Estimation of Absorbed Doses on the Basis of Cytogenetic Methods

Vladimir A. SHEVCHENKO, Galina P. SNIGIRYOVA*, Alexander V. RUBANOVICH

N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences
Gubkin st. 3, 117809 Moscow, Russia: shevchenko@vigg.ru
**Moscow Research Institute of Diagnostics and Surgery,*
Ministry of Health and Medical Industry of the Russian Federation, Moscow

Introduction

Long-term studies in the field of radiation cytogenetics have resulted in the discovery of relationship between induction of chromosome aberrations and the type of ionizing radiation, their intensity and dose. This has served as a basis of biological dosimetry as an area of application of the revealed relationship, and has been used in the practice to estimate absorbed doses in people exposed to emergency irradiation [1,2]. The necessity of using the methods of biological dosimetry became most pressing in connection with the Chernobyl accident in 1986, as well as in connection with other radiation situations that occurred in nuclear industry of the former USSR.

The materials presented in our works [3-8] demonstrate the possibility of applying cytogenetic methods for assessing absorbed doses in populations of different regions exposed to radiation as a result of accidents at nuclear facilities (Chernobyl, the village Muslyumovo on the Techa river, the Three Mile Island nuclear power station in the USA where an accident occurred in 1979). Fundamentally, new possibilities for retrospective dose assessment are provided by the FISH-method that permits the assessment of absorbed doses after several decades since the exposure occurred [7]. In addition, the application of this method makes it possible to restore the dynamics of unstable chromosome aberrations (dicentrics and centric rings), which is important for further improvement of the method of biological dosimetry based on the analysis of unstable chromosome aberrations [9].

The purpose of our presentation is a brief description of the cytogenetic methods used in biological dosimetry, consideration of statistical methods of data analysis and a description of concrete examples of their application.

1. Analysis of chromosome aberrations for the purposes of biological dosimetry

Under the action of radiation the genetic materials of the human organism, as well as any living organisms, experience damages of DNA molecules resulting in chromosome breakage and further in various chromosomal rearrangements. Such rearrangements of chromosomes are observed visually in metaphases of dividing cells, for example, in stimulated human peripheral blood lymphocytes. Single breaks of DNA molecules result in the occurrence in metaphases of chromosome fragments clearly revealed by the cytogenetic analysis. It is important to note that ionizing radiation induces damages affecting both chromosome chromatids, which leads to the appearance of paired fragments (in contrast to single fragments resulting from the damage of one chromatid, which is most often observed under the influence of UV-rays and chemical mutagens). In case there are two and more breaks of DNA molecules in a cell, the broken ends can recombine, creating new rearranged chromosomal structures (dicentric chromosomes, ring chromosomes, translocations, inversions, deletions). With the usual methods of chromosome staining, the most clearly recorded under a microscope are dicentric chromosomes (a chromosome rearrangement with two centromeres taking place due to the association of two broken chromosomes) and centric ring chromosomes (a chromosome closed in a ring as a result of two breaks at the ends of this chromosome). As a rule, such chromosome rearrangements are accompanied by paired fragments (acentric terminal parts of chromosomes). Analysis of the frequency of such easily detectable chromosomal rearrangements in metaphases has formed the basis for the development of methods of biological dosimetry.

Two types of chromosome aberrations can occur under the influence of radiation: unstable (dicentrics, centric rings, acentric fragments) and stable (symmetric translocations, pericentric inversions etc.). The occurrence frequency of unstable aberration, namely dicentrics [10], is most often used to estimate absorbed doses. Sometimes the analysis takes into account centric rings, but their frequency is insignificant in comparison with the frequency of dicentrics (approximately 5-10%) [11].

The advantage of the analysis of the level of dicentrics in biodosimetric studies is that they are easily found with a optical microscope without using special methods of processing and staining. One of the requirements for correct application of this method of dosimetry is to analyse metaphases of the first cell cycle. This is due to the fact that during cell proliferation about 50% of dicentrics are lost during the first postradiation division. The control of the cell cycle

with the use of special differential staining of chromosomes (FPG-method) allows us to avoid this mistake [12].

