ORIGINAL ARTICLE

The Frequency of Lymphocytes Containing Dumbbell-Shaped Nuclei Depends on Ionizing Radiation Dose and Correlates with Appearance of Chromosomal Aberrations

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ABSTRACT

Nuclear anomalies of different types appear in cells in response to the action of ionizing radiation after the passage of the first mitotic division. In this article, we present the results of the study of the frequency of occurrence of three types of nuclear anomalies ("tailed" nuclei, nucleoplasmic bridges, and dumbbell-shaped nuclei) *in vitro* in human lymphocytes cultured with cytochalasin B when exposed to X-rays at doses of 0.0, 0.1, 0.2, 0.4, 0.5, 0.7, 1.0, 1.5, and 2.0 Gy. To stop the cell cycle of cultured lymphocytes after the first mitotic division, a cytokinesis block was performed using cytochalasin B. Dose-dependent curves of the occurrence of lymphocytes containing "tailed" nuclei, nucleoplasmic bridges, or dumbbell-shaped nuclei after irradiation have been constructed. At the same time, frequencies of occurrence of chromosomal aberrations (dicentric and ring chromosomes) in the culture of lymphocytes exposed to the same radiation doses were studied. Comparison of the frequencies of occurrence of dicentric and ring chromosomes with frequencies of occurrence of nuclear anomalies allows us to conclude that these nuclear anomalies are formed as a result of chromosomal aberrations arising in lymphocytes under the action of ionizing radiation. More than that, most of the chromosomal aberrations are converted into dumbbell-shaped nuclei *in vitro* in the culture of lymphocytes in the cytochalasin block.

Key words: Chromosomal aberrations, dumbbell-shaped nuclei, ionizing radiation, nuclei aberrations, nucleoplasmic bridges, "tailed" nuclei

Introduction

Ionizing radiation effects on cell populations as a genotoxic agent could lead to the appearance of cells with morphologically anomalous nuclei.[1-3] Among these anomalies, micronuclei, nucleoplasmic bridges, and "tailed" nuclei are distinguished. Such anomalies arise after pathological mitotic divisions and are clearly distinguishable in a light microscope. In this case, the mechanism of their occurrence caused by ionizing radiation is associated with the formation of double-strand breaks of DNA and as a consequence ring and dicentric chromosomes.[4]

Micronuclei are the most studied form of nuclei pathology. They appear as fragments of the cell nucleus, which carry an incomplete part of the genome.[5-10] Micronuclei may contain either an acentric region of the chromosome or an entire chromosome that has not been distributed to one of the opposite poles during anaphase of mitosis. Fragments or whole chromosomes eventually become covered with a nuclear envelope and morphologically look similar to the cell nuclei, not exceeding one-third of its diameter. At the same time, several variants of micronucleus "fate" are possible: degradation of the micronucleus, its removal from the cell, apoptosis of the whole cell, or cooperation of the micronucleus with the basic cell nucleus.[11]

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In addition to micronuclei, another types of nuclei pathology, generally accepted in radiobiology as markers of DNA damage, are nucleoplasmic bridges.[12] Such bridges arise when the centromere of the dicentric chromosomes diverges to the opposite poles of the cell during anaphase.[13] During the formation of two new nuclei of daughter cells in the telophase, the formed nucleoplasmic bridge is also covered with a nuclear envelope. Usually, it undergoes a rupture during cytokinesis, resulting in the formation of so-called "tailed" nuclei. In this connection, it is possible to observe nucleoplasmic bridges while studying cells in the cytochalasin block, where further division of the cytoplasm does not occur.[14]

"Tailed" nuclei were found in the cells of different fish species,[15,16] as well as in humans,[17] after exposure to ionizing radiation. The formation of "tails" is closely related to the formation of nucleoplasmic bridges. It can be said that a cell exposed to ionizing irradiation undergoes a "breakage–fusion–bridge" cycle during repeated mitotic divisions. The resulting nucleoplasmic bridges break during cytokinesis with the formation of "tailed" nuclei. The end sections of such broken chromosomes are recognized by the DNA repair system as double-strand breaks and are cross-linked to form dicentric chromosomes. As a result, after anaphase, nucleoplasmic bridges are newly formed.[13]

