Quantitative Evaluation of DNA Single-Strand Breaks in Irradiated P-388 Leukemia Cells Using the Method of Alkaline Denaturation

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UDC 616.155.392-092.4-085.849-07

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 115, № 4, pp. 416-418, April, 1993
Original article submitted November 19, 1992

Key Words: *DNA; P-388 leukemia cells; irradiation*

One of the most sensitive methods of assessing single-strand breaks of DNA is the method of alkaline denaturation first described by Ahnstrom and Rudberg [2,5]. Its essence lies in an indirect measurement of the rate of DNA denaturation in cell lysates judged by the accumulation of single-stranded DNA (or loss of double-stranded DNA). Quantitative assessment of double-stranded DNA is carried out after separation of single-stranded molecules (e.g., using hydroxyl-apatite chromatography [4]) or by fluorimetry [3]. The denaturation conditions are chosen so that the process is initiated at the ends of a duplex or at the break-containing sites. After the optimal level is reached, the process is terminated by mild acidification of the solution. The specific content of denatured or native DNA in the cell lysates serves as an indicator of the initial number of single-strand breaks.

The aim of this study was to determine the optimal conditions for measuring DNA single-strand breaks in P-388 leukemia cells exposed to various doses of radiation.

MATERIALS AND METHODS

P-388 leukemia was maintained *in vivo* by serial transplantations on male mice of the DBA/2 strain. Five to six days after intraperitoneal injection of 10^6 tumor cells (the end point of the exponential growth phase in this tumor model) cells were harvested, freed of ascitic fluid, washed, and resuspended in 0.85% NaCl in a concentration of 5×10^6 cells per ml.

In order to perform controlled DNA denaturation after Birnboim [3], aliquots of 200 μl of cell suspension were transferred to three series of tubes (A, B, and C) followed by the addition of solution 1, containing 9 M urea, 10 mM NaCl, 5 mM EDTA, and 0.1% Na-dodecylsylfate. Cells were lysed for 10 min at 0°C. Lysates of groups A and B served as the internal control, while the C group samples were used for controlled denaturation. Following the completion of lysis, to the specimens of control group A 400 μl of solution 2 were added (1 M glucose, 14 mM 2-mercaptoethanol) for the prevention of denaturation. DNA of control group B was pulverized by ultrasound. 100 μl of each of the two following alkaline denaturing solutions were then carefully added to the samples of all groups, the first one consisting of 4 volumes of solution 1 and 6 volumes of 0.25 M NaCl, and the second one of 4.5 volumes of solution 1 and 0.55 volume of 0.25 M NaCl. The samples were first incubated without stirring for 30 min at 0°C and then for various controlled periods at 25°C (see Results). DNA denaturation in the samples of groups A and B was terminated by adding 400 μl of solution 2, after which DNA fragmentation in groups A and C was performed in order to reduce the viscosity of the solutions. Thus, in the group A samples DNA is double-stranded, as denaturation does not proceed after the addition of solution 2; the samples of group B contain denatured DNA only, while

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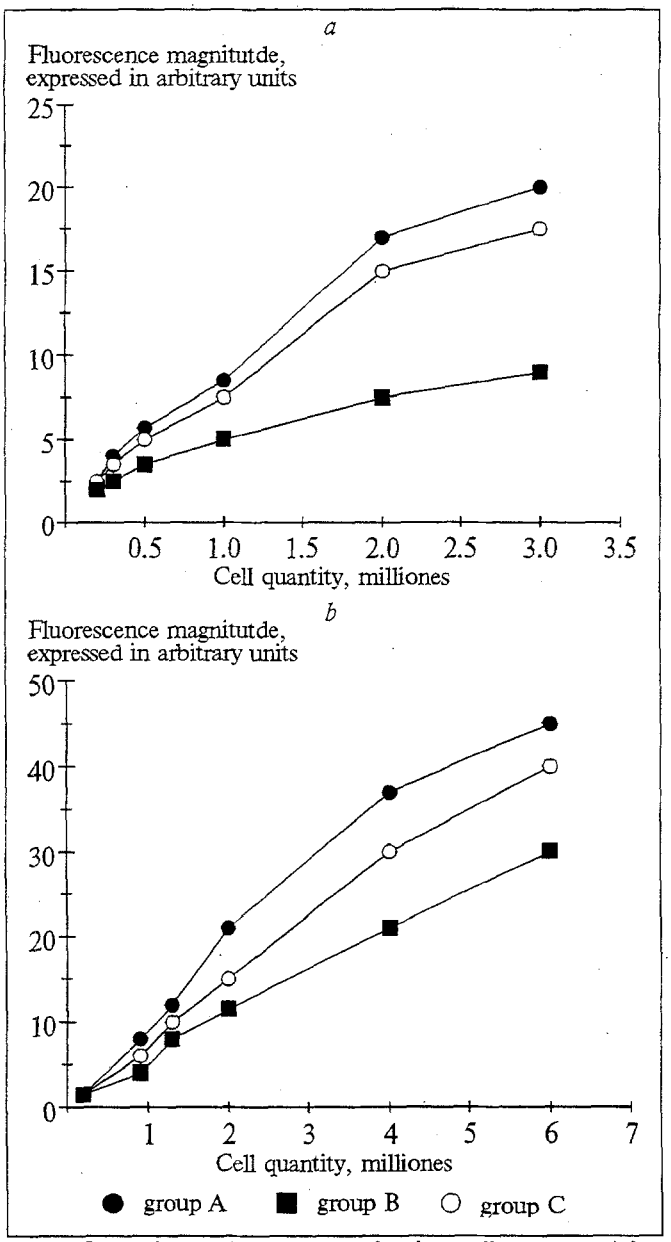