The spontaneous level of dicentrics is characterized by rather low values. Thus, the data on a relative level of dicentrics widely reported in publications show a significant interlaboratory variability of this value. The dicentric frequency in control groups may vary from 0 to $2.35 \cdot 10^{-3}$ per cell [11]. The average value of the frequency of dicentrics is within the range of $0.3\text{-}0.5 \cdot 10^{-3}$ per cell. Lloyd et al. [13] analyzed the data on the frequency of dicentrics in control groups of donors that were obtained in 65 different laboratories. An average dicentric level (the extreme values were not taken into account) made up $0.55 \cdot 10^{-3}$ per cell. In our experiments the spontaneous frequency of dicentrics for 82 donors having no contact with ionizing radiation made up 0.1-0.2 per 1,000 cells (26,849 metaphases analyzed). Thus, it is necessary to obtain reliable results on the spontaneous frequency of dicentrics in laboratories engaged in biological dosimetry in order to do correct assessment of radiation doses.

In experiments with animals [14] and in cytogenetic examinations of patients subjected to radiation therapy [15,16], it was shown that the frequency of dicentrics in lymphocytes of peripheral blood was comparable upon irradiation in vivo and in vitro. This fact is the main prerequisite for the use of calibration «dose - effect» curves obtained in vitro in biological dosimetric studies .

Special cytogenetic methods have been developed to register other exchange chromosome aberrations (inversions, deletions, translocations). The best known methods are G-banding and FISH. The latter was applied in our studies and therefore we shall discuss here the potentialities of this method.

In our experiments lymphocyte cultures and chromosomes preparation were prepared according to standard procedures [1]. Slides prepared for FISH analyses were stored at -20° C under nitrogen atmosphere until use. Slides for conventional analyses were stored for 5 days at room temperature and were then subjected to standard fluorescence plus Giemsa (FPG) staining.

Plasmid DNA of chromosome specific *Hind III* pBS libraries of human chromosomes 1, 4 and 12 [17] was biotinylated [18]. A degenerate a-satellite DNA pancentromeric probe was produced by in vitro amplification using a polymerase chain reaction (PCR) and labeled with digoxigenin [19]. Hybridization, detection of bound biotin-labeled painting probes for the target chromosomes with streptavidin-fluorescein isothyocyanate (FITC) conjugate and the bound digoxigenin-labeled pancentromeric probe with AMCA-labeled (7-amino-4-methylcoumarin-3-acetic acid) antibodies were carried out [19]. Counterstaning was performed with propidium iodide (PI) in antifade solution.

Conventional chromosome analysis was carried out exclusively in complete first division metaphases (M1) identified by uniformly stained sister chromatids. Recorded were all types of chromosomal damage but data are presented only for dicentrics and ring chromosome. About 300 cells were scored from each examined clean-up worker, each irradiated individual from contaminated regions and each control subject. For scoring FISH-painted exchanges, each metaphase spread was analyzed with a filter set allowing a simultaneous observation of FITC and PI fluorescence. The blue AMCA fluorescence of the bound digoxigenin-labeled pancentromeric probe was visualized by using an ultraviolet excitation filter. Chromosome morphology was additionally checked with a filter set providing only PI fluorescence. Depending on the quality of FISH-painting between 300 and 2,000 complete metaphases were scored from each donor. Rearrangements involving painted target chromosomes (yellow) and any other PI stained (red) chromosomes can be easily detected as two-colored structures (yellow/red). Two-colored chromosomes with one blue AMCA centromeric signal were classified either as complete or incomplete symmetrical translocations or insertions. Two-colored chromosomes with two centromeric signals were classified as dicentrics, dic(AB).

Genomic frequencies, F_G, for symmetrical translocations or dicentrics were calculated from the frequencies, F_p, of painted translocations or dicentrics for the target chromosomes by inversion of the equation $F_p = 2.05 f_p (1-f_p) F_G$ earlier applied by Lucas et al. [20] where $f_p = 0.192$ is the fraction of DNA contained in the painted chromosomes.