The least studied markers of radiation exposure are the dumbbell-shaped nuclei. However, these forms of pathology of the cell nucleus were repeatedly detected in the peripheral blood lymphocytes in the liquidators of the consequences of the Chernobyl accident but were rarely described.[18] The same form of nuclei can be observed in granulocytes of the peripheral blood of patients with the Pelger-Huet anomaly.[19] The two nuclei were fused together, resembling a dumbbell or a figure "eight." Morphologically, this form of nuclei differs from the nuclei connected by a nucleoplasmic bridge. The appearance of dumbbell-shaped nuclei is associated with the formation of dicentric and ring chromosomes.[20]

Thus, all nuclear anomalies appearing in the cell in response to the effects of radiation have a similar mechanism of formation. This mechanism is based on the introduction of double-strand breaks in DNA molecules by ionizing radiation. Only such powerful defects in the structure of DNA can lead to the formation of chromosomal aberrations, namely dicentric and ring chromosomes. Various defects in the distribution of dicentric chromosomes to the poles of the cell during the anaphase of mitosis lead to visually distinct cellular defects – nuclear anomalies. Considering all of the above, nuclear anomalies are a simple marker for bioindication and possibly biodosimetry even at small doses (from 0 to 2 Gy). The detection of micronuclei has already formed the basis for the widespread micronucleus test.[10] However, other types of nuclei pathology have a mechanism of formation similar to the micronuclei, arising from chromosomal aberrations as a manifestation of their pleiotropic effect. Thus, in some cells, several anomalies can be formed at once. For example, in cells with dumbbell-shaped nuclei, one or more micronuclei can appear. These cells are not considered in modern biodosimetric tests despite their multi-aberration nature. This study contains the results of analyzing of "tailed" nuclei, nucleoplasmic bridges, and dumbbell-shaped nuclei. We described the dependence of their appearance in human lymphocytes cultured by the cytokinesis block method from the dose of X-ray radiation from 0 to 2 Gy. In parallel with nuclear anomalies formed after the first mitosis after radiation exposure, metaphase frequencies with dicentric and ring chromosomes were studied.

Materials and Methods

In the experiment peripheral blood of female, 30-year-old, not smoking, not drinking donor was used. Heparinized blood was placed sterilely in 3-ml plastic syringes. Nine blood samples were irradiated with nine different doses: 0.0, 0.1, 0.2, 0.4, 0.5, 0.7, 1.0, 1.5, and 2.0 Gy. Irradiation was carried out at room temperature on a RUM-17 apparatus (200 kW, 13 mA, 1.0 mm Cu filter, a focal distance of 30 cm) at a dose rate of 0.6788 Gy/min. An hour after irradiation, blood samples were divided for two experiments and cultured: 0.5 ml of the blood was added to 4.0 ml of the medium Roswell Park Memorial Institute 1640 enriched with 10% fetal calf serum (Sigma, Missouri, USA) and phytohemagglutinin (PHA; Gibco, Waltham, MA) in sterile glass tubes (0.15 ml/5 ml of medium).

To prepare slides with binuclear cells for nuclei anomalies analysis, cytochalasin B (Sigma, Missouri, USA) at a final concentration of 3 μg/ml was added to the first part of blood samples at the 44th h of the incubation. The stock solution of cytochalasin B was prepared by dissolving 1.0 μg of the lyophilized material in 1.1 ml of dimethyl sulfoxide just before the experiment. After that, the cultivation was stopped at the 72nd h. The cells were treated with a 0.1 M KCl solution for 3 min and then centrifuged for 10 min. The supernatant was then removed, and the precipitate was fixed with a freshly prepared mixture of methanol and glacial acetic acid in a 3:1 ratio and then centrifuged. The procedure was repeated four times. All supernatants were discarded, and drops of fixed cell suspension were applied to clean glasses. The preparations were dried and stained with Azure II-eosin according to Romanovsky. The nuclear anomalies were scored in at least 2000 cells at each of nine doses of irradiation (0.0, 0.1, 0.2, 0.4, 0.5, 0.7, 1.0, 1.5, and 2.0 Gy) using the magnification of ×1000.