Fig. 1. Dependence of fluorescence level on cell quantity. *a*) 0–3 million cells per sample; *b*) 0–6 million cells per sample.

in the group C the DNA is partially denatured. To each sample 1.5 ml of a solution containing 6.7 μg per ml ethidium bromide and 13.3 mM NaOH were added, and the fluorescence was measured at 590 nm wavelength using an excitation wavelength of 518 nm. The percentage of double-stranded DNA was calculated as follows: [(Fc-Fb)/(Fa-Fb)]×100%.

RESULTS

As is shown in Fig. 1, the magnitude of the fluorescence signal is proportional to the DNA concentration in the sample and corresponds to the fluorescence of the DNA-ethidium bromide complex present in a given concentration.

The initial part of the curves approximates a straight line. In our study this rectilinear region corresponded to 20 μg DNA per sample at ethidium bromide concentration of 6.7 μg per ml, and 40 μg DNA per sample at a fluorochrome concentration of 13.5 μg per ml.

The intensity of fluorescence of the DNA-ethidium bromide complex in all examined groups depens largely on several parameters, including the alkali concentration [1]. It was found that by varying the alkali concentration, it was possible to influence the fluorescence intensity in samples from different control groups (Fig. 2). As was described earlier, the method of estimating DNA single-strand breaks by controlled alkaline denaturation is based on measuring the fraction of double-stranded DNA in the samples by the intensity of fluorescence of the DNA-ethidium bromide complex. Used as internal standards are the control series A and B, which contain complexes of ethidium bromide with single- and double-stranded DNA, respectively, as well as various other possible fluorescing complexes and free ethidium bromide in concentrations equal to those in the experimental series (C). Thus, the range of fluorescence values in the samples of the C series is limited by maximal and minimal levels, corresponding to the indexes of groups A and B, respectively. Therefore, the maximal sensitivity of this method should be observed under conditions of a maximal difference between the A and B fluorescence levels.

As shown in Fig. 2, these conditions correspond to an alkali concentration of 0.029 M (at a constant temperature of denaturation).

The fluorescence of samples from the A and B series proved to be independent of the duration of incubation in the range of 10 min to 4 hours (Fig. 3).

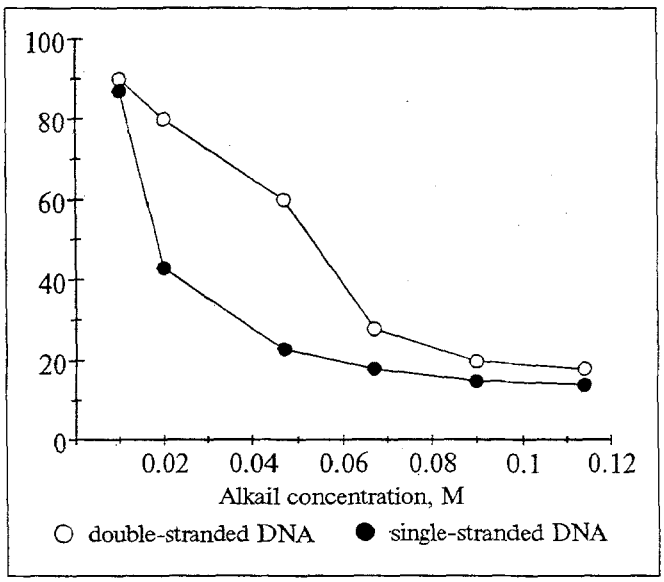

Fig. 2. Effect of NaOH concentration on fluorescence. Designations as in Fig. 1.

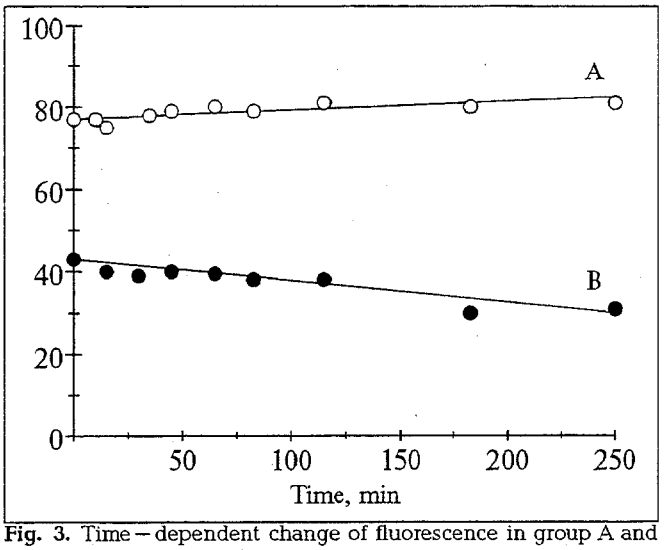

Fig. 3. Time-dependent change of fluorescence in group A and group B samples. Designations as in Fig. 1.

