EFFECT OF FRACTIONAL ACTION OF UNEQUAL CONCENTRATIONS OF CHEMICAL MUTAGENS

A. N. Tulina

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Recent investigations [1, 4-13] have shown the ability of the initial preliminary concentration of a mutagen to significantly weaken the mutagenic action of the basic concentration, which may be tens and hundreds of times greater than the preliminary concentration. This effect can be regarded as the manifestation of a unique adaptive response of the cell to external environmental mutagenic factors as a result of the activity of cellular repair mechanisms. In the publications cited the effect was assessed on dividing cells. Because of the considerable time interval between treatments (from 2 to 8 h) it is therefore impossible to identify the stage of the cell cycle in which this effect takes place. The possibility likewise cannot be ruled out that the cell cycle may have been delayed as a result of the procedures of treatment and washing of the mutagen, which may lead to treatment of other stages of the cell cycle, with a lower level of cytogenetic sensitivity. These technical difficulties can be avoided by using an unstimulated culture of lymphocytes in the G_0 stage. This paper gives data on the cytogenetic effect of fractional exposure to unequal concentrations of mutagens differing in their chemical structure on unstimulated human lymphocytes.

EXPERIMENTAL METHOD

Experiments were carried out on a culture of human lymphocytes at the G_0 stage before stimulation with phytohemagglutinin (PHA). The basic concentration of the mutagens thiotepa (a single-site mutagen) and dipin (a two-site mutagen) was 20 μg/ml. The preliminary concentrations were 10, 10^2, 10^3, 10^4, and 10^5 times less than the basic concentration. The schedule of the experiments was the same for both mutagens and was as follows: 10 ml of culture mixture (1 part of whole blood, 3 parts of bovine serum, 12 parts of Eagle's medium) was treated with five preliminary concentrations of the mutagen (in the version without preliminary treatment, 1 ml of Hanks' solution was added to the culture mixture) and the sample was incubated at 37°C for 1 h. The cells were then washed twice with Eagle's medium, the culture mixture was changed, and incubation without the mutagen was carried out at 37°C for 2 h. Treatment with the basic concentration of the mutagen was then given with an exposure of 1 h at 37°C. The cells were then washed 3 times with Eagle's medium to remove the mutagen, the culture mixture was changed, and PHA ("Difco P") was added at the rate of 0.015 ml to 10 ml of the culture mixture. Colchicine was added 2 h before fixation in a dose of 0.5 μg/ml. The culture was fixed at the 56th hour of incubation with a mixture of methanol and glacial acetic acid in the ratio of 3:1. The resulting preparations were stained with azure-eosin. In each version from 125 to 250 metaphases were analyzed. The 95% confidence intervals for the percentage of aberrant metaphases and the number of aberrations were estimated by formulas taken from [2]. The significance of differences was estimated by the ψ^2 test.

EXPERIMENTAL RESULTS

The experimental results are given in Fig. 1 for thiotepa and Fig. 2 for dipin. The vertical lines correspond to 95% confidence intervals. The zone bounded by broken lines corresponds to the 95% confidence interval for versions treated with

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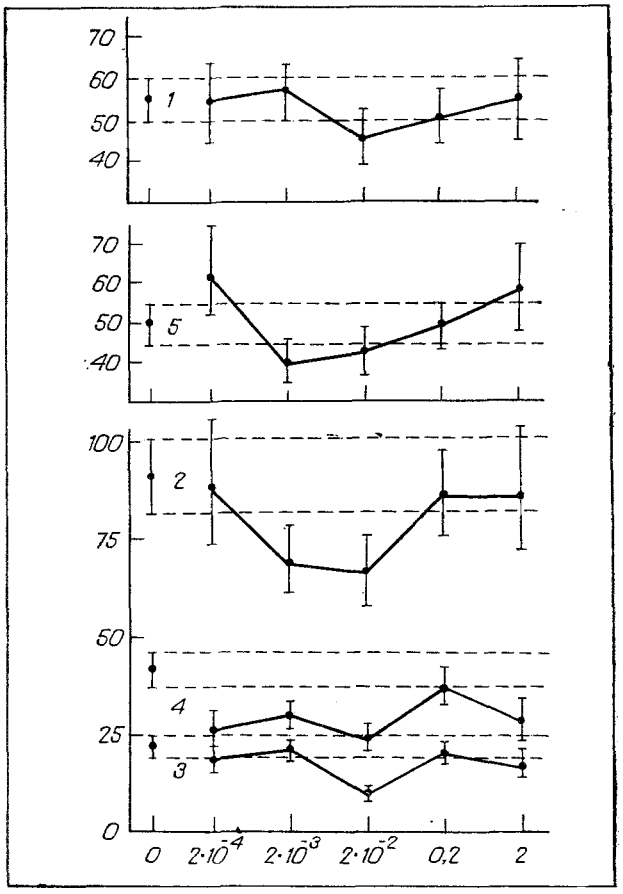

