Evaluation of Organ Specificity of Mutagenic Effects of Cyclophosphamide in Mice by Micronucleus Test

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In vivo cytogenetic effects of cyclophosphamide was simultaneously evaluated in 7 mouse organs. Cyclophosphamide produced most pronounced changes in the urinary bladder and bone marrow, the main target organs for this carcinogen. Mutagenic effects of the preparation were also detected in the lungs, large intestine, and stomach. This approach can be used for evaluation of organ specificity of mutagens and for prediction of the carcinogenic effects of chemical compounds.

Key Words: *cyclophosphamide; mutagenic effect; micronucleus test; lungs; urinary bladder; organ specificity*

The micronucleus test with bone marrow cells is currently used for evaluation of the mutagenic properties of chemical compounds and for prediction of their carcinogenicity [3,4]. At the same time, many substances produce changes in various organs and tissues, but not in the bone marrow [6]. *In vivo* micronucleus test on various mammalian organs allow us to estimate mutagenic activity of substances with regard to their organ specificity [6]. These assays are performed only on 2-3 organs, while for comparative analysis it is important to evaluate changes simultaneously in various organs. It is necessary to develop simple methods for preparation of reagents and to elaborate experimental designs that can be applied in studies of various organs and tissues.

Microscopic assay should be performed on smears prepared from cell suspensions, but not on histological slices of organs. This method allows us to obtain monolayers of undamaged and separately localized cells. Alkaline dissociation of cells that follows fixation of organs in formalin can also be used to prepare suspensions [1]. This procedure allows us to obtain and examine preparations from various organs at any convenient time. During elaboration of the experimental design, it is necessary to take into account proliferative activity of tissues. Subacute experiments with daily exposures to a test factor in 3 doses (standard for studies of mutagens) should be conducted for 1-2 weeks. Under these conditions a sufficient number of cells can pass through the mitotic cycle. We used this approach in experiments with 1,2-dimethylhydrazine and cyclohexene derivatives [7,8,11]. Here we performed a combined study of cytogenetic effects caused by the mutagen and carcinogen cyclophosphamide (CP) [10] in the lungs, various portions of the gastrointestinal tract, and urinary bladder.

MATERIALS AND METHODS

Experiments were performed on male (CBA×C57Bl6) F_1 mice weighing 20 g and obtained from the Kryukovo nursery. Water solution of CP (0.2 ml/20 g) was administered intragastrically through a probe at 24-h intervals (4 times). The doses of CP were 10 or 20 mg/kg (0.07 or 0.15 LD_{50}, respectively) [9]. Each experimental group included 6 mice. Ten mice served as the control. Bone marrow cells were isolated 24 h after mutagen administration [5].

The prestomach, stomach, small intestine (medium portion of the ileum, 4 cm in length), rectum, 4-5-cm portion of the large intestine proximal to the anus, urinary bladder, and lungs were fixed in 10%

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neutral formalin. Suspension preparations were obtained by alkaline dissociation of cells [1] modified to reveal micronuclei (MN). The samples were fixed in formalin for 6 months, washed with flowing water for 8 h, and kept in 50% KOH for 16 h. The mucosa was removed from hollow organs. The lungs were cut with scissors to a gruel state. The scrape or lung samples were washed with distilled water for 2 h and centrifuged at 1500 rpm for 6 min. The supernatant was removed, and smears were prepared from the precipitate. The samples were fixed with ethanol and acetic acid (3:1) for 15 min and stained with 2.5% acetorsein at 37°C for 1 h; the cytoplasm was stained with light green. The preparations were examined using an immersion lens (magnification 10×90). We analyzed 2000 cells from each mouse organ. Bone marrow cells with MN were counted. The ratio between the count of polychromatophilic erythrocytes and the total number of erythrocytes was estimated by calculating 200 cells. The mitotic index (MI) and count of cells with MN were estimated in other organs.

The results were analyzed by nonparametric Mann—Whitney *U* test (Statistica for Windows software).

RESULTS

In control mice the count of cells with MN was 1‰ (similarly to previous experiments) [6]. CP in doses of 10 and 20 mg/kg increased the count of bone marrow cells with MN by 4 and 9 times, respectively (Table 1). This dose-dependent effect of CP was described by a linear equation:

$$y=1.75\pm0.82x \ (p<0.001),$$

where y is the dose of CP (mg/kg) and x is the count of cells with MN (‰). The ratio between the count of polychromatophilic erythrocytes and the total number of erythrocytes in the control was 0.45. CP in doses of 10 and 20 mg/kg increased this parameter to 0.51 and 0.55, respectively, which indicated suppression of erythropoiesis (*i.e.*, toxicity of the preparation).

CP in both doses produced significant mutagenic effects in the urinary bladder: the count of epithelial cells with MN 8-fold surpassed the control.

