occupationally exposed males (M.ex.) and females (F.ex.) *versus* the controls (C.m. or C.f.) is presented in Fig. 1.

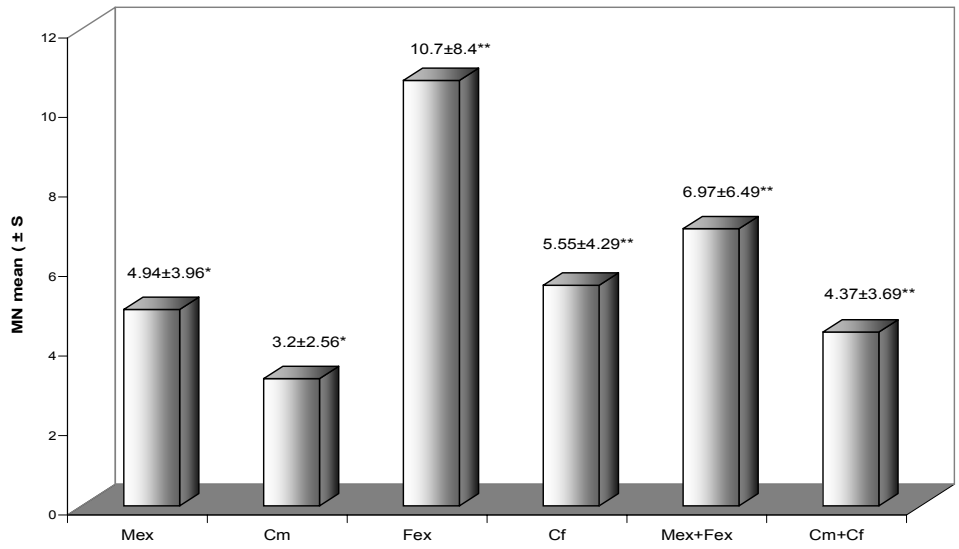


Fig. 1. MN mean (±SD) values in occupationally exposed males (M.ex), females (F.ex) and in control males (C.m) and females (C.f); *significantly different from control ($p < 0.01$); **significantly different from control ($p < 0.001$).

The MN averages in exposed workers were 6.97 (±6.49) meaning 4.94 (±3.96) in M.ex. and 10 (±8.40) in F.ex.; in controls (C.m. + C.f.) the parameter was 4 (±3.69), being 3.2 (±2.5) in males and 5.5 (±4.29) in females. In this respect

the two-tailed Student's "t"-test showed a significant difference in micronuclear induction between the total number of exposed versus the control group as well as in females ($p < 0.001$) and in males $p < 0.01$.

Fig. 2 shows the data concerning the mean of micronuclei analyzed in 1000 binucleated cells/individual, in males occupationally exposed to ionizing radiation *versus* controls, sub-divided according to age-group.

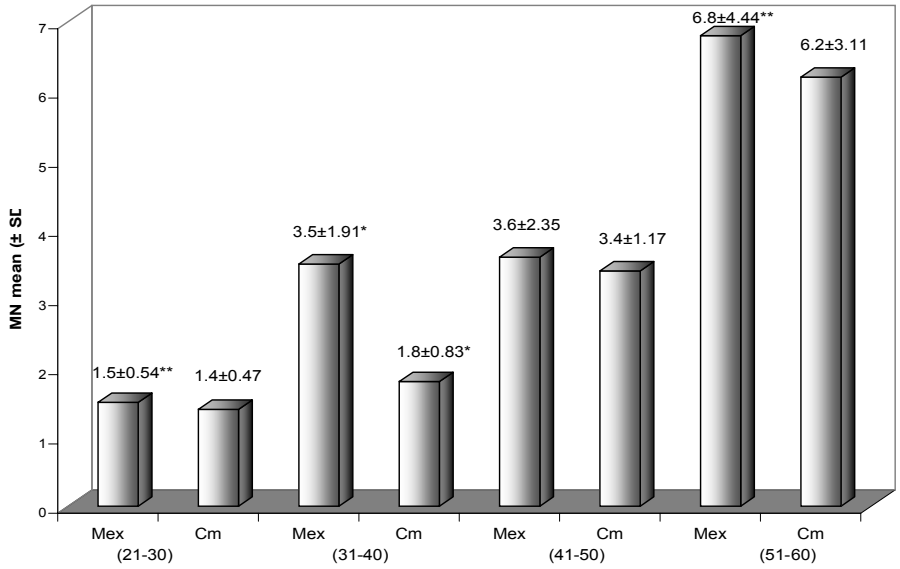


Fig. 2. MN mean (±SD) values in occupationally exposed males (M.ex) in ionizing radiation and control males (Cm) subdivided according to age group; *significantly different from control ($p < 0.001$); **M.ex. 20–30 *versus* M.ex. 51–60 years old ($p < 0.0001$).

