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Nuclear Abnormalities of Lymphocytes as the Simplest Markers for Bioindication Test in Case of Mass Casualty Events Involving Radiation Exposure

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Abstract

Radiation exposure leads to a large number of victims who seek medical help in the first hours. To provide medical care, it is necessary to correctly establish the fact and dose of radiation by bioindication and biodosimetry methods. Lymphocytes containing nuclear anomalies are easily detected cells of peripheral blood and are suitable as objects of bioindication in the case of radioactive exposure to a large number of people. Among these anomalies we distinguish micronuclei, "tailed" nuclei, nucleoplasmatic bridges, dumbbell-shaped nuclei, etc. This review observes the main types of these nuclear anomalies of lymphocytes in the light of their common origin from dicentric chromosomes. We recommend using these alterations in peripheral blood lymphocytes nuclei as the simplest biomarkers in the framework of bioindication tests when it is necessary to work with a large number of victims.

Keywords: Radiation exposure; Bioindication; Biomarkers; Lymphocytes nuclei abnormalities; Dicentric chromosomes

Introduction

Sources of ionizing radiation are now widely used in all areas of human life, which greatly increases the likelihood of a radiation emergency and the possibility of radiation injury to a large number of people. Within a relatively short time upon such radiation accidents, many victims and alleged victims seek medical care, and such a situation results in dramatic difficulties in the operation of various medical institutions. The most important methods used to confirm the fact of irradiation and to measure the dose of radiation are different indication and dosimetry techniques, which in the initial stages of liquidation of radiation accidents actually determine opportunities to provide health care.

Quantitative estimation of the dose of irradiation received by the human body is carried out by means of physical dosimetry (using dosimeters and radiometers) or biological dosimetry. Physical dosimetry shows the exact dose of ionizing radiation absorbed by the human body. However, this method has several disadvantages: a high error in the equipment used to measure the dose of radiation; the measurements are limited to the area where the dosimeter is attached to clothing, measuring is concerned only to gamma radiation, etc. In addition, in the case of a radiation emergency, physical dosimetry data may be absolutely inaccessible.

The methods of biological dosimetry are based on dose-dependent reactions of the body tissues to the effect of radiation and on the detection of specific radiation biomarkers. The field of activity of biodosimetry has significantly expanded with the methods of genomics [1,2], proteomics [3,4], metabolomics [5] and transcriptomics [6]. At the same time, methods of cytogenetics that require the cultivation of lymphocytes *in vitro* or the use of special equipment still remain the classical methods [7-13]. These include the dicentric chromosome assay (DCA), the micronucleus assay or cytokinesis-block micronucleus assay (CBMN), the premature chromosome condensation assay (PCC), fluorescence in situ hybridization (FISH) and the recent addition of cH2AX scoring as a cytogenetic tool. Among them, the dicentric chromosome assay is considered to be the gold standard in the field of cytogenetic biodosimetric studies, but it is unlikely to be used in emergency situations with a large number of victims. Recently a number of improvements have been proposed in this regard for the dicentric chromosome assay. One of these attempts is the creation of an international network of laboratories to participate in a web-based platform for counting of images of the dicentric chromosome assay [12].

The appearance of dicentric chromosomes in lymphocytes as a result of radiation leads to the appearance of the following anomalies: micronuclei, "tailed" nuclei, nucleoplasmatic bridges, dumbbell-shaped nuclei, etc. Such anomalies arise at the cellular level and are well distinguished in a light microscope. Thus, nuclear anomalies are the simplest biomarker for bioindication, and we also assume that in connection with the dose-dependent effect of their appearance, established *in vitro* and *in vivo*, they can also become an object of biodosimetry. An analysis for the detection of such biomarkers of radiation can be carried out in any country, in any laboratory of a medical institution, where it is possible to determine a differentiated white blood cell in blood smears. Smears of peripheral blood taken from a finger should be prepared by a routine method, air dried, fixed with 96% ethanol, and stained with Giemsa. After that, smears can be observed to detect nuclear anomalies in the cells of lymphocytes. In this case, all types of karyopathology should be taken into account in a

complex manner in connection with their pleiotropic origin. In the laboratory, one analysis will take 40-60 minutes. With the introduction of the method into use and the creation of an express test, this time can be reduced to 15 minutes. We would recommend conducting a test for nuclear anomalies within a period of 72 hours to 3 months after irradiation. This is due to the fact that all known dose-dependent effects were shown in lymphocyte populations 72 hours after irradiation.