Fig. 1. Frequency of chromosomal aberrations after treatment with 20 μg/ml of thiotepa preceded by preliminary treatment with various concentrations of thiotepa. Abscissa, thiotepa concentration for preliminary treatment, μg/ml. Here and in Fig. 2: ordinate, number of aberrations per 100 cells (for the fraction of aberrant metaphases, in %); 1) fraction of aberrant metaphases; 2) total number of breaks; 3) number of breaks in exchanges; 4) number of chromatid breaks; 5) number of chromosomal and isochromatid breaks.

mutagens, but without preliminary treatment. The mutagenic effects of only the preliminary concentrations were not significant. Under the influence of 2 μg/ml of thiotepa the fraction of aberrrant metaphases was 9.8% and the number of chromosomal breaks per 100 cells was 12.3; for a concentration of 0.2 μg/ml the corresponding values were 3.1% and 4.7, and for a concentration of $2 \cdot 10^{-2}$ μg/ml they were 5% and 5. For lower concentrations the level of chromosomal aberrations was virtually identical with the control (0.5%). Dipin (2 μg/ml) induced 6% of aberrant metaphases and seven chromosomal breaks per 100 cells, a concentration of 0.2 μg/ml induced 2.5% and 2.5 respectively and a concentration of $2 \cdot 10^{-2}$ μg/ml caused 1.5% and 1.5. For lower concentrations the level of chromosomal aberrations was the same as in the control (0.75%).

It will be clear from Fig. 1 that the effect of the basic concentration of thiotepa was reduced after preliminary treatment with $2 \cdot 10^{-2}$ μg/ml of thiotepa for the total number of chromosomal breaks ($p < 0.01$) and also the number of single breaks and the number of breaks in chromatid exchanges ($p < 0.001$). No decrease in the effect was found for paired breaks (chromosomal and isochromatid) and the fraction of aberrant metaphases. The same also was observed in the case of dipin, but there were two distinguishing features. First, a wider pretreatment dose range was observed at which the effect of the basic dose was reduced (between $2 \cdot 10^{-3}$ and 0.2 μg/ml). Second, there was a decrease in the number of paired breaks but not of single (chromatid) breaks. This is perfectly natural because thiophosphamide causes mainly chromatid lesions, whereas dipin caused paired lesions. The experimental results are definite evidence that activation of repair actually takes place in the G_0 stage.

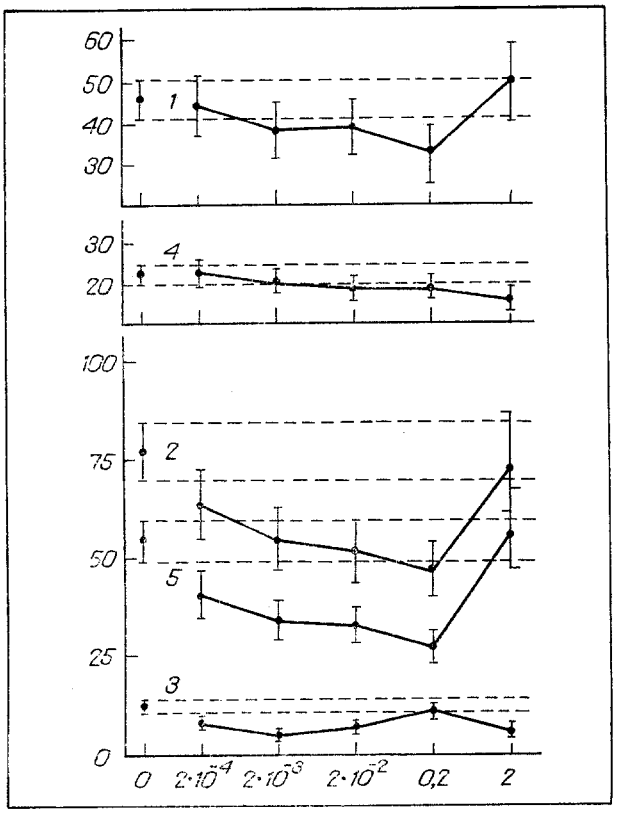

Fig. 2. Frequency of chromosomal aberrations after treatment with 20 μg/ml of dipin preceded by preliminary treatment with different concentrations of dipin. Abscissa, dipin concentration for preliminary treatment, μg/ml.