CP increased the count of lung cells with MN by 2-3 times. Lung cells were presented mainly by type I and II pneumonocytes and alveolar macrophages, whose contents were practically similar. MN were revealed in all types of cells.

In the large intestine, CP 2-fold increased the count of epithelial cells with MN compared to the control. The dose-dependent effect of CP was described by the linear equation:

$$y=0.11\pm0.60x \ (p<0.005).$$

CP slightly increased the count of epithelial cells with MN in the stomach ($y=0.02\pm0.56x$, $p<0.05$), but had no effect on this parameter in the prestomach and small intestine.

MI in the prestomach, stomach, small intestine, large intestine, and lungs of control animals were 0.62, 0.65, 0.73, 1.04, and 0.09%, respectively. CP did not change this parameter. CP increased MI only in the urinary bladder (from 0.2 to 0.57%).

There was a good correlation between mutagenic and carcinogenic effects of CP. CP displayed cytogenetic activity in 5 of 7 organs. The mutagenic effect of this preparation was most pronounced in the urinary bladder and bone marrow, the main target organs for carcinogenic activity of perorally administered CP [10]. The increase in MI in epithelial cells of the urinary bladder confirmed carcinogenic activity of CP in this organ. The count of cells with MN also increased in the lungs, the main target organs for carcinogenic action of inhaled CP [10].

The cytogenetic effect of CP was less pronounced in the large intestine and stomach, which are not the targets for carcinogenic activity of CP. The effect of CP in the stomach is in doubt, since the differences in the count of cells with MN are obviously related to their low number in the control (0.1‰). Previous studies showed that the mean count of cells with MN in control mice is 1.32‰ [6]. However, it can not be excluded that CP is hydrolyzed at 30°C with the release of Cl^- [10], which acidifies the medium and contributes to the formation of mutagenic components. The cytogenetic effect of CP in the large intestine was reported previously [2].

Thus, the micronucleus test performed simultaneously on several mammalian organs holds much promise for predicting mutagenic and carcinogenic effects of chemical compounds and for evaluating of organ specificity.

TABLE 1. Number of Cells (‰) with MN in Various Organs of Mice Treated with Cyclophosphamide ($X\pm SE$)

Organ	Control	CP, mg/kg	
		10	20
Bone marrow	1.15±0.39	5.00±0.98*	10.50±0.89*
Prestomach	0.25±0.11	0.33±0.11	0.17±0.11
Stomach	0.1±0.1	0.33±0.11***	0.58±0.15**
Small intestine	0.35±0.15	0.16±0.11	0.33±0.17
Large intestine	1.5±0.2	3.25±0.51**	3.50±0.67**
Urinary bladder	1.65±0.60	13.50±5.04**	12.75±3.12*
Lungs	1.00±0.64	3.00±0.94***	1.75±0.59

Note. *$p<0.001$, **$p<0.01$, and ***$p<0.05$ compared to the control.

REFERENCES

1. L. N. Belov, M. E. Kogan, M. E. Leont'eva, *et al*., *Tsitologiya*, **17**, No. 11, 1332-1337 (1975).
2. I. F. Vyskubenko, E. G. Fel'dt, N. I. Sherenesheva, and V. S. Zhurkov, *Methodical and Methodological Problems of Genetics* [in Russian], Tomsk (1990), pp. 107-113.
3. *Methodical Recommendations for Studies of Mutagenic Activity of Chemical Substances after Estimation of their Maximum Permissible Concentrations in Water* [in Russian], Moscow (1986).
4. *Methodical Recommendations for Estimation of Carcinogenic Activity of Medicinal Preparations and Accessory Substances in Express Tests. Manual on Experimental (Before-Clinical) Studies of Pharmacological Substances* [in Russian], Moscow (2000), pp. 47-60.
5. *Methodical Recommendations for Estimation of Mutagenic Activity of Chemical Substances by the Micronucleus Test* [in Russian], Moscow (1984).
6. L. P. Sycheva and V. S. Zhurkov, *Vestn. Ros. Akad. Med. Nauk*, No. 7, 14-18 (1997).
7. L. P. Sycheva, Z. I. Zholdakova, E. E. Polyakova, *et al*., *Byull. Eksp. Biol. Med.*, **29**, No. 6, 683-685 (2000).
8. G. A. Belitsky, T. A. Lytcheva, I. A. Khitrovo, *et al*., *Cell Biol. Toxicol*., **10**, 265-279 (1994).
9. *Cyclophosphamide. Registry of Toxic Effects of Chemical Substances*, Eds. R. J. Lewis and R. L. Tatken, Cincinnati (1980), p. 219.
10. *IARC. Cyclophosphamide. Evaluation of the Carcinogenic Risk of Chemicals to Humans. Some Antineoplastic and Immunosuppressive Agents*, Lyon (1981), Vol. 26, pp. 165-202.
11. V. S. Zhurkov, L. P. Sycheva, O. Salamatova, *et al*., *Mutat. Res*., **368**, 115-120 (1996).