Expression of the c-Fos Gene in Hypothalamic Cells and Cytotoxic Activity of Natural Killer Cells in the Spleen of Rats after Treatment with Cytoxan

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In experiments on rats we studied the effect of cyclophosphamide-containing drug Cytoxan on activation of neurons in hypothalamic structures involved in the regulation of natural killer cell activity in the spleen and changes in cytotoxicity of these cells. Administration of Cytoxan in a dose of 60 mg/kg increased the number of c-Fos-positive cells in the ventromedial hypothalamus and lateral hypothalamic area and reduced interferon-α-induced cytotoxic activity of natural killer cells. Our findings attest to the involvement of central mechanisms of regulation of splenic natural killer cells into side effects of Cytoxan.

Key Words: *Cytoxan; natural killer cells; c-Fos protein*

Cytostatics (*e.g.*, cyclophosphamide-containing drugs) widely used in clinical practice produce pronounced side effects, which limits the duration or even makes impossible their use [2,5,6,8,9]. Deciphering of the mechanisms of side effects of these drugs will allows us to develop new approaches to diminish their adverse impact. This work was designed to study the effect of Cytoxan on the functions of the nervous and immune system. We evaluated the influence of Cytoxan on activation of neurons in the ventromedial hypothalamus (VMH) and lateral hypothalamic area (LHA) involved in the regulation of activity of splenic natural killer cell (NKC) [11] and studied changes in cytotoxicity of these cells.

MATERIALS AND METHODS

Experiments were performed on 20 male Wistar rats weighing 180-220 g. The animals were adapted to experimental conditions for 5 days before the start of the study. Cytoxan (Brystol-Myers Squibb) containing cyclophosphamide and diuretic mannitol was injected intraperitoneally in doses of 60 and 100 mg/kg.

The animals were divided into 4 groups. Some rats remained intact. Control rats received intraperitoneal injection of 1.5 ml physiological saline. The animals of 2 experimental groups were intraperitoneally treated with Cytoxan in doses of 60 and 100 mg/kg.

The brain was removed 2 h after intraperitoneal injection of Cytoxan and fixed by intracardiac perfusion [7]. c-Fos-like protein was identified in frozen sections by the immunoperoxidase method using primary polyclonal antibodies against c-Fos proteins and secondary antibodies labeled with horseradish peroxidase (Sigma) [4]. Taking into account morphological heterogeneity of the test structures, the brain sections from all animals were analyzed at the same levels and with demarking relevant zones within the structures [3]. c-Fos-positive cells were counted as described elsewhere [7] using Ista-Video-Test software.

Cytotoxic activity of splenocytes was measured 24 h after Cytoxan treatment by the ability of splenocytes to lyse human myeloleukemia K562 cells

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in vitro. The interaction between rat spleen NKC and target cells (effector/target ratios 25:1 and 50:1) was determined by ^{3}H-thymidine incorporation into unlysed target cells.

Additional stimulation of splenocytes included *in vitro* treatment with interferon-α (IFN-α, Institute of Vaccines and Sera, St. Petersburg). IFN-α in a dose of 1.25 U/ml was added to the mixed suspension of splenocytes and target cells before cell incubation.

The results were analyzed by Student's *t* test.

RESULTS

c-Fos protein, product of c-Fos gene expression, serves as a marker of activation of nerve cells. Therefore, the degree of activation of hypothalamic structures was determined by the number of c-Fos-positive cells. The number of these cells was estimated in 3 zones of VMH (ventrolateral, central, and dorsomedial zones; Fig. 1, *b*) and 2 zones of LHA (basal and perifornical zones; Fig. 2, *b*). The number of c-Fos-positive cells in VMH and LHA of control rats increased compared to intact animals. After administration of Cytoxan in a dose of 60 mg/kg the number of c-Fos-positive cells decreased in the ventrolateral zone, but increased in the dorsomedial zone of VMH (Fig. 1, *a*). The number of c-Fos-positive cells increased in both zones of LHA (Fig. 2, *a*). Treatment with Cytoxan in a dose of 100 mg/kg was followed by the appearance