The increase of this parameter in accordance with the age, in M.ex is as follows: in individuals with age between 20 – 30 years old, the MN mean is 1.5 (±0.54); it becomes 3.6 (±.35) for those between 41 – 50 years old and 6.8 (±4.44) in individuals of 51–60 years old. In C.m. the MN number increases as the age increases, but to a lower extent, showing a value proximity to M.ex. in the field of 51–60 years old (6.2±3.11). The two-tailed Student's "t"-test shows significant differences in micronuclear induction between the M.ex. and C.m. in 31–40 age domain ($p < 0.001$).

In Fig. 3 we present the frequency of micronuclei in females occupationally exposed to ionizing radiation versus controls, sub-divided according to age-group. An increase of this parameter with the age is seen in F.ex. as well as in C.f. The MN mean is 3 (±2.27) in F.ex. in ages between 20–30, and becomes 14 (±5.71) in F.ex. in ages between 51–60. In the cases of the unexposed subjects (C.f.) aged between 20–30 years old, MN mean is 1.6 (±1.51) and 11.4 (±2.40) in C.f. aged

between 51–60 years old. Significant differences in the cases of F.ex., between the 31–40 and 41–50 years old domain versus control ($p < 0.01$) was found, the higher significance being between F.ex. 20–30 years old domain and F.ex. 51–60 years old domain ($p < 0.0001$).

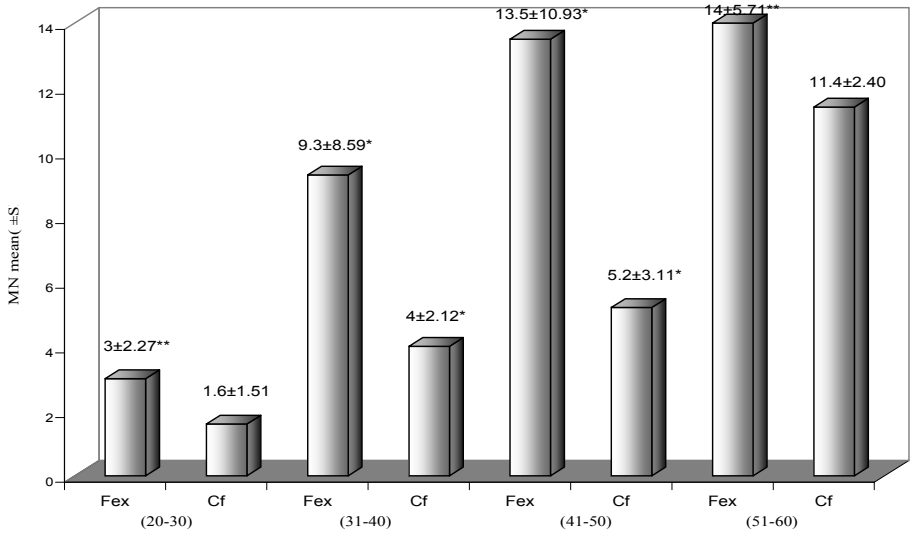


Fig. 3. MN mean (±SD) values in occupationally exposed females (F.ex) in ionizing radiation and control females (C.f) subdivided according to age group; *significantly different from control ($p < 0.01$); **F.ex. 20–30 *versus* F.ex. 51–60 years old ($p < 0.0001$).

The micronucleus averages in occupationally exposed subjects (M.ex. or F.ex.) sub-divided by time of exposure expressed in years of work in an environment exposed to ionizing radiations proves the increase of micronuclei frequency in correlation with the exposure years in both genders, more obvious in F.ex. The MN incidence in M.ex. who just started their work activity (0–1 years old) is 1.2 (±0.83), 4.6 (±4.03) for 11–20 years of activity and 8.4 (±4.76) in those having more than 31 years of activity in noxious environment (Fig. 4).

The comparison between the micronuclei frequency in the exposed subjects at the beginning of their professional career (0–1 years old) and the other intervals of lengths in service led to a significant difference ($p < 0.001$).

