Effect of the Triterpenoid Miliacin on the Sensitivity of Lymphocytes in the Thymus and Spleen to Dexamethasone-Induced Apoptosis

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We studied the effect of plant triterpenoid miliacin on dexamethasone-induced apoptosis in thymocytes and splenocytes. Miliacin produced a protective effect on splenocytes by decreasing the degree of DNA fragmentation due to blockade of the cascade cell death distally to the intramembrane phosphatidylserine translocation.

Key Words: *lymphocytes; dexamethasone; apoptosis*

Glucocorticoids (GC) produce various effects on the system of immunogenesis and modulate different cells and stages of the immune response [5,7,11]. The effects of GC are realized via induction of apoptosis in lymphocytes [2,4,8]. The inhibition of GC-induced death in lymphoid cells is a new approach to the therapy and prevention of secondary immunodeficiencies associated with excessive effects of these compounds during severe stress [1].

Here we studied the protective effect of plant triterpenoid miliacin on dexamethasone-induced apoptosis in lymphocytes of the thymus and spleen.

MATERIALS AND METHODS

We used miliacin isolated from millet oil crystals precipitated in cold (kindly provided by Prof. L. E. Olifson). The preparation was purified by recrystallization from chloroform. After mass spectrometry, study of nuclear magnetic resonance and infrared spectra, and analysis of chromatographic homogeneity and qualitative composition this preparation was assigned to the group of pentacyclic triterpenoids with a structure of 3-β-methoxygermanycene (3-β-methoxy-Δ^{18}-oleanene, Fig. 1) [3].

Thymocytes and splenocytes were obtained from 3 groups of (CBA×C57BL6)F_1 mice weighing 18-20 g and obtained from the Stolbovaya nursery (Russian Academy of Medical Sciences). Group 1 animals were intact before isolation of cells. In group 2 mice the solvent Tween-21 (final concentration 160 nmol/kg) in 0.9% NaCl was injected intraperitoneally 3 times at 3-day intervals. Group 3 animals received 2 mg/kg miliacin in the solvent. Thymocytes and splenocytes were isolated 1 day after treatment with the solvent or miliacin.

Thymocytes were obtained in a Potter homogenizer with a Teflon pestle by the method of squeezing. Splenocytes were routinely isolated in a Ficoll-Verografin gradient. The cells were washed and resuspended in Hanks solution (2×10^6 cells/0.5 ml). The cell

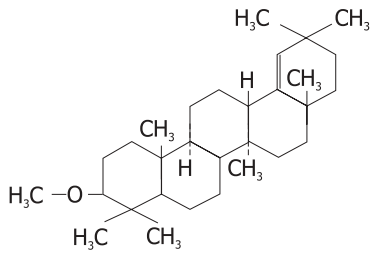

Fig. 1. Chemical structure of pentacyclic triterpenoid miliacin.

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suspension (4×10^5, 0.1 ml) was placed in 6-well Costar plates with 2.9 ml DMEM medium (PanEko) containing 10% fetal bovine serum (BioWhittaker), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Dexamethasone (Sigma) in a final concentration of 60, 300, or 3000 nM was added to wells to induce apoptosis. Cell death was studied after 16-h incubation in a humid atmosphere at 37°C and 5% CO_2. Control suspensions of thymocytes and splenocytes were incubated without glucocorticoid. In special series we studied apoptosis in freshly isolated cells.

After incubation cell samples from each well were divided into 2 equal portions for the analysis of early and late stage of cell death. The early stage of apoptosis was determined by the ability of cells to bind annexin V. The results obtained by this method illustrate changes in membrane asymmetry of phosphatidylserine (translocation from the inner to the outer leaflet of the plasma membrane) [6]. After incubation the cells were washed with Hanks solution and resuspended in the buffer to bind annexin V (PharMingen). Annexin V (1.5 μl) conjugated with fluorescein isothiocyanate (annexin V-FITC) was added to 3×10^5 cells (0.3 ml). The mixture was incubated at room temperature for 15 min, washed from unbound conjugate with Hanks solution, and resuspended in the same solution. Fluorescence (FL1) was determined on a FACSCalibur flow cytofluorometer (Becton Dickinson). Another portion of cells was used to study the late stage of apoptosis characterized by fragmentation of genomic DNA and accumulation of hypodiploid cells. Dexamethasone-treated and control cells were fixed in 70% ethanol in 0.9% NaCl, washed with Hanks solution, and resuspended in a buffer containing 0.15% propidium iodide, 0.3% NP-40, 50 μg/ml RNase A, and 0.1% sodium citrate (Sigma). FL2 of the suspension was recorded on a flow cytofluorometer. The index of hypodiploidy reflecting degradation of genomic DNA was estimated as the ratio between the incidence of events to the left of the peak corresponding to hypodiploid cells (sub-G1 area) and total count of cells. The results of cytofluorometry (not less than 5000 events for each sample) were analyzed using CellQuest™ software (Becton Dickinson). The significance of differences was evaluated by Student's *t* test.