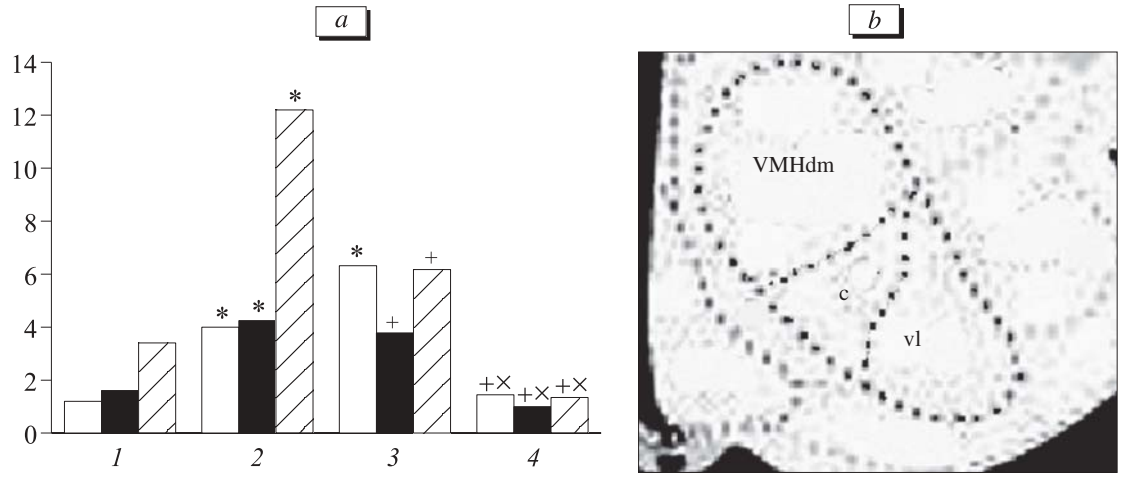

Fig 1. Number of c-Fos-positive cells (*a*) in VMH zones (*b*) after intraperitoneal injection of Cytoxan in various doses. *a*) ordinate: number of c-Fos-positive cells in VMH zones (10×10^3 μ^2). Light bars, dorsomedial zone; dark bars, central zone; shaded bars, ventrolateral zone. *b*) dm, dorsomedial zone of VMH; c, central zone of VMH; vl, ventrolateral zone of VNH (according to the rat brain atlas [2]). Here and in Figs. 2 and 3: intact animals (*1*); control animals (*2*); administration of Cytoxan in a dose 60 mg/kg (*3*); administration of Cytoxan in a dose 100 mg/kg (*4*). Here and in Fig. 2: $p<0.05$: *compared to intact animals; +compared to control animals; ×compared to animals receiving 60 mg/kg Cytoxan.

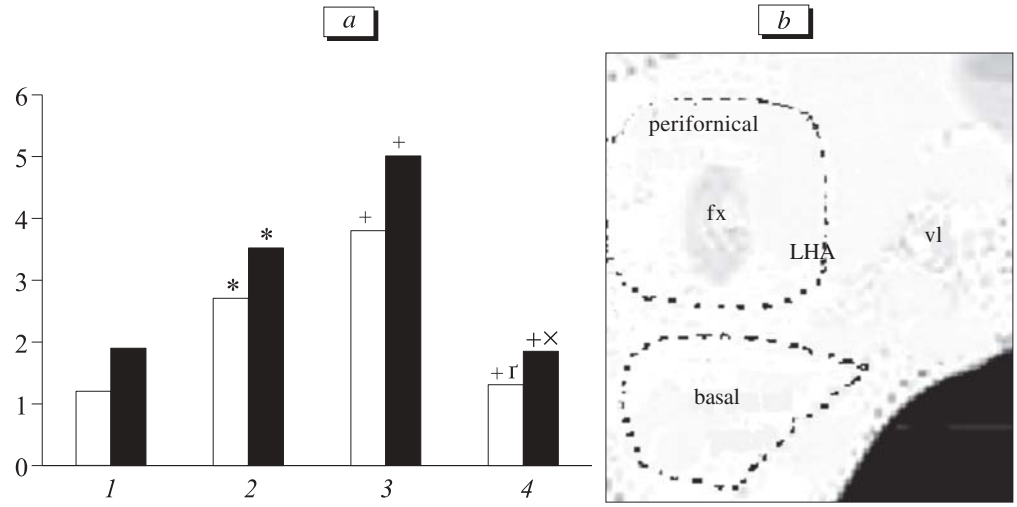

Fig. 2. Number of c-Fos-positive cells (*a*) in LHA zones (*b*) after intraperitoneal injection of Cytoxan in various doses. *a*) ordinate: number of c-Fos-positive cells in LHA zones (10×10^3 μ^2). Light bars, perifornical zone of LHA; dark bars, basal zone of LHA. *b*) perifornical, perifornical zone of LHA; basal, basal zone of LHA (according to the rat brain atlas [2]).