To obtain metaphase chromosomes, another part of the donor peripheral blood samples was cultured for 51 h. During the entire time of cultivation, bromodeoxyuridine (Sigma, Missouri, USA) was administered at a concentration of 10 μg/ml to indicate the first division of mitosis. To obtain metaphase plates, colchicine (Sigma, Missouri, USA) was added into the peripheral blood culture 3 h before fixation at a concentration of 0.06 μg/ml. Then, cells were treated hypotonically with 0.55% KCl for 40 min at 37°, with a subsequent three time change of chilled fixator (methanol/glacial acetic acid in the 3: 1 ratio) for 1 h. Preparations of chromosomes were made by placing the cell suspension in humidified glasses. Then, the slides were stained with Azure II-eosin according to Romanovsky and were calculated for dicentric and ring chromosomes. At least 500 metaphase plates were analyzed for each of seven doses (0.0, 0.2, 0.4, 0.7, 1.0, 1.5, and 2.0 Gy).

Statistical analysis was performed with the use of Student's *t*-test for confidence intervals calculation and also Pearson's correlation coefficient to establish correlation between frequencies of nuclei abnormalities and chromosomal aberrations.

Results

Establishment of the dose dependence of the frequency of occurrence of lymphocytes containing dicentric and ring chromosomes

Results of studies of chromosomal aberrations are presented in Table 1. The dependence of the frequency of metaphases with dicentric and ring chromosomes on the dose of X-ray irradiation is described by the equation of the linear-quadratic function:

$$Y = 0.007 + 0.01D + 0.046D^2,$$

Where D is the dose in Gray and Y is the frequency of lymphocytes with dicentric and ring chromosomes.

Remarkably, there are no statistically significant differences between values of frequencies of metaphases with dicentric and ring chromosomes observed on 0.2 and 0.4 Gy. It is quite possible that this dose range corresponds to the plateau. The dose-dependent curve of occurrence of metaphases with dicentric and ring chromosomes at different doses from 0.0 to 2.0 Gy is shown in Figure 1.

Along with the dicentric and ring chromosomes, we also registered marker chromosomes characterized by elongated long shoulders. It turned out that the frequency of metaphases with such markers was 0.0 Gray – 0.0000, after irradiation with doses of 0.2 Gy – 0.0000, 0.4 Gy – 0.0010, 0.5 Gy – 0.0025; 0.7 Gy – 0.0026, 1.0 Gy – 0.0050; 2.0. Gy – 0.0046; 3.0 Gy – 0.038, and 4.0 Gy – 0.025.

Establishment of the dose dependence of the frequency of occurrence of lymphocytes containing "tailed" nuclei

"Tailed" nuclei appeared as the outgrowths of the cell nuclei of lymphocytes, coinciding with the nucleus itself in the intensity of the staining [Figure 2].

Results of analysis of "tailed" nuclei in the peripheral blood lymphocytes are presented in Table 2. The frequency of occurrence of binuclear cells with "tailed" nuclei depends on the dose of X-ray irradiation; the character of the dependence is described by a linearly quadratic function:

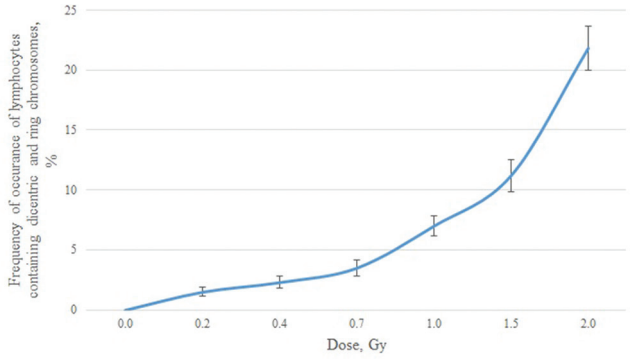

Figure 1: Dependence of frequency of peripheral blood lymphocytes containing dicentric and ring chromosomes on X-radiation dose

$$Y = 0.009 + 0.05D + 0.003D^2,$$

Where D is the dose in Gy and Y is the frequency of occurrence of lymphocytes with "tailed" nuclei.

Figure 3 shows the dose-dependent curve of occurrence of lymphocytes with "tailed" nuclei at different doses from 0.0 to 2.0 Gy.