Since in the experimental series (C) DNA denaturation should be blocked at the stage ensuring maximal sensitivity of the method of DNA strand break detection in the damaged cells (as compared to intact cells), the next step in our study was to seek the optimal duration of alkaline treatment of the samples. Figure 4 shows that denaturation of irradiated samples occurs even in the cold. Thus, the process of alkaline denaturation may be divided into two stages. On the first stage the lysates are treated with the denaturing solution and are stored in the cold for 30 min in order to level the alkali concentration; the second stage consists of warming to 25°C. Strictly speaking, during the incubation on ice denaturation is undesirable, as it

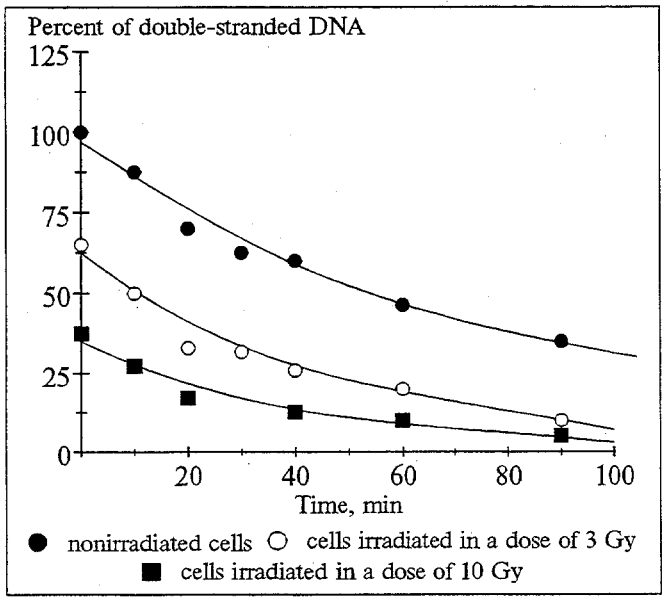

Fig. 4. DNA denaturation in irradiated and nonirradiated cells.

first proceeds unevenly throughout the volume of the sample. After the samples have been transferred to the incubator, they undergo warming to 25°C, and thus the second stage begins. It follows from Fig. 4 that for radiation doses up to 10 Gy it is sufficient to incubate the samples at 25°C for 10-20 min. Prolonged denaturation leads to a decrease of the fraction of double-stranded DNA in both irradiated and intact cells, thus limiting the opportunity to identify the damage. In the course of measuring the percentage of double-stranded DNA remaining after controlled denaturation in cells exposed to various doses of ionizing radiation, we disclosed a dependence of the specific amount of double-stranded DNA on the radiation dose (Fig. 5). This relationship ties in with the idea that the appearance

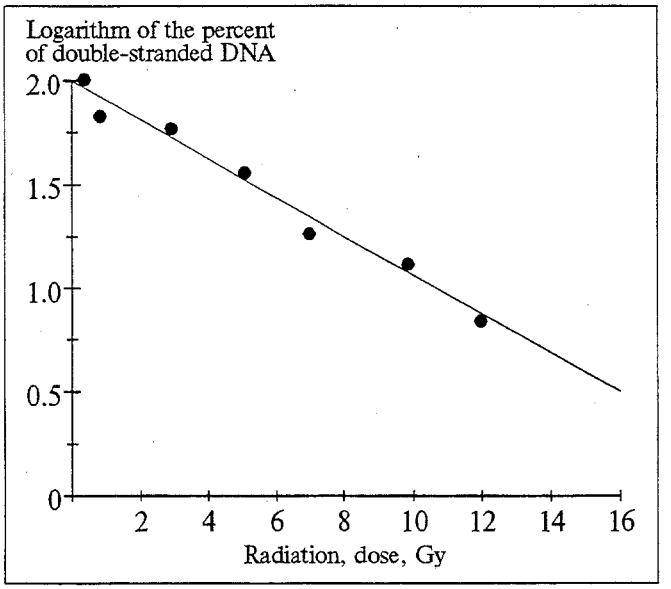

Fig. 5 Quantity of double-stranded DNA as a function of the dose of radiation. Cell quantity is 0.25 mln per sample; concentration of ethidium bromide solution 3.3 μg per ml.

of single-strand breaks is a one-shot process, and it proves the applicability of our methods to the study of DNA single-strand breaks.

The authors wish to express their gratitude to N. P. Yavorskaya, I. S. Golubeva, and M. V. Shalygina for their help in maintaining the cell lines.

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