Regarding F.ex. who have just started their activity (0–1 years old) the MN mean is 2.8 (±3.11), in F.ex. with 11–20 years of professional activity it is 13.6 (±12.09) and the F. ex. in those with more than 31 years of professional activity it is 15 (±4.63) (Fig. 5).

From a statistical point of view for the MN frequency comparison when starting the professional career, in F. ex. compared with up to 10 years old in professional work, the statistical difference is $p < 0.01$. This one increases to $p < 0.001$ in the case when a maximal length of work is taken into account.

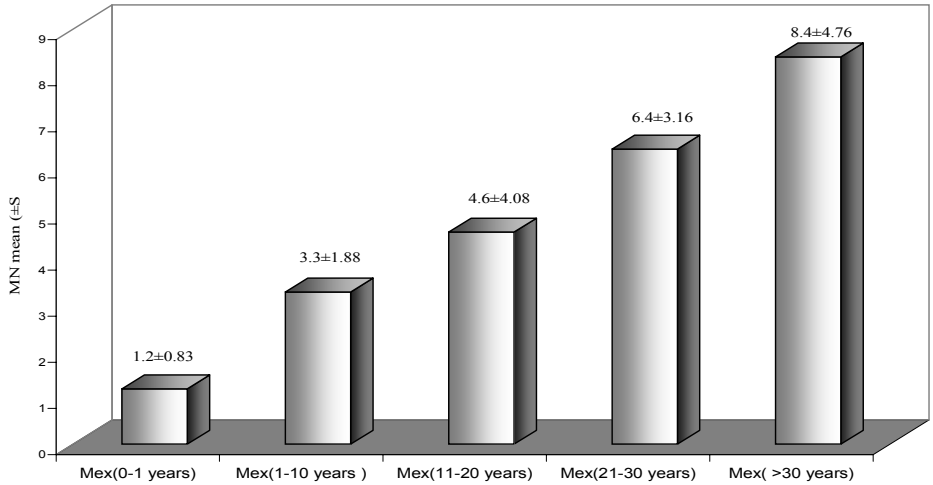


Fig. 4. The MN mean (±SD) values in male workers exposed to ionizing radiation, subdivided according to time-exposure group (years) ($p < 0.001$).