Establishment of the dose dependence of the frequency of occurrence of lymphocytes containing nucleoplasmic bridges

Nucleoplasmic bridges appeared as sections of chromatin, connecting two nuclei in a cell [Figure 4]. Results of analysis of nucleoplasmic bridges in the peripheral blood lymphocytes are presented in Table 3. The frequency of occurrence of binuclear cells with nucleoplasmic bridges depends on the dose of X-ray

Table 1: Chromosome aberrations observed after *in vitro* irradiation of the human peripheral blood at doses from 0.0 to 2.0

Dose (Gy)	Number of metaphases scored	Number of cells with dicentric and ring chromosomes	Frequency of cells with dicentric and ring chromosomes (%)
0.0	1000	0	0.0
0.2	1000	15	1.5
0.4	1000	23	2.3
0.7	782	28	3.5
1.0	857	60	7.0
1.5	535	60	11.2
2.0	500	109	21.8

Table 2: Binuclear lymphocytes with "tailed" nuclei after X-irradiation

Dose (Gy)	Number of lymphocytes scored	Number of cells with "tailed" nuclei	Frequency of cells with "tailed" nuclei (%)
0.0	5632	51	0.91
0.1	5380	43	0.80
0.2	5680	80	1.40
0.4	4150	55	1.33
0.5	5022	78	1.55
0.7	3640	107	2.94
1.0	4089	97	2.37
1.5	2780	85	3.06
2.0	2016	80	3.97

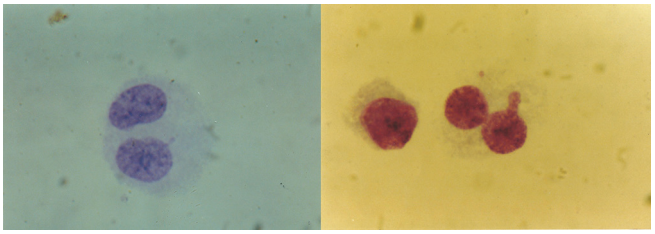

Figure 2: Lymphocytes containing "tailed" nuclei (Azure II-eosin staining, ×1000)

irradiation; the character of the dependence is described by a linearly quadratic function:

$Y = 0.000722 + 0.004D + 0.001D^2$,

Where D is the dose in Gy and Y is the frequency of lymphocytes with nucleoplasmic bridges.

Figure 5 shows the dose-dependent curve of the occurrence of lymphocytes with nucleoplasmic bridges at different doses from 0.0 to 2.0 Gy.

Table 3: Binuclear lymphocytes with nucleoplasmic bridges after X-irradiation

Dose (Gy)	Number of lymphocytes scored	Number of cells with nucleoplasmic bridges	Frequency of cells with nucleoplasmic bridges (%)
0.0	5632	0	0.0
0.1	5380	3	0.06
0.2	5680	8	0.14
0.4	4150	13	0.31
0.5	5022	19	0.38
0.7	3640	18	0.49
1.0	4089	23	0.56
1.5	2780	18	0.65
2.0	2016	31	1.54

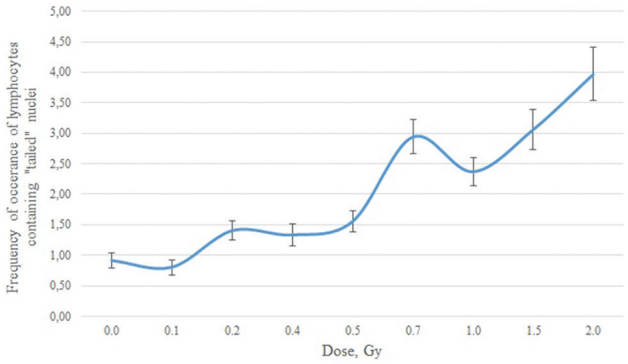

Figure 3: Dependence of frequency of peripheral blood lymphocytes containing "tailed" nuclei on X-radiation dose

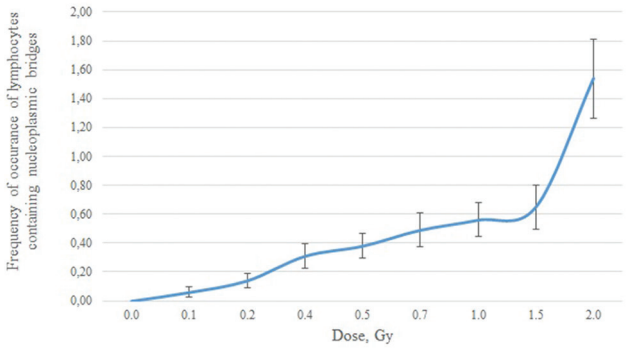

Figure 5: Dependence of frequency of peripheral blood lymphocytes containing nucleoplasmic bridges on X-radiation dose

Establishment of the dose dependence of the frequency of occurrence of lymphocytes with dumbbell-shaped nuclei

Dumbbell-shaped nuclei represent two closely spaced nuclei of the interphase cell, reminiscent of the shape of a figure "eight" or dumbbell [Figure 6].