2. Statistical methods of biodosimetry

When processing the results of cytogenetic studies of exclusive importance is the choice of adequate methods of mathematical statistics. This is associated with specific difficulties faced when comparing the frequencies of rare events. Numerous discussions about the methods of biodosimetry and radiobiology of small doses concerned, in the majority of cases, the problems of reliability of statistical interpretations [21].

Statistical processing of the results of cytogenetic analysis includes two necessary stages:

1) Calculation of the level of significance of differences between irradiated and control groups of persons.

2) Evaluation of irradiation doses at the individual and group levels using calibration.

2a. Statistical significance of observed differences in aberration frequencies

Statistical analysis of cytogenetic data should begin with considering the question about the reliable interval of the aberration frequency in the irradiated group. In the case of processing cytogenetic data, this standard procedure has a number of specific features.

We shall enter necessary designations. Let N_0 cells be examined for the control group, and n_0 cells with chromosome aberrations be found among them. We shall designate the corresponding numbers for the irradiated persons as N_1 and n_1. Then the frequencies of

$$u = |\varphi_0 - \varphi_1| \sqrt{\frac{N_0 N_1}{N_0 + N_1}}$$

cells with chromosome aberrations are $p_0 = n_0/N_0$ and $p_1 = n_1/N_1$. For their comparison various modifications of the Student's criterion are frequently used. For example, u-statistics is calculated by the formula where

$$\varphi_i = 2\arcsin\sqrt{p_i}, \quad i = 0, 1.$$

The differences can be considered as significant, if the obtained value u is higher than 2.

However, the real aberration frequencies are so small that the standard parametric criteria become too conservative. For example, with the same number of estimated metaphases ($N_0 = N_1$) the value u is approximately

$$u \approx \sqrt{2}(\sqrt{n_1} - \sqrt{n_0}).$$

It means, that 9 cells with aberrations in the experiment do not statistically differ from 4 in control cells with any volume of examined material. At the same time the use of finer statistical tests can lead to opposite results. It seems that in processing of cytogenetic data one should completely abandon the Student's criterion and use nonparametric methods.

In some cases, it is enough to use the χ^2 - test which reduce to the calculation of the value

$$\chi^2 = \frac{(N_0 + N_1)(N_0 n_1 - N_1 n_0)^2}{N_0 N_1 (N_0 + N_1 - n_0 - n_1)(n_0 + n_1)}.$$

The differences between the control and the experiment are significant if the calculated value of χ^2 is higher than 4. Otherwise, the observed differences can be attributed to casual reasons.

In the analysis of cytogenetic data the number of examined metaphases significantly exceeds the cells with chromosome aberrations. In this case the expression for χ^2 is simplified:

$$\chi^2 \approx \frac{(N_0 n_1 - N_1 n_0)^2}{N_0 N_1 (n_0 + n_1)}$$

In practice, with same volume of examined material in the control and in the experiment, the primary estimation of χ^2 can be reduced to a very simple formula:

$$\chi^2 \approx \frac{(n_1 - n_0)^2}{n_0 + n_1}$$

There is a very essential restriction for the application of the χ^2 criterion in processing the results of cytogenetic analysis. The number of cells with aberrations both in the experiment and in the control (n_0 and n_1) should be more than 5. If this condition is not met, the χ^2 values may be overestimated and, thus, the significance of differences. On the other hand, with sufficiently large values of n_0 and n_1, the χ^2 criterion is as conservative as the Student's test. Therefore, when processing the results of cytogenetic analysis, it is preferable to use the exact Fisher criterion, which although complicated permits any doubts to be removed in all situations.