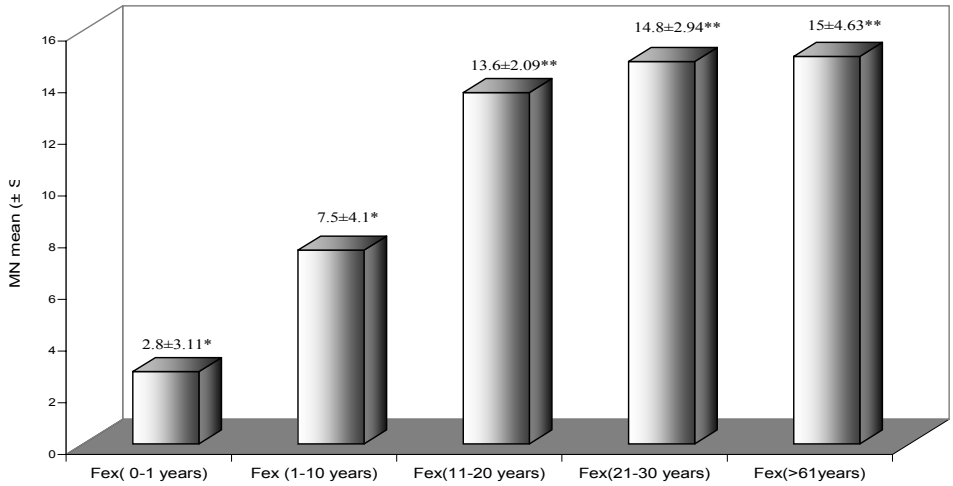
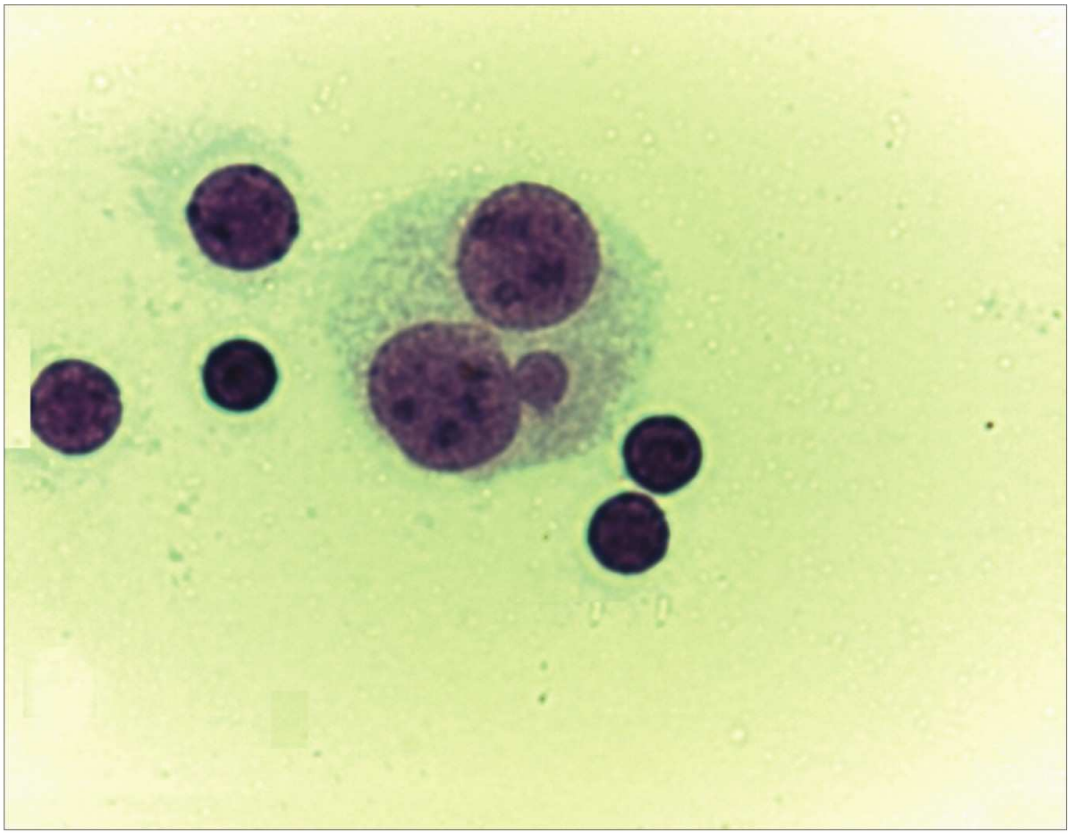
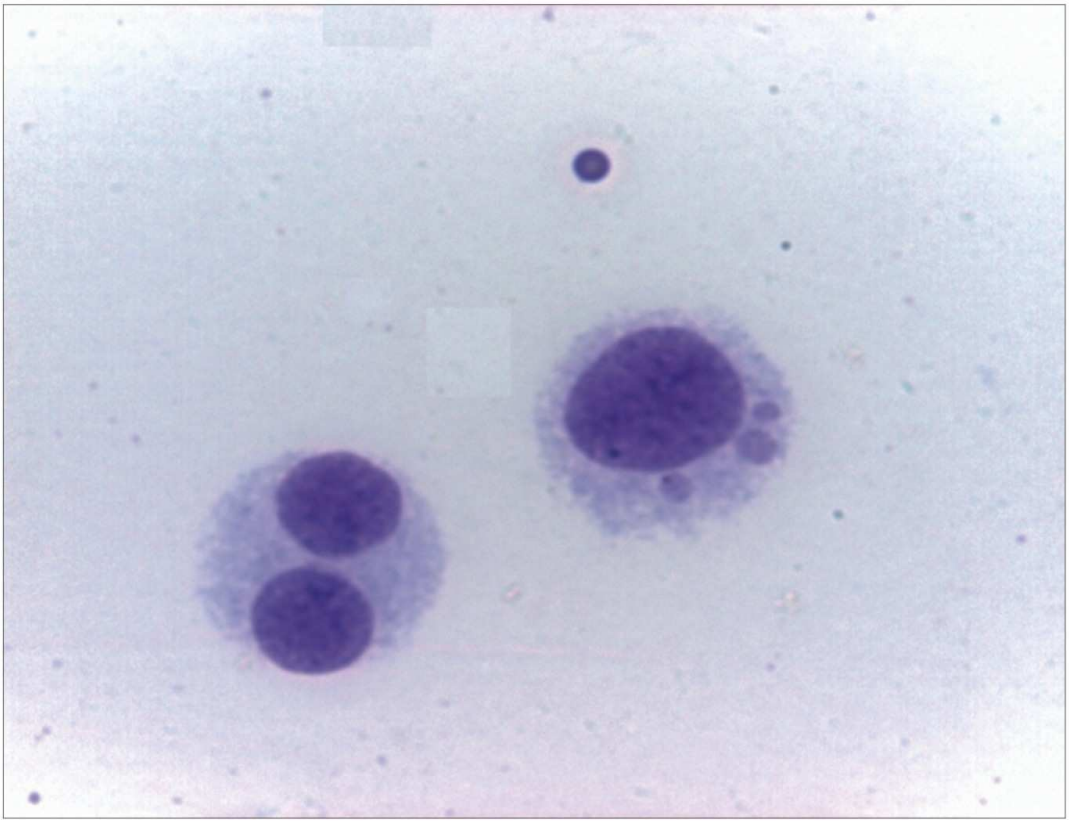