The cell size is not increased comparing to the cells with normal nuclei, and the area occupied by the dumbbell-shaped nuclei is visually equal to the area occupied by the two separated cell nuclei. It turned out that in more than half of the cases of observing dumbbell nuclei in such cells, there is also one or several micronuclei (data not shown).

Results of analysis of dumbbell-shaped nuclei in the peripheral blood lymphocytes are presented in Table 4. The frequency of occurrence of lymphocytes with dumbbell-shaped nuclei depends on the dose of X-ray irradiation; the character of the dependence is described by a linear-quadratic function:

$Y = 0.003 + 0.014D + 0.019D^2$,

Where D is the dose in Gy and Y is the frequency of lymphocytes with dumbbell-shaped nuclei.

Figure 7 shows the dose-dependent curve of the occurrence of lymphocytes with dumbbell-shaped nuclei at different doses from 0.0 to 2.0 Gy.

Discussion

Results indicate that the frequency of occurrence of cultured lymphocytes with "tailed" nuclei positively correlates with the frequency of occurrence of lymphocytes with dicentric and ring chromosomes ($r = 0.89$, $P < 0.005$) and does not correlate with the frequency of occurrence of lymphocytes containing chromosomes with elongated shoulders. It is important to note

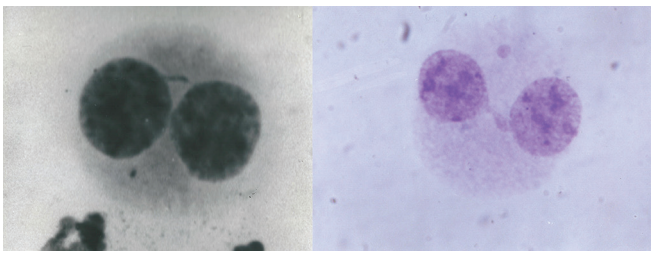

Figure 4: Lymphocytes containing nucleoplasmic bridges (Azure II-eosin staining, ×1000)

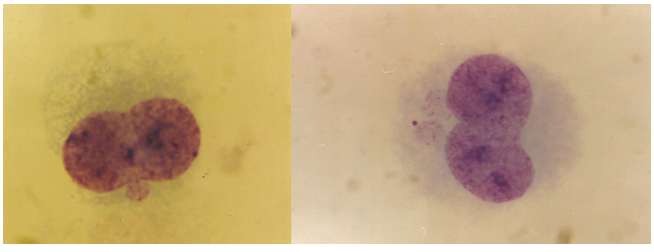

Figure 6: Lymphocytes containing dumbbell-shaped nuclei (Azure II-eosin staining, ×1000)

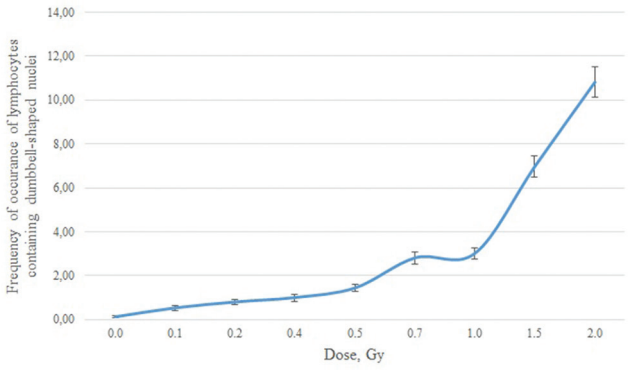

Figure 7: Dependence of frequency of peripheral blood lymphocytes containing dumbbell-shaped nuclei on X-radiation dose