The essence of this universal method consists in exact calculation of the probability of occurrence of observed differences under the assumption that the irradiated and control groups are indiscernible by the aberration frequency. According to the combinatorial theory of probabilities with the given assumption, the probability of detecting in the control no more than n_0 of cells with aberrations is equal to

$$P = \sum_{k=0}^{n_0} \frac{C_{N_0}^{k} C_{N_1}^{n-k}}{C_N^n}$$

where $\tilde{N}_n^N$ - is the number of combinations from N of elements on n, $N = N_0 + N_1$ and $n = n_0 + n_1$. This probability defines the level of significance of difference by exact Fisher criterion [22]. With $P < 0.05$, the aberration frequency in the control is significant lower than in irradiated group (differences are essentially nonrandom).

The formula for P includes the probabilities of hypergeometric distribution, which describes the probability of sample size n without returning to the set of size N. Upon sampling from a large set ($N >> n$), samples with returning can be replaced by samples without returning. Thus, hypergeometric probabilities are replaced by binomial ones, which considerably reduces calculations. In the given approximation the level of significance is equal:

$$P \approx \sum_{k=0}^{n_0} C_n^k q^k (1-q)^{n-k}$$

where $q = N_0/N$. In this sum, the value of the term with the number $k = n_0$ exceeds the rest approximately by one

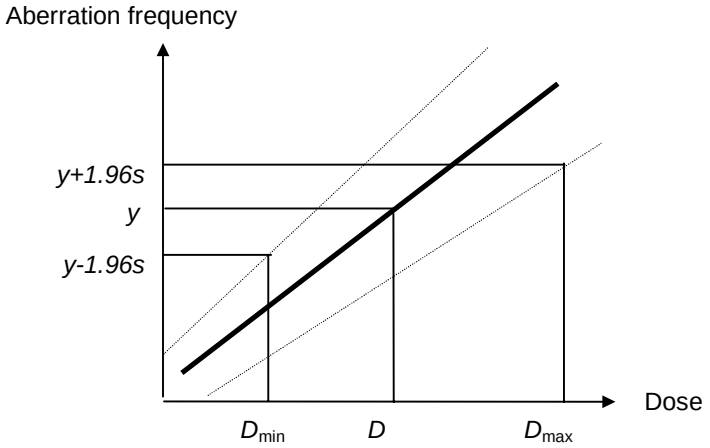

Fig 1. Calibration dose - effect curve with confidential intervals. A scheme of determination of 95%-confidential interval of dose estimation, corresponding to aberration frequency equal to $y \pm s$ is given.

order of magnitude. It can be used for primary estimations of the significance of differences:

$$P \approx \frac{n!}{n_0!n_1!}\left(\frac{N_0}{N}\right)^{n_0}\left(\frac{N_1}{N}\right)^{n_1}$$

If this probability is higher than 0.05, the differences between the experiment and the control cannot be considered significant.

If the sample sizes in the experimental and control groups are identical, the exact Fisher criterion allows the following interpretation: P is the probability of occurrence of no more than n_0 'heads' upon n throwings of a symmetric coin. Let, for example, two aberrations be found in the control and 8 chromosome aberrations in experimental group with identical numbers examined metaphases. Can such differences arise for purely casual reasons? What is the probability to make an error when asserting that the control group differs from the experimental one? According to the last formula, this probability is exactly equal to the probability of occurrence of no more than two "heads" in 10 throwings of a symmetric coin.

2b. Estimation of irradiation doses on the basis of calibration data

Estimation of individual doses of irradiation by the aberration frequency can be conducted on the basis of comparison with previously obtained calibrated data. As a rule, these data are presented by equations of linear or linear-square regression. In this case, the dose estimate is obtained as a result of simple substitution of the observed aberration frequency in a corresponding equation of regression. The procedure of determination of confidence intervals for dose estimates is shown in Fig. 1 taken from [1].

Let the observed aberration frequency is equal to $y \pm s$, where

$$s = \sqrt{\frac{y(1-y)}{N}}$$

is an average error in the aberration frequency y when a sample size is N. Then the upper (lower) estimate is at a point of crossing of the level y+1.96 s (y-1.96 s) with the lower (upper) confidential curve of regression.