Fig. 5. The MN (± SD) values in female workers exposed to ionizing radiation, subdivided according to time-exposure group (years); *$p < 0.01$ in 0–1/1–10 years of professional exposure; ** $p < 0.001$ in other times of exposure.

By using lymphocyte cytokinesis-block micronucleus (CBMN) assay as an ex *in vivo* measure of chromosome breakage and loss of genetic material in individuals [9], we have the opportunity to discover other nuclear events like: mononucleated, binucleated and multinucleated lymphocytes and their morphological characteristics, nucleoplasmatic bridges, nuclear buds, apoptotic and necrotic cells.



a.



b.

Fig. 6. Photomicrographs of typical binucleated lymphocytes with micronucleus near nuclear membrane (**a**); a mononucleated lymphocyte having three micronuclei in the cytoplasm (**b**).

Fig. 6 (a, b) shows typical binucleated and mononucleated lymphocytes having one or more micronuclei. They were rendered evident according to the Fenech *et al*. [11] criteria. We remarked the integrity of the cell's cytoplasm, the two nuclei dimensional equality inside the binucleated lymphocytes, the MN dimensions (usually they vary between 1/16 and 1/3 of the mean diameter of the main nuclei), as well as their distribution in the cytoplasm. Fig. 7 illustrates a binucleated lymphocyte having a "nuclear bud" which arises from the elimination of amplified DNA [17]. In Fig. 8 the nucleocytoplasmic bridges inside of binucleated lymphocyte are present; these are thought to originate from rearranged chromosomes with more than one centromere, e.g. dicentric chromosomes.

DISCUSSIONS

The hypothesis of a direct correlation between MN frequency and cancer development is supported by a number of aspects: a) increased frequency of MN in target tissue as well as in peripheral lymphocytes in cancer patients b) the congenital diseases like the Bloom syndrome or ataxia telangiectasia have both high MN frequency and an increased risk of cancer c) the MN frequency is much more correlated with the blood concentration of vitamins and folates whose deficiency is associated with an increased risk of some cancers, etc. [9]. Therefore, it could be considered that the micronucleation test validation as a biomarker with predictive value for the estimation in due time of the high increased cancer risk has numerous reasons.

This study's target is to obtain information about two main aspects:

A. The first one refers to the genetic material response being under the impact of the physical noxa in the occupational exposure, namely the nuclear structural changes (the micronuclei appearance, nucleoplasmatic bridges and "nuclear buds"). There are some accepted mechanisms by which micronucleus can arise: loss of acentric fragments during mitosis, mitotic loss of whole chromosomes and apoptosis. Apoptosis is known as a nuclear destruction phenomenon in which nuclear fragments appear as a consequence of nucleus disintegration in an intact cytoplasm [5]. The micronucleus test used in this study is not an innovation, it was extensively used to screen individuals exposed to various chemical and physical noxae [1, 3, 4, 6]. The cytokinesis-block technique using cytochalasin-B, a microfilament assembly inhibitor, arrests division of cytoplasm without stopping the nuclear division, making possible the appearance of binucleated lymphocytes. The binucleated cells that have accomplished one nuclear division will express their induced micronucleus.