Table 4: Binuclear lymphocytes with dumbbell-shaped nuclei after X-irradiation

Dose (Gy)	Number of lymphocytes scored	Number of cells with dumbbell-shaped nuclei	Frequency of cells with dumbbell-shaped nuclei (%)
0.0	5632	6	0.11
0.1	5380	28	0.52
0.2	5680	45	0.79
0.4	4150	41	0.99
0.5	5022	72	1.43
0.7	3640	102	2.80
1.0	4089	123	3.01
1.5	2780	194	6.98
2.0	2016	218	10.81

that at all the doses studied, the values of the frequencies of dicentric and ring chromosomes were significantly higher than the values of the frequencies of the "tailed" nuclei. Apparently, a significant part of the nucleoplasmic bridges formed by dicentric and ring chromosomes under the conditions of our experiment are not realized in "tailed" nuclei. At the same time, the frequency of chromosomal aberrations increased sharply from 0.7 Gy, and the frequency of "tailed" nuclei was insignificant. This suggests that the formation of dicentric and ring chromosomes leads to the formation of not only "tailed" nuclei but also other nuclear anomalies.

Attention is drawn to the similarity of the nucleoplasmic bridges and the "tails" of the nuclei. This confirms that one of the reasons for the appearance of "tailed" nuclei is just the breakage in the nucleoplasmic bridge. In many cases, cells with nucleoplasmic bridges contained one or more micronuclei, which confirms the pleiotropic nature of the appearance of nuclear anomalies.

The frequency of occurrence of lymphocytes with nucleoplasmic bridges also positively correlates with the frequency of occurrence of dicentric and ring chromosomes ($r = 0.97$, $P < 0.05$); however, the total frequency of occurrence of "tailed" nuclei and nucleoplasmic bridges is still lower than the frequency of chromosomal aberrations at all doses. This is due to the fact that in the anaphase of mitosis with the divergence of chromosomes, the dicentric chromosome can form a bridge between the daughter nuclei only if its two centromeres are oriented to different poles of the dividing cell. However, if both centromeres of dicentric chromosome are oriented to one pole, then after the first division of mitosis, the nucleoplasmic bridge will not be formed. The probability of these events is approximately the same, which indicates that two cells with chromosomal aberrations should have one cell with a bridge or "tail." However, for example, with irradiation with a dose of 1 Gy, 7.0% of cells containing chromosomal aberrations are observed, but in reality, only 3% of cells with "tailed" nuclei (2.4%) and bridges (0.56%) were formed. When irradiated with a dose of 2 Gy, 21.8% of cells were with chromosomal aberrations and only 5.51% of cells were with "tailed" nuclei (3.97%) and bridges (1.54%). This suggests that there is another anomaly into which chromosomal aberrations are transformed under the action of ionizing radiation, which is especially often encountered at high doses. Such an anomaly is likely dumbbell-shaped nuclei.

As a result of our studies, a correlation was found between the frequency of occurrence of dumbbell-shaped nuclei and the frequency of occurrence of dicentric and ring chromosomes in irradiated lymphocytes ($r = 0.99$, $P < 0.05$). A small plateau is observed on the dose-dependent curve of the occurrence of dumbbell-shaped nuclei from the irradiation dose between 0.2 and 0.4 Gy. The same plateau can be seen at these doses on the frequency curve of the occurrence of dicentric and ring chromosomes. Among other anomalies, dumbbell-shaped nuclei have the curve of the dependence of the frequency most coinciding with the corresponding curve for dicentric and ring chromosomes. A general diagram showing the frequencies of all nuclear anomalies and chromosome aberrations from the radiation dose is shown in Figure 8. Frequencies of dumbbell-shaped nuclei were proportionally lower than the frequencies of lymphocytes carrying chromosomal aberrations at all radiation doses, but keeping a positive correlation between both of them. More than that, the frequencies of occurrence of dumbbell-shaped nuclei on the last two doses of radiation (1.5 and 2.0 Gy) were significantly higher than earlier ones. The same rise was observed on the curve of frequency of chromosomal aberrations on high doses. We consider this to be especially important since it most likely means that at high doses, the majority of dicentric chromosomes formed are realized precisely as dumbbell-shaped nuclei. Thus, this could be the main nuclear anomaly, which should be paid attention to during biometric tests. Probably, these results indicate that the biometric and biodosimetric tests widely used today require a serious correction at high doses of radiation with allowance for dumbbell-shaped nuclei.