An alternative approach consists in complete abandoning the application of regression equations and a direct use of calibration data presented in tabulated form. The appropriate mathematical theory is rather complex and uses Bayes's approach [2]. The theory permits a complete construction of the density distribution of probability of dose estimation corresponding to the observable frequency of aberrations. Numerical examples show that this method, though preferable in mathematical rigidness, does not essentially change evaluations obtained by the regression method [11].

3. Estimation of absorbed doses on the basis of analysis of unstable and stable chromosome aberrations

The basis for application of cytogenetic methods in biological dosimetry is a clear-cut dependence of the frequency of chromosome rearrangements on the doses of ionizing radiation, which has been repeatedly demonstrated in experiments. Having at disposal such dose dependencies obtained in vitro under the action of different kinds of ionizing radiation on cells (calibration curves), it is possible to attack the problem to estimate absorbed doses in irradiated people by analyzing the frequency of chromosome aberrations [1].

The pattern of a «dose - effect» curve for dicentrics and other chromosomal aberrations depends on the

Table 1. Coefficients of the equation $y = \alpha D + \beta D^2$ for dicentrics obtained after irradiation of human peripheral blood lymphocytes by various types of radiation.

Kinds of radiation	$\alpha \pm$ S.E. [$10^{-1}Gy^{-1}$]	$\beta \pm$ S.E. [$10^{-2}Gy^{-2}$]
^{60}Co γ, (0.017 Gy/m)	0.090 ± 0.400	4.17 ± 0.28
^{60}Co γ, (0.5 Gy/m)	0.107 ± 0.041	5.55 ± 0.28
220 kV X-ray (0.5 Gy/m)	0.404 ± 0.030	5.98 ± 0.17
14.5 MeV neutrons	1.790 ± 0.150	7.40 ± 1.39
Fission spectrum neutrons, E=1.6 MeV	3.690 ±0.210	13.34 ± 1.73
Fission spectrum neutrons, E=0.7 MeV	8.350 ± 0.100	-

types of ionizing radiation and its energy characteristics. The analysis of the generalized data of different authors on the dependence of the frequency of dicentrics in peripheral blood lymphocytes on the dose and type of radiation is presented in the work by D.C. Lloyd et al. [13].

For low-energetic radiation this dependence is usually described by the linear-quadratic equation $y = c + \alpha D + \beta D^2$, where c is the spontaneous level of dicentrics. For radiation with high LET the dose-effect dependence is linear $y = c + \alpha D$. As an example, the mean coefficients of the equations of calibration curves [11] are given in Table 1. The biological efficiency of different kinds of ionizing radiation can be judged by the ratio of coefficients.

The biological dose calculated on the basis of the frequency of dicentrics with the use of a calibration curve suitable for a given radiation situation is equivalent to the dose of acute uniform irradiation. The lower limit allowing a reliable (95 % probability) estimation of a dose (as a value equivalent to the dose of acute irradiation) depends on the number of analyzed cells, which is clearly demonstrated by Prof. Bauchinger in Table 2. In reality, with the analysis of about 5,000 cells the lower limit of reliable dose estimation is about 100 mGy for gamma - radiation and 50 mGy for fission spectrum neutrons.

Successful application of the analysis of the frequency of unstable aberrations (dicentrics and centric rings) for estimation of an irradiation dose is possible mainly after a single rather uniform radiation exposure in early (3-4 months) periods. So, in the work of Brewen et al. [23] the case of an emergency irradiation from a ^{60}Co source is described. Using the phantom and film-dosimetry the dose received by the injured person was reconstructed. It made up 127 R. On the basis of cytogenetic analysis the dose made up 144 R. The biological dose corresponded well to the data physical dosimetry.

Retrospective estimation of doses by the frequencies of unstable chromosome aberrations seems to be problematic today. The basic reason is the elimination of cells with unstable aberrations from circulating blood. However there are works where possible approaches for solving this problem are discussed. These approaches are based on the analysis of the processes of elimination of cells with aberrations, or on the analysis of the distribution of aberrations among cells [24,25,9].