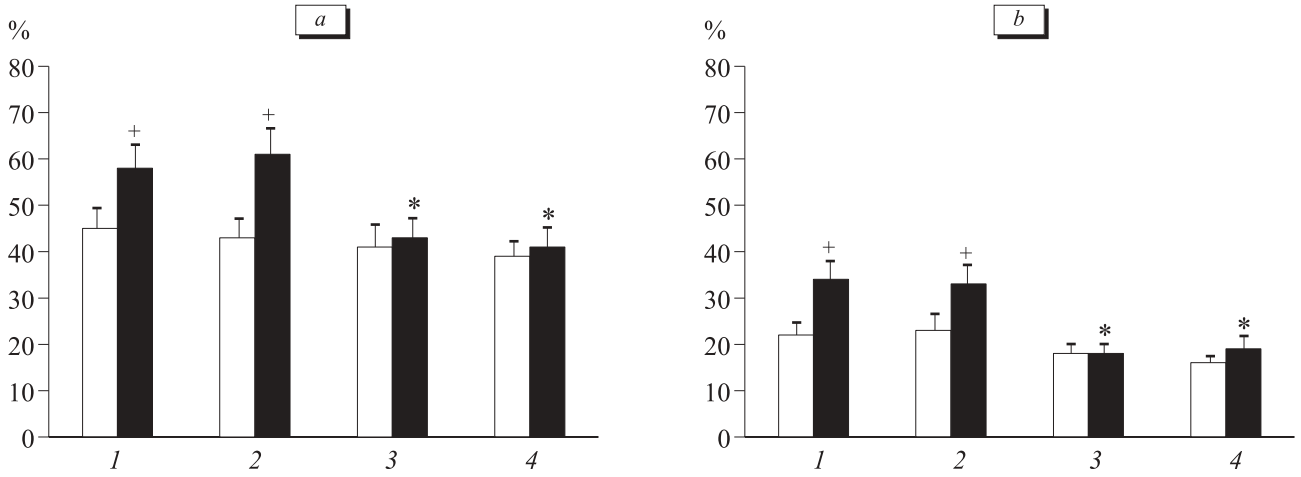

Fig. 3. Cytotoxic activity of natural killer cells (NKC) from rat spleen 24 h after administration of Cytoxan in various doses. Light bars, cytotoxic activity of NKC without additional stimulation; dark bars, after additional stimulation of splenocytes with IFN-α *in vitro* (1.25 U/ml). $p<0.05$: *compared to intact and control animals; +compared to cytotoxic activity of rat splenocytes without additional stimulation of cells with IFN-α.

of morphologically different cells with large vacuoles. Under these conditions the number of c-Fos-positive cells decreased in all zones of VMH and LHA (Figs. 1, *a*; 2, *a*).

Cytotoxic activity of splenic NKC remained practically unchanged 24 h after administration of Cytoxan in doses of 60 and 100 mg/kg (Fig. 3). IFN-α is a cytokine that regulates cell activity and stimulates NKC [1]. Addition of IFN-α to cultured cells from control animals increased cytotoxic activity. Specific cytotoxic activity of cells from rats receiving Cytoxan did not increase in response to stimulation with IFN-α (Fig. 3). Therefore, Cytoxan in doses of 60 and 100 mg/kg prevents the increase in cytotoxic activity of NKC induced by IFN-α treatment.

Our findings suggest that the number of c-Fos-positive cells in VMH and LHA increases during the early period after administration of Cytoxan in a dose of 60 mg/kg. These brain structures are involved in the regulation of NKC activity in the spleen. It should be emphasized that stimulation of VMH decreased cytotoxicity of these cells [11]. Cytoxan decreases IFN-α-induced cytotoxicity of NKC. There changes reflect impaired activation of cells (reaction to the regulatory signal). This methodical approach allowed us to reveal a change in cytotoxic activity of NKC during the early stage after cytostatic administration. It should be emphasized that standard methods are effective only on days 5-14 after treatment [5,9,10].

Activation of brain cells in VMH is most pronounced after administration of Cytoxan in a dose of 60 mg/kg. The degree of activation differs in various zones of this nucleus. The number of c-Fos-positive cells increases in the dorsomedial zone of VMH, which has an inhibitory effect on cytotoxicity of spleen NKC. It may be suggested that the decrease in NKC cytotoxicity is partly related to activation of cells in this zone of VMH.

These data attest to possible involvement of central mechanisms of regulation of splenic NKC into realization of side effects of Cytoxan. It cannot be excluded that Cytoxan produces a direct antimetabolic effect on these cells. Our results illustrate the importance of functional tests for cell activation in response to regulatory signals. These tests hold promise for the individual selection of appropriate drugs, study of variations in the patient's reaction to treatment, and search for new methods to diminish the side effect of cyclophosphamide preparations.

REFERENCES

1. F. I. Ershov, *Interferon System under Normal and Pathological Conditions* [in Russian], Moscow (1996).
2. H. Ben-Hur, G. Kossoy, Y. Tendler, *et al.*, *In Vivo*, **16**, No. 5, 287-292 (2002).
3. *Brain Maps: Computer Graphic Files*, Ed. L. W. Swanson, Amsterdam (1992).
4. *Handbook of Immunoperoxidase Staining Methods*, Ed. J. A. Bourue, Santa Barbara (1983).
5. V. R. Makar, M. K. Logani, A. Bhanushall, *et al.*, *Bioelectromagnetics*, **26**, 10-19 (2005).
6. R. Martinelli, L. S. Pereira, E. S. Santos, and H. Rocha, *Nefron*, **74**, No. 2, 313-317 (1996).
7. N. S. Novikova, T. V. Kazakova, V. J. Rogers, and E. A. Korneva, *Neuroendocronol. Lett.*, **23**, No. 4, 315-320 (2002).
8. C. A. O'Reilly and J. H. Exon, *Physiol. Behav.*, **37**, 759-764 (1986).
9. H. W. Smith, C. J. Winstead, K. K. Stank, *et al.*, *Toxicology*, **194**, Nos. 1-2, 129-145 (2003).
10. L. S. Stewart, L. S. Sewell, and A. W. Thompson, *Clin. Exp. Immunol.*, **79**, No. 3, 416-423 (1990).
11. M. Wenner, N. Kawamura, and T. Ishikawa, *Neuroimmunomodulation*, **7**, No. 1, 1-5 (2000).