The mechanism of the appearance of dumbbell-shaped nuclei is discussed. Thus, the appearance of dumbbell-shaped suspensions in the cell nucleus was attributed to the morphological features of amitosis, the direct method of cell division, in which the components of the nucleus are distributed unequally between daughter nuclei.[21,22] Later, dumbbell-shaped nuclei were found

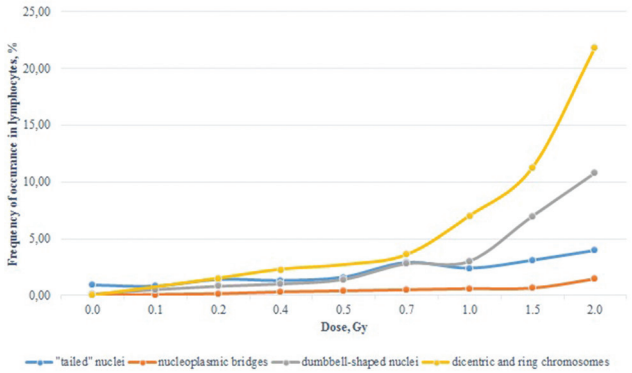

Figure 8: Dependence of frequencies of peripheral blood lymphocytes containing different nuclei anomalies on X-radiation dose

in intensively dividing cells at low temperature and under nutrient deficiency.[23] Thus, the mechanism for the appearance of such a form of the nucleus is unclear.

We managed to find out that the nature of dumbbell-shaped nuclei is connected with several nucleoplasmic bridges that are part of their composition. Figure 9 shows a cell with a dumbbell-shaped nucleus with nucleoplasmic bridges detected in its composition. More than that, bridges start in the first nucleus and end in the second. In our understanding, these bridges are dicentric chromosomes, the centromeres of which during the anaphase of mitosis were distributed to the opposite poles of the dividing cell. This is confirmed by the presence of micronuclei, which may contain isolated paired acentric fragments corresponding to these dicentrics. Probably, the main factor, due to which the dicentric chromosomes in our experiment passed precisely to dumbbell-shaped nuclei, and not to other types of nuclear anomalies, is the effect of cytochalasin B.

It remains to note that the counting of cells with dumbbell-shaped nuclei is simpler than the cytogenetic analysis of metaphase chromosomes, which makes them attractive as biometric and biodosimetric markers. When dumbbell-shaped nuclei are formed at high radiation doses, micronuclei are also formed, which are not taken into account in the traditional protocol of the micronucleus test. Accounting for dumbbell-shaped nuclei and their micronuclei is necessary, since the nature of their occurrence at high doses is common and is associated with the formation of several dicentric chromosomes that make up the dumbbell-shaped nuclei, as well as several acentric fragments that separate themselves in the form of micronuclei.

Conclusion

The data presented confirm the general nature of the origin of different types of nuclear anomalies due to chromosomal aberrations arising in human peripheral blood lymphocytes under the influence of ionizing radiation under cultivation with cytochalasin B. In addition to the widely known "tailed" nuclei and nucleoplasmic bridges in human lymphocyte culture under the action of cytochalasin B, a large number of such nuclear anomalies as dumbbell-shaped nuclei are formed. Moreover, the

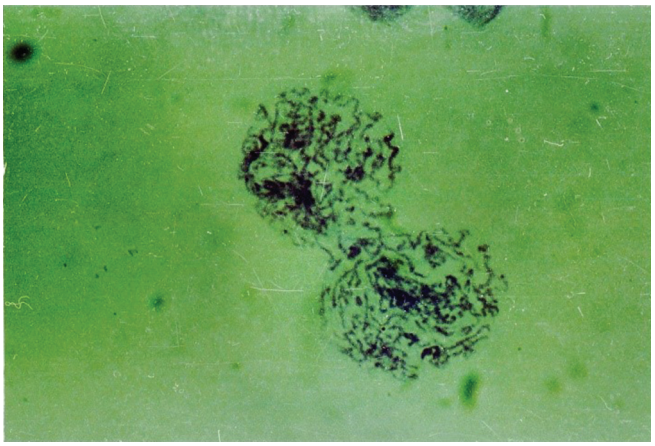

Figure 9: Dumbbell-shaped nuclei containing several nucleoplasmic bridges. (Azure II-eosin staining, with the addition of a green filter in transmitted light, ×1000)

presented results suggest that in most cells in which chromosomal aberrations have arisen under the action of ionizing radiation, they do lead to the formation of dumbbell-shaped nuclei, and this trend increases with increasing radiation dose.

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Conflicts of interest

There are no conflicts of interest.

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