The data collected from cytogenetic examinations of patients subjected to radiation therapy [26,27] and people injured as a result of accidents at nuclear enterprises [28-31] have allowed the quantitative description of the processes of elimination of cells with unstable chromosome aberrations and to estimate the duration of the lymphocyte life. Unfortunately, these data are not unambiguous. The parameters characterizing the elimination of lymphocytes

Table 2. Cell number and significantly elevated dicentric frequencies as compared to a background level (14 per 35,500 cells) required for the detection of a dose of ^{60}Co γ-rays (0.5 Gy•min^{-1}) with $p = 0.05$.

Cell number	Dicentrics significant	Dose (Gy)
100	1	0.63
200	2	0.56
500	2	0.31
1000	3	0.24
2000	4	0.17
5000	6	0.10
10000	10	0.074
20000	17	0.056
50000	37	0.041
100000	70	0.033

considerably vary depending on individual radiosensitivity of the donors, conditions of irradiation and the action of various additional environmental factors. There is no universal curve, especially for cases of partial irradiation. Also there is a lot of uncertainty in the estimation of the half-life period of cells with unstable chromosome aberrations which varies from 110 days to 4 years according to different authors. In our studies on the estimation of the half-life period of cells with unstable chromosome aberrations by calculating the ratio of the frequency of stable aberrations (estimated by the FISH method) to the frequency of unstable aberrations, this period is 4 years [9]. Apparently, retrospective dose estimation on the bazis of the frequency of unstable aberrations can be used taking into account the elimination process of cells with aberrations, when it is necessary to estimate an average dose for a group of irradiated individuals. In case of individual dosimetry, the dose can be determined only with a rather wide confidence interval.

For the purposes of retrospective dose estimation the FISH method is most often recommended at present. Stable chromosome aberrations revealed with this method are not eliminated in time, therefore, the dose estimation based on the analysis of the frequency of such stable chromosome aberrations (translocations) is possible within many years after exposure to ionizing radiation.

Biological dose estimates were derived from an acute in vitro ^{137}Cs gamma-ray calibration curve (dose rate 0.5 Gy/min) for FISH-painted translocations (chromosomes 1, 4 and 12) obtained in the laboratory of M. Bauchinger [19] modyfied taking into the data for the control material obtained in our laboratory:

$$y = (0.96\pm0.27)\cdot10^{-3} + (0.95\pm0.21)\cdot10^{-2}\cdot D + (1.45\pm0.14)\cdot10^{-2}\cdot D^2,$$

where y is the frequency of observed FISH-painted translocations (chromosomes 1,4 and 12), D - the absorbed dose, Gy. On the basis of this calibration curve with the application of the FISH method, the estimation of absorbed doses in the Chernobyl liquidators within 8-10 years from the irradiation was carried out [7]. To this and, an experimentally obtained value of the frequency of FISH-painted translocations is substituted in the presented linear-quadratic equation and a corresponding D value is derived by solving the equation.

For instance, in case 1.6 translocations per 100 cells (the frequency 0.016) were observed in an irradiated patient on the basis of a calibration curve, it corresponded to the absorbed dose of 0.77 Gy (95% confidence interval from 0.3 up to 1.10 Gy). If 5 translocations per 100 cells (the frequency 0.05) were observed, the absorbed dose made up to 1.54 Gy.

It should be noted that this calibration curve concerns only a part of cell translocations including FISH-painted chromosomes (chromosomes 1, 4 and 12 in the given case). In principle, the study may involve other painted chromosomes as well. Depending on the size of painted chromosomes and their number, the analysis may include different portions of the entire genome. The calculation of the translocation frequency per whole genome is made using (as indicated in section 1 of this work) the formula of Lucas et al. [20] which takes into account the size and number of painted chromosomes.

Using this methodology the absorbed doses have been evaluated for a group of 52 liquidators, inhabitants of the Altai territory irradiated about 45 years ago from nuclear explosions on the Semipalatinsk nuclear test site, and people living in the region of the Three Mile Island power station at which there was a nuclear accident in 1979 accompanied by an emission of radionuclides to the environment. The results of these studies are presented in a special work.

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