RESULTS

Apoptosis in freshly isolated thymocytes and splenocytes was practically undetectable in the test with propidium iodide. Incubation for 16 h led to the appearance of hypodiploid cells in all groups. The mean indexes of hypodiploidy for thymocytes from intact mice and animals receiving the solvent and miliacin were 3.9, 1.9, and 2.2%, respectively. For splenocytes these indexes were 11.9, 12.8, and 9.6%, respectively. The count of hypodiploid splenocytes increased more significantly than the number of hypodiploid thymocytes, which was probably related to more complex and long procedure of splenocyte isolation. The ratios of annexin V-positive cells in freshly isolated thymocyte and splenocyte suspensions were 6.5 and 9.5%, respectively. After 16-h incubation the numbers of these cells increased to 21.3-24.1 and 26.9-29.2%, respectively (differences between groups were insignificant). These data confirm low informativeness of measurements of spontaneous apoptosis [6] and suggest that miliacin does not modulate cell viability after 16-h incubation without the hormone.

Thymocytes and splenocytes treated with 60 nM dexamethasone were characterized by pronounced an-

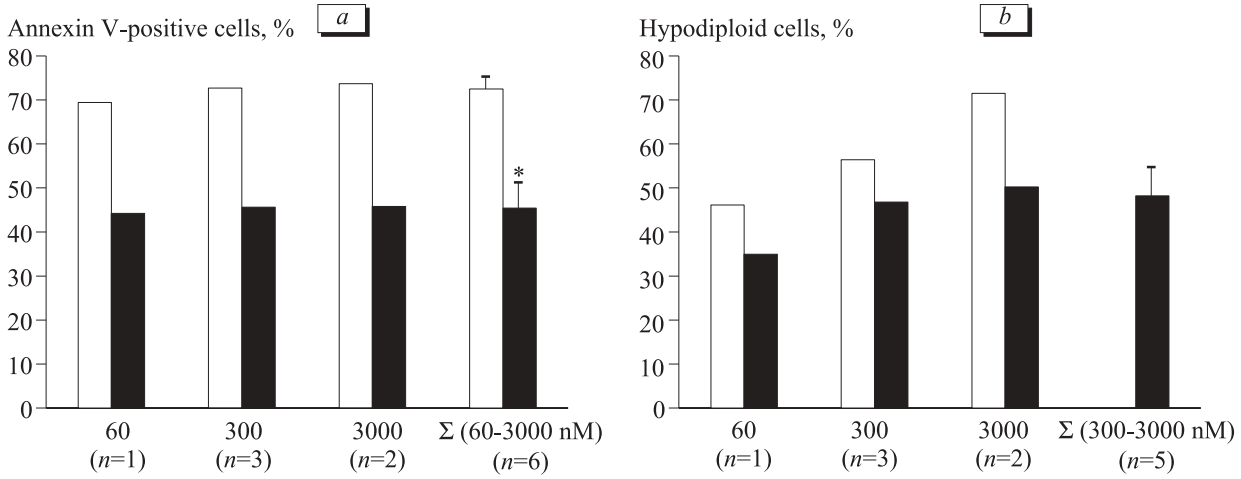

Fig. 2. Apoptosis in thymic and splenic lymphocytes induced by dexamethasone in various doses: hormone-specific death. Difference in apoptosis after 16-h incubation of control and dexamethasone-treated cells. *n*, number of animals. Light bars: thymocytes. Dark bars: splenocytes. **p*<0.05 compared to thymocytes.