More than that, these types of karyopathology have been found, in addition to human cells (including blood lymphocytes in individuals exposed to the Chernobyl disaster in 1986), as well as in various animals and plants. This makes these biomarkers universal, permitting to use them not only to estimate people's health damage but also the state of ecosystems located in the zone of radiation pollution.

In this review, the main types of nuclear anomalies of human lymphocytes are observed in the light of their common origin from dicentric chromosomes in response to radiation exposure.

Nuclear Anomalies of Human Lymphocytes

Micronuclei

Micronuclei are the most studied nuclei anomaly that manifests under the influence of ionizing radiation. Initially, they were found in human erythrocytes, where their appearance was induced by vitamin B12 deficiency [14]. Micronuclei represent fragments of the cell nucleus, which carry an incomplete part of the genome. The frequency of detection of micronuclei correlates with an increase in the dose of irradiation, as well as with the frequency of occurrence of double-stranded DNA breaks [15,16]. Thus, the mechanisms of micronuclei formation are closely related to this form of aberrations.

The micronucleus can contain either an acentric region of the chromosome or an entire chromosome that was not distributed to one of the opposite poles during anaphase of mitosis. Fragments or whole chromosomes eventually become covered with a nuclear envelope and morphologically appear to be similar to the cell nuclei, not exceeding 1/3 of its diameter [17]. The ratio of the frequency of the two mechanisms of the micronuclei appearance in human lymphocytes under the influence of genotoxic agents lies in the range from 70: 30 to 30:70, depending on age and sex [17]. To determine the nature of micronuclei containing an acentric region or an entire chromosome, a pancentromeric DNA probe is used.

After introducing double-stranded DNA breaks under the influence of radiation, acentric DNA fragments can be formed as a result of non-restoration of these ruptures by DNA reparation systems or as a result of disrupting the work of this system. Thus, in the case of damage to ATM, BRCA1, BRCA2, RAD54 proteins of the reparation system, two chromosome segments bearing centromeres may merge to form a dicentric chromosome with a simultaneous separation of the two acentric fragments [18]. Another way for acentric fragments to form is a damage of DNA excision reparation system which corrects the mismatched nucleotide pairs. In this case DNA double-strand breaks can also be formed [19,20] and, as a consequence, the acentric fragments of chromosomes.

A reason of the entire chromosome isolation inside the micronucleus might be its misconnection with spindle microtubules during the karyokinesis. Among possible mechanisms are hypomethylation of centromeric and pericentromeric sites, kinetochore proteins damage, histones hypomethylation with the following decondensation of centromeric region, spindle microtubules defect, defect of mitosis phases checkpoint proteins and the failure of the amplification in centromeric regions [21]. Among other chromosomes, X-chromosome is included in micronuclei more often [22].

Micronuclei in peripheral blood lymphocytes most often correspond to cell nuclei by color, chromatin grain and intensity of staining (Figure 1). Sometimes micronuclei chromatin in lymphocytes appears darker and condensed than the chromatin of the main nucleus, and sometimes, on the contrary, a lumen is seen in the center of the micronucleus. The absolute sizes (diameters) of micronuclei in lymphocytes vary from 0.5 to 4.0 μm. Micronuclei of small sizes can sometimes be taken as basophilic granules in the cytoplasm. The shape of micronuclei in lymphocytes is usually round or oval. Much more often micronuclei are observed in single-nucleated cells, although there are also binuclear lymphocytes with micronuclei. Quite often there are micronuclei closely adjacent to the main nucleus, but without an obvious connection with it. The presented microphotographs were obtained by us in 1994 from the archive of hematological samples taken from the liquidators of Chernobyl disaster [23].