The micronuclei from the images taken by light microscopy are captive within the binucleated lymphocyte cytoplasm which has a well defined outline; they present an exact delimitation regarding the nuclei, the same tinctoriality and

their dimensions do not exceed 1/3 of the nuclear volume. Their number in the binucleated cells can vary [1, 2, 3, etc.] and they can also be found within the lymphoid cells with a multiple number of nuclei. An ex *in vivo*/ *in vitro* analysis of lymphocytes with CBMN assay allows to easily distinguish between mononucleated cells which did not divide and binucleated lymphocytes which performed their nuclear division during the *in vitro* culture. Thus, the presence of mononucleated lymphocytes carrying MN (a various number, as in Fig. 6.b) brings an indication on the background level of chromosome/genome alterations accumulated *in vivo* and the frequency of MN binucleated cells brings an indication on the accumulated damage before cultivation plus alterations expressed during the first *in vitro* mitosis. MN incidence estimation through CBMN assay may not identify all events at the chromosomal level; e.g., aberrations such as symmetrical reciprocal translocations are not expressed as MN, but asymmetrical translocations such as dicentric chromosomes and the acentric fragments may be signaled as nucleoplasmatic bridges (Fig. 8) and MN, respectively.

B. The second aspect consists in a lot of 105 occupationally exposed to ionized radiations individuals and of a control group composed of 40 individuals, on which the micronuclei test by using the techniques and the micronucleation estimation criteria offered by M. Fenech *et al*. [11] was applied. The individual response to the physical noxa was analyzed taking into account the gender and age fields, as well as a phasing in accordance with the time spent in the environment of radioactive exposure; it was statistically provided by the Two-tailed Student "t"-test. The ionizing radiations obviously induce micronucleation when the MN mean of the exposed group subjects is compared to those of controls ($p < 0.001$) (Fig. 1). The separation on genders shows that MN mean is more significant in females ($p < 0.001$) than in males ($p < 0.01$).

At this moment there is no explanation about this molecular behavior between genders, but it is very clear that the physical noxa pursued is capable of inducing damage in the human genome, determining thus the appearance in the lymphocyte nucleus of some microstructures which provide exact information regarding the degree of chromosomal material fragmentation, micronuclei, respectively.

An analysis on age groups of the MN mean in the exposed males in comparison with the controls shows a significant increase of the micronucleation index in the field 31–40 years old ($p < 0.001$) (Fig. 2). For the rest of the age groups the statistical comparison does not show significant values, suggesting thus the attenuation in differences through the appearance, to some extent, of micronuclei in controls, too, simultaneously with the growth in age. The comparison between micronucleation induced by radiation in young subjects (M.ex. 20–30 years old) which shows values very close to unexposed persons of the same age and the exposed ones (51–60 years old) presents an obvious statistical

relevance ($p < 0.0001$). A MN mean analysis on age groups in the exposed females in comparison with controls presents statistical differences in the age fields 31–50 years old (Fig. 3) ($p < 0.01$). At a MN mean inter-comparison within the age groups in the occupationally exposed females the irradiation source harmful effect is recorded after 51 years old versus 20–30 years old with a statistical relevance of $p < 0.0001$.

Also the micronuclei appearance as a result of some breakage in the chromosomal material has a more significant statistical relevance with the increase of the activity duration in the harmful environment ($p < 0.001$) between 0–1 years old / more than 31 years exposure age in males (Fig. 4). In females $p < 0.01$ between 0–1 years / 1–10 years exposure age and $p < 0.001$ in other times of exposure (Fig. 5).

Micronuclei frequency increases as the age increases naturally; there are well known ageing events which take place in the genetic material, too; the ageing process is stimulated to become an indicator of noxiousness with practical direct application in the case of the occupational exposures to chemical or physical noxae. In the case of occupationally exposed subjects to ionizing radiation, a cumulative effect takes place, too. As we could notice in our study, in males the process is going differently than in females, where the MN frequency is even more intense, the events taking place in direct connection with the exposure duration to the harmful environment.

CONCLUSIONS

1. The micronucleus test could be considered as a biomarker with predictive value for the estimation in due time of the high increased cancer risk in subjects occupationally exposed to ionizing radiation.

2. The cytokinesis-block technique using cytochalasin-B permits the structural and morphological effects evidence on cell nucleus, showing the following events: the appearance of micronuclei as a consequence of acentric fragments loss during mitosis, nucleoplasmic bridges originated from chromosomal rearrangements, “nuclear bud” arising from the elimination of amplified DNA.

3. There is an individual response to the physical noxa, depending on the gender, age and length of work in the radioactive environment. When the control and the exposed groups were compared, the induced micronuclei show an increase with the age and with the time spent by the individual in the harmful environment, in both genders. This effect becomes more pronounced in females.

4. As a conclusion, it is obvious that these studied individuals belong to a risk group; therefore, we recommend biological monitoring and work protection norms to be reinforced.

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