Effect of Activation of Ca^{2+},Mg^{2+}-Dependent Endonuclease on Proliferative Response of Human Peripheral Blood Lymphocytes

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Activity of Ca^{2+},Mg^{2+}-dependent endonuclease in human peripheral blood mononuclears is studied. Intact cells exhibit extremely low activity of the enzyme. Treatment with the synthetic hexapeptide imunofan considerably stimulates endonuclease activity in mononuclears. This activation does not depend on additional protein synthesis. An 1-h incubation in the presence of cycloheximide also activates Ca^{2+},Mg^{2+}-dependent endonuclease. These data suggest that imunofan and/or cycloheximide activate the apoptotic cascade. This leads to activation of endonuclease, which is not synthesized *de novo* but persists in the cell in the form of inactive precursor.

Key Words: *apoptosis; mononuclears; Ca^{2+},Mg^{2+}-dependent endonuclease; proliferation*

A number of agents and factors such as glucocorticoids, Ca^{2+} ionophores, heat shock, ATP, γ-irradiation, and cultivation under adverse conditions [6] induce apoptotic death of lymphocytes. Apoptosis is characterized by internucleosomal fragmentation of DNA [4]. It was demonstrated that DNA fragmentation in glucocorticoid-treated thymocytes is accomplished by Ca^{2+},Mg^{2+}-dependent endonuclease (CME), which is constitutively expressed in thymocyte nuclei and can be activated *in vitro* in the presence of Ca^{2+} and Mg^{2+} [4]. Then "silent" endonuclease was found in the nuclei of some cell lines (CTLL-2 [7], X-63 and MLC [8]) and in the nuclei of mouse splenocytes [8]. "Silent" endonuclease was readily activated by incubation with Ca^{2+} and Mg^{2+}.

In the present study we analyze the activity of "silent" endonuclease in human peripheral blood mononuclears (MN). The effect of the novel immunomodulator imunofan (IF), a modified analog of the active center of thymopoietin II [1], on CME activity was also studied.

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MATERIALS AND METHODS

Mononuclears were isolated from peripheral blood of healthy donors by gradient centrifugation. The cells were twice washed with sterile Hanks' solution and resuspended in complete RPMI-1640 medium (ICN) containing 10% fetal calf serum (ICN), 2 mM L-glutamine, 40 μg/ml gentamicin, and 5×10^{-5} M mercaptoethanol.

Preincubation with 0.05-1.25 μg/ml IF (Bionoks, Moscow) was carried out in serum-free RPMI-1640 at 37°C for 1 h. The cells were then washed, counted, transferred to 96-well plates (Nunc) in a concentration of 2×10^5 cells per well, and cultured in complete culture medium in the presence of concanavalin A or phytohemagglutinin in a concentration of 5 μg/ml each for 72 h at 37°C and 5% CO_2. ^{3}H-thymidine (40 kBq/well) was added 4-6 h before the end of incubation. Mononuclears were transferred onto fiberglass filters with a cell harvester (Flow). The intensity of labeling was determined on a scintillation spectrophotometer.

For determination of CME activity, MN were incubated with IF (0.05 and 0.25 μg/ml) and/or

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cycloheximide (30 μg/ml) in serum-free RPMI-1640 at 37°C for 1 h. Cells were then washed, resuspended in the same medium, and cultured for 4-6 h under the same conditions. After incubation, they were sedimented, resuspended in 1 ml buffer I (0.9 M sucrose, 5 mM $CaCl_2$, 0.01% Triton X-100, and 50 mM Tris-HCl, pH 8.0). The cell suspension was homogenized in a glass homogenizer. Nuclei were isolated by centrifugation in sucrose density gradient (14,000*g*, 30 min, 4°C) [8]. The pellet was resuspended in buffer II (0.25 M sucrose, 50% glycerol, 5 mM $CaCl_2$, pH 8.0). All manipulations were carried out on the cold.

The nuclear suspension was stored at −20°C for no more than 2 months. The DNA concentration in the nuclear extracts was measured spectrophotometrically.

Samples containing 5 μg DNA per sample were washed and resuspended in incubation buffer III (0.25 M sucrose, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 8.0). The samples were incubated at 37°C for 2 h. The reaction was terminated by NaCl (final concentration 1 M). For purification of preparation the samples were treated with ribonuclease (20 μg/ml) and proteinase K (1 μg/ml) at 37°C. The samples were deproteinated by adding an equal volume of chloroform:isoamyl alcohol (24:1), vigorously shacked, and aqueous DNA-containing phase was collected.

The samples were analyzed by electrophoresis in 1.2% agarose gel. The gels were stained by ethidium bromide and photographed in UV. The negatives were analyzed by densitometry, and endonuclease activity was calculated [2,8].

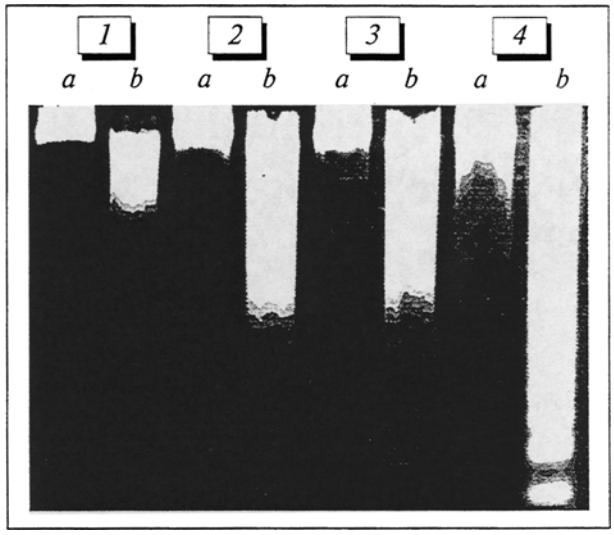

Fig. 1. Electrophoresis of DNA in nuclear extracts incubated in the absence (*a*) and presence (*b*) of Ca^{2+} and Mg^{2+}. *1*) control; *2*) cycloheximide, 30 μg/ml; *3*) imunofan, 0.25 μg/ml; *4*) imunofan+cycloheximide.

The data were processed statistically using the Student's *t* test; the differences were significant at $p<0.05$.

RESULTS

Electrophoresis showed that 2-h incubation of MN nuclei in the presence of Ca^{2+} and Mg^{2+} did not result in considerable nuclease activation and degradation of chromatin. On the other hand, DNA from IF-treated cells degraded in a typical staircase manner yielding nucleosome-sized fragments (Fig. 1). In intact nuclei (not exposed to