When the results are analyzed, attention is drawn to one circumstance: whereas if certain doses were used for preliminary treatment a decrease was observed in the total number of chromosomal breaks and breaks participating in exchanges, on account of lesions typical for each mutagen, the total number of injured cells was unchanged ($p < 0.05$). This can be interpreted as evidence that, irrespective of the dose of preliminary treatment, the level of injury to the cells due to the action of the basic dose was the same. However, after preliminary treatment with relatively small doses, the repair systems were evidently activated. As a result, the number of breaks per cell was reduced and, as a result, the number of exchanges also was reduced. This is evidence that quantitative relationships governing the formation of chromosomal aberrations were modified. Changes in the type of distribution of cells for number of chromosomal breaks can therefore be predicted, and this is a matter for separate investigation.

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ANALYSIS OF THE SECONDARY STRUCTURE OF DNA DURING ACTIVATION OF LYMPHOCYTES BY MITOGENS

E. Yu. Moskaleva

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Key words: DNA; secondary structure; mitogens; proliferation; lymphocytes.

The development of the immune response is accompanied by differentiation and proliferation of immunocompetent cells under the influence of the antigen. To study the molecular mechanisms of lymphocyte activation, stimulation of peripheral blood mononuclear leukocytes by mitogens is used as a model of the immune response in vitro [9]. The state of the structure of DNA of the lymphocytes during activation has not been studied, although it is known that the secondary structure of DNA of resting and proliferating cells differs [1, 2], and their differentiation also is accompanied by changes in the structure of DNA [5].

The aim of this investigation was to study the secondary structure of DNA of human peripheral blood lymphocytes during activation by mitogens on the rate of alkaline denaturation of DNA in cell lysates. The process of activation was monitored by determining the increased intensity of DNA and RNA biosynthesis, by the use of ^{3}H-thymidine and ^{3}H-uridine.

EXPERIMENTAL METHOD

Human mononuclear leukocytes were isolated from fresh heparinized blood by the method in [4], using centrifugation through a Ficoll—Paque gradient ("Pharmacia"), and after washing with Hanks' medium, they were used as lymphocytes, for those cells accounted for 80-95% of the preparations obtained. To obtain highly purified lymphocytes the fraction of mononuclear leukocytes was centrifuged through fetal bovine serum (FBS) with 5 mM EDTA to remove platelets, after which they were subjected to adhesion on plastic to remove monocytes [7], and then to filtration through a plastic column with nylon wadding to remove any contaminating monocytes and B lymphocytes [6]. Lymphocytes isolated in this way did not proliferate on the addition of concanavalin A (conA), confirming the high degree of purification of the monocytes. The intensity of DNA and RNA synthesis was determined by measuring incorporation of ^{3}H-thymidine (1 μCi/well, 40 mCi/mmole) and ^{3}H-uridine (10 μCi/well, 40 mCi/mmole), during incubation for 2 h with cells in a 96-well panel (Costar) in complete medium RPMI-1640 with 10 mM HEPES and 10% FBS, and in special cases, in Iscov medium without FBS in a CO_2 incubator. ConA was added to the lymphocytes in a concentration of 4 μg/ml. For radiometry the cells were collected on filters and washed to remove acid-soluble products with 5% TCA by means of an automatic cell harvester (Flow Laboratories). The structure of DNA of the intact cells and at various times after addition of conA was studied by the direct fluorometric method using ethidium bromide as described in [3], in the modification [8]. The rate of alkaline denaturation of DNA was estimated as a percentage of DNA remaining in the double-stranded form (dsDNA) after incubation of the cell lysate at 15°C for 1 h after establishment of the denaturing pH of 12.8 for 30 min at 0°C, calculated by the equation $D = [(P - B)/(T - B)] \cdot 100$, where B denotes the background fluorescence of the samples determined after treatment of the lysates with ultrasound and alkali (pH 12.8) for complete denaturation of DNA, T denotes total fluorescence, determined by dsDNA and fluorescent contaminants without treatment with alkali, and P denotes fluorescence determined by DNA fractions remaining in the dsDNA form after alkaline

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