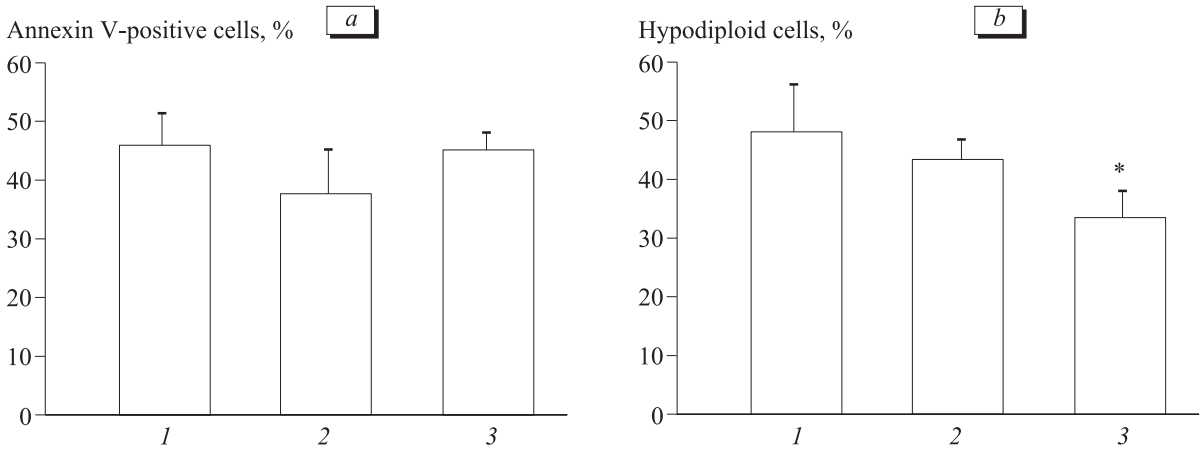

Fig. 3. Effect of miliacin on dexamethasone-induced apoptosis in splenocytes: intact animals (n=5, *1*) and mice receiving the solvent (n=3, *2*) and miliacin (n=8, *3*). *p<0.05 compared to groups *1* and *2*.

nexin V-reactivity and this parameter did not increase after incubation with 300 and 3000 nM dexamethasone (Fig. 2, *a*). This finding allowed us to combine the results of experiments with annexin V in different doses for each type of cells. It should be emphasized that the intensity of apoptosis in thymocytes was much lower than in splenocytes, which probably reflects high sensitivity of immature thymocytes to GC [4]. The ratio of hypodiploid thymocytes increased with increasing the concentration of dexamethasone and reached maximum after treatment with the hormone in a dose of 3000 nM (Fig. 2, *b*). Due to differences in the index of hypodiploidy for thymocytes, it was impossible to combine the results of studies with dexamethasone in various doses. The number of dead splenocytes was maximum after treatment with dexamethasone in a dose of 300 nM. The index of hypodiploidy did not increase after increasing dexamethasone concentration to 3000 nM. No differences were revealed after administration of dexamethasone in doses of 300-3000 nM, which allowed us to combine the results of experiments with splenocytes treated with the hormone in various concentrations. The count of died thymocytes estimated by DNA fragmentation was higher than the number of died splenocytes (similarly to the test with annexin V).

The study of apoptosis in cells with annexin V and propidium iodide showed that this process depended on the dose of dexamethasone. After treatment with dexamethasone hypodiploid cells were accumulated more rapidly than annexin V-positive cells. The number of annexin V-positive splenocytes surpassed that of hypodiploid cells even after administration of dexamethasone in a dose of 60 nM. The differences became less pronounced with the increasing the dose of dexamethasone. It was probably associated with the increase in the index of hypodiploidy. Thymocytes treated with dexamethasone in doses of 60-300 nM were also characterized by predominance of annexin V-reactivity over DNA fragmentation. These differences disappeared, when the dose of dexamethasone was increased to 300 nM due to the increase in hypodiploidy. These differences in the parameters of apoptosis reflect not only higher sensitivity of the test with annexin V (characterizing early stages of cell death), but also incompleteness of this process (absence or insignificant degradation of genomic DNA). In other words, processes leading to destruction of the nuclear material are activated not in all annexin V-positive cells undergoing apoptosis. Mobilization of the cellular protective system probably blocks the initial stage of cell death and, therefore, inhibits subsequent processes. They include considerable decrease in the transmembrane electric potential of mitochondria, release of cytochrome *c* into the cytoplasm, and activation of effector caspases and nucleases. These irreversible changes cause cell death [12]. The initial predominance of annexin V-reactivity over hypodiploidy is followed by the disappearance of differences due to an increase in the degree of DNA fragmentation. It illustrates the sequence of events during dexamethasone-induced death of lymphocytes and reflects the possibility of blocking cascade apoptotic reactions.

Miliacin had no effect on death of thymocytes in the presence of dexamethasone. By contrast, the degree of dexamethasone-induced DNA fragmentation in splenocytes from mice receiving this triterpenoid was much lower compared to intact and solvent-treated animals (Fig. 3). The study with annexin did not reveal between-group differences. These results indicate that miliacin blocked the early stage of cascade apoptotic reactions distal to phosphatidylserine translocation and proximal to DNA fragmentation.

The protective effect of miliacin was probably related to activation of antiapoptotic mechanisms or blockade of signals for cell death. Protective activity

of this compound could be due to its antioxidant properties and prevention of GC-induced apoptosis [9, 10]. This triterpenoid can act as a membrane protector that prevents action of free oxygen radicals and other highly reactive metabolites on lipids and proteins in the plasma membrane. Miliacin produced a specific protective effect on splenocytes, which suggests the existence of different mechanisms underlying GC-induced death in various cells. The ability of miliacin to protect splenocytes from GC-induced death indicates that cyclic triterpenoids hold much promise for prevention of immune imbalance under stress conditions.

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