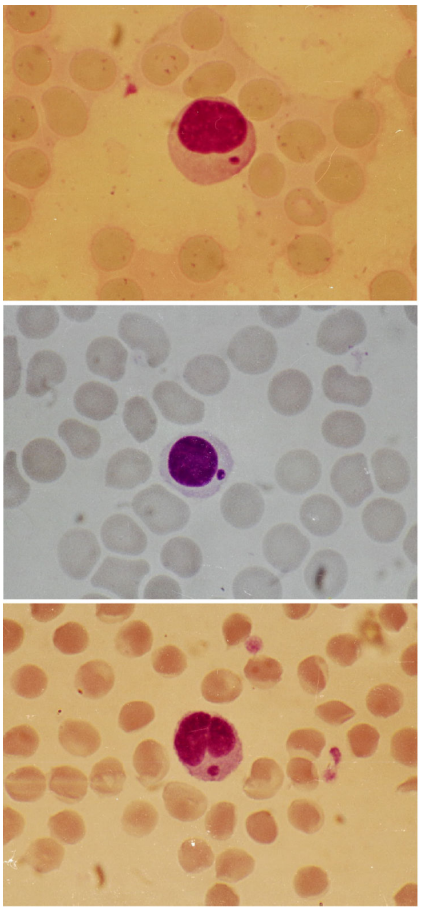

Figure 1: Micronuclei in human lymphocytes. Staining of azur II-eosin by Romanovskii. Magnification 1000x.

Livingston et al. [24] demonstrated that the relationship between the frequency of detection of micronuclei and the radiation dosage is

described by a non-threshold linear function: y= 2.78x + 3.71. This formed the basis for the widespread micronucleus test [17,25], validated in ionizing radiation biodosimetry. In this test micronuclei detection serves as an indicator of the genotoxicity of different agents. Later the test protocol was expanded, and along with the definition of micronuclei in human lymphocytes, nucleoplasmatic bridges and nuclear protrusions ("tailed" nuclei) were taken into account [26,27]. Today, there is no indication that genotoxicity tests of this kind should determine any other forms of nuclear anomalies, along with micronuclei, bridges and protrusions. However, other types of karyopathology, found in cells with micronuclei, also arise due to chromosomal aberrations as a manifestation of their pleiotropic effect. Such cells should be taken into account in the micronuclear test and, moreover, be considered as multiaberrant cells. Thus, modern protocols of the erythrocyte micronuclear test (EMNA), the analysis of micronuclei in erythrocytes by flow cytometry (EMNCA), and the analysis of micronuclei in the culture of human lymphocytes using the cytochalasin block (CBMN) require substantial revision in connection with the account of other nuclear anomalies that are also genotoxicity indicators.

Nucleoplasmatic bridges

Nucleoplasmatic chromatin bridges arise when centromeres of the dicentric chromosomes are pulled to the opposite poles of the cell during anaphase [21]. During the formation of two new nuclei of daughter cells in the telophase, the formed nucleoplasmatic bridge is also covered by a nuclear envelope. It usually undergoes a rupture during cytokinesis, resulting in the formation of "tailed" nuclei. One of the reasons for the appearance of a nucleoplasmatic bridge may be a disruption in the preparation of double-strand DNA breaks and the fusion of two formed chromosome regions, each containing a centromere. In this case, micronuclei that are formed from the acentric fragments of chromosomes that remain after "cross-linking" are often found in the cell. Another mechanism for the appearance of nucleoplasmatic bridges is the fusion of two chromosomes in the telomere region with a dicentric chromosome formation. Such a fusion can occur in the case of disruption of the formation of a complex of telomeric proteins protecting the terminal regions of chromosomes. In this case, the repair enzymes recognize telomeric regions as double-strand breaks and "sew" them [28-30].

Frequency of occurrence of cells with nucleoplasmatic bridges depends on the dose of irradiation and this correlation is described by a linear-quadratic function with the following equation: $y = 0.002+0.002x+0.0009x^2$ [31]. Binuclear lymphocytes with chromosomal bridges (Figure 2) were also observed in Chernobyl disaster liquidators' blood with an average frequency of 0.057%, while in the control male donors similar cells were recorded with a frequency of 0.005% [23].

In proliferating cells during the subsequent division the bridge breaks to form two daughter cells containing so-called "tailed" nuclei. That is why the appearance of bridges in human peripheral blood lymphocytes after irradiation is a much rarer event compared to "tailed" nuclei, despite of their common origin. In this connection, it is more convenient to observe bridges when studying cells in the cytochalasin block, where further division of the cytoplasm does not occur. For comparison, rarely proliferating epithelial cells in people exposed to radiation have more bridges than "tailed" nuclei. For the same reason, bridges can persist for decades in thyroid cells exposed to radiation, due to their low proliferative capacity. Thus, the stretched bridges are a more convenient biomarker of ionizing radiation in the cells of the follicular epithelium of the thyroid gland, although "tailed" nuclei are also observed in the thyrocytes [32].

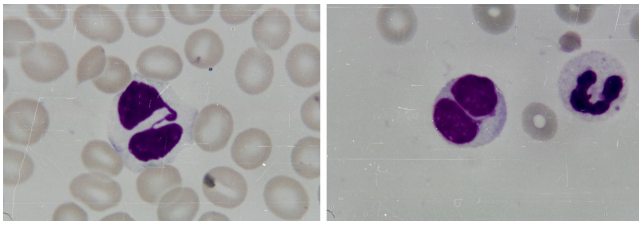

Figure 2: Micrographs of lymphocytes with nucleoplasmatic bridges. Staining of azur II-eosin by Romanovskii. Magnification 1000x.

"Tailed" nuclei

"Tailed" nuclei represent a thin protrusion or outgrowth of the nucleus into the cytoplasmic space. Such "tails" often have terminal expansion in the form of oval or round micronuclei. The length of these tails varies in lymphocytes of peripheral blood from 2 to 7 μm. Color, chromatin structure and intensity of "tails" staining usually correspond to the colors of the nucleus [33]. It should be noted that sometimes the "tail" chromatin is more compacted at the periphery of terminal expansion than the chromatin of the nucleus, while the terminal expansion has a clear center. Sometimes the cytoplasm of cells with a "tailed" nucleus also contains small individual micronuclei, which confirms the common origin of these events.

Nuclear "tails" can be classified into 16 different morphological types [23] in human lymphocytes. They are presented in Figure 3. A brief description of the main types:

- Types 1-3 have relatively thick "tails" with terminal expansion. The chromatin of both "tails" and nuclei is slightly condensed, without accumulations or signs of pycnosis. Types 1-3 differ from each other only in length (1- long, 2- medium, 3- short).
- Type 4 is an elongated "tail" without constriction at the nucleus end and with no terminal enlargement.
- Type 5 is similar in size and shape to type 4, except for the presence of a constriction where it emerges from the nucleus.
- Types 6-8 have a thin stalk of different lengths and a terminal enlargement in the form of a drop-like or round micronucleus, with chromatin more condensed at the periphery. Types 6-8, as with types 1-3, differ from each other only in the length of the stalk.
- Type 9 differs from types 6-8 only by the presence of breaks or discontinuities of the chromatin strand forming the stalk.
- Type 10 is a relatively thin and long "tail" without a terminal enlargement.
- Type 11 emerges from the nucleus at a point where the nucleus has a convex protuberance.
- Type 12 has two consecutively located chromatin enlargements.
- Type13 is a "tail" with a thin bifurcated stem with terminal enlargements.
- Type 14 has one enlargement in the form of a drop-like or round micronucleus connected to the nucleus by two stems.

- Type15 has a thin distal chromatin strand extending out of the enlargement.
- Type 16 has two nuclear "tails" which could be all of the above types.

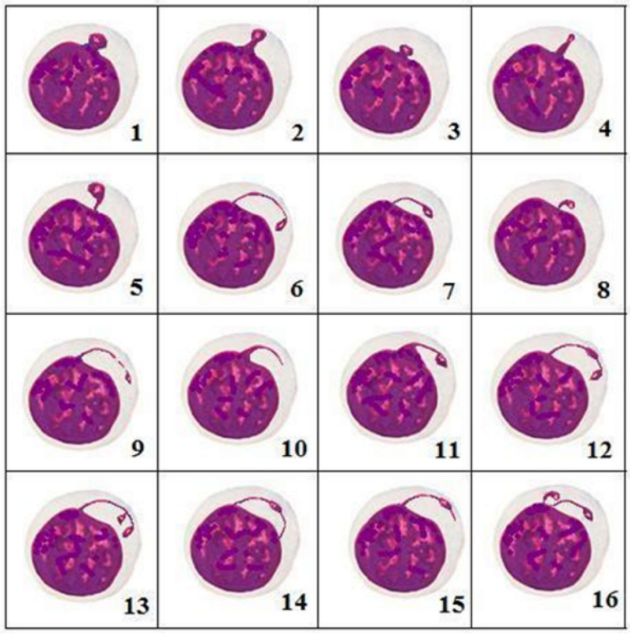

Figure 3: Drawings of 16 types of "tailed" nuclei observed in human lymphocytes.

The most common types of "tails" of lymphocytes are shown in Figure 4. The presented micrographs were obtained by us in 1994 from the archive of hematological samples taken from the liquidators of Chernobyl disaster.

Frequency of occurrence of cells with tailed nuclei depends on the dose of irradiation and this correlation is described by a linear-quadratic function with the following equation: y = 0.009+0.005x +0.003x2 [31]. A positive statistically significant correlation was also found between the presence of "tailed" nuclei and dicentric chromosomes in cells from the same individuals. Thus, the appearance of "tailed" nuclei is a predictable and universal manifestation of the formation of dicentric chromosomes and, as a consequence, the fact of irradiation. In cases of radiation exposure, it is possible to observe "tailed" nuclei *in vivo*, not only in human lymphocytes, but even in embryonic fish erythrocytes [34].

In the investigation of peripheral blood of the Chernobyl accident liquidators the differences between the irradiated people and the control groups were statistically significant. The mean frequency of "tailed" nuclei in control men was 0.14%, in control women 0.17%, and in control children 0.05%. On the other hand, irradiated subjects had lymphocytes with nuclear "tails". The maximum value in this group was 3.2%, with an average value of 0.50% [23].

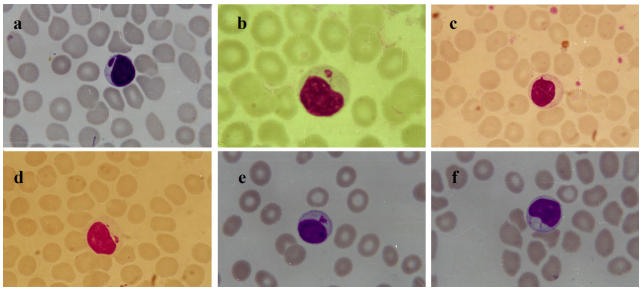

Figure 4: Micrographs of lymphocytes with "tailed" nuclei. (a) "tailed" nucleus of type 5, (b) "tailed" nucleus of type 6, (c) "tailed" nucleus of type 10, (d) "tailed" nucleus of the type 12, (e) "tailed" nucleus of type 13, (f) "tailed" nucleus of type 16. Staining of azur II-eosin by Romanovskii. Magnification 1000x.

Different fish species [34,35] and human [33] cells demonstrate "tailed" nuclei after exposure to radiation. There is a strong correlation between the frequency of occurrence of nucleoplasmatic bridges and "tailed" nuclei [31], which indicates that the latter are formed by rupture of bridges during cytokinesis. The cell disposed to ionizing radiation with the following mitotic divisions undergoes the "rupture-fusion-bridge" cycles. The resulting nucleoplasmatic bridges break during cytokinesis with the formation of "tailed" nuclei. The end regions of such broken chromosomes are recognized by the reparation system as double-strand breaks and are cross-linked to form dicentric chromosomes. As a result, after anaphase, nucleoplasmatic bridges are newly formed [36,37].

"Tailed" nuclei and nucleoplasmatic bridges can be the most specific indicators of radiation, allowing one to distinguish its effects from the effects of other genotoxic agents. Thus, with the simultaneous action of gamma radiation and agrochemicals, the fish formed the following anomalies: micronuclei, "tailed" nuclei, nucleoplasmatic bridges, deformed nuclei and vacuolated nuclei. However, the combination of "tailed" nuclei and bridges appeared only after exposure to ionizing radiation [17,26,33,34,37]. This was also confirmed by studies in which the appearance of "tailed" nuclei and nucleoplasmatic bridges was observed in the thyroid cells of mammals, in various cell lines and in peripheral blood lymphocytes of liquidators of the Chernobyl accident [26,38,39]. The simultaneous appearance in the cells of nucleoplasmatic bridges and "tailed" nuclei under the influence of radiation is also a consequence of the pleiotropism of chromosomal aberrations arising as a result of double-strand DNA breaks.

Dumbbell-shaped nuclei

The least studied biomarkers of radiation exposure are the dumbbell-shaped nuclei (Figure 5). However, these forms of pathology of the cell nucleus were repeatedly detected in peripheral blood lymphocytes in the liquidators of the consequences of the Chernobyl accident [40]. The two nuclei were fused together, resembling a dumbbell or a figure "eight". Morphologically this form of nuclei differs from the nuclei united by a nucleoplasmatic bridge. The appearance of dumbbell-shaped nuclei is associated with the formation of dicentrics and ring chromosomes [31]. In addition, the appearance of dumbbell-like nuclear suspenders was also attributed to the morphological features of amitosis, the direct method of cell division, in which the components of the nucleus are distributed unevenly between the

daughter nuclei [41,42]. However, later it was demonstrated that dumbbell-shaped nuclei can be formed in intensively dividing cells at low temperature and under nutrient deficiency [43]. Thus, the mechanism for the appearance of such a form of the nucleus is not clear. However, dumbbell-shaped nuclei can be considered a marker of genomic instability due to the fact that there is a correlation of their frequency with the effects of radiation and other genotoxic agents. Frequency of occurrence of cells with dumbbell-shaped nuclei depends on the dose of irradiation and this correlation is described by a linear-quadratic function with the following equation: $y = 0.003+0.014x +0.005x^2$ [31].

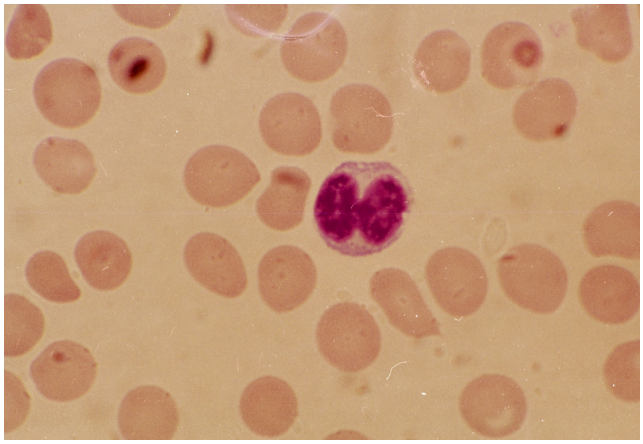

Figure 5: Lymphocyte with a dumbbell-shaped nucleus. Staining with Azure II-eosin by Romanovsky. Magnification 400x.

We would like to emphasize that the simplest biomarkers (nuclei abnormalities) we suggest for radiation exposure detection *in vivo* is not an alternative to classical radiation biomarkers *in vitro* [17], as well as to other modern approaches [2-5,44-46]. Detection of chromosomal aberrations (dicentrics) and the cytochalasine B micronucleus and "Cytome" methods are reliable bioindication and biodosimetry methods. However, in an emergency situation, when hundreds or even thousands of people can be exposed to radiation, these testing methods are unlikely to be available to all exposed subjects that require the cultivation of lymphocytes *in vitro*.

In this article, we describe biomarkers that have the same origin as dicentrics (chromosomal aberrations with a double DNA break), but are encountered and persisted *in vivo*. These biomarkers can be detected in blood smears from the finger, and the simplicity and low cost of the method make it practical in the emergency bioindication of the effects of radiation factors.

Conclusion

Lymphocytes with different types of karyopathology that are easily observed with regular peripheral blood smears are a biological response to radiation. In emergency situations, we recommend using lymphocytes with such anomalies as a simple biomarker in conjunction with hematological studies of blood smears of irradiated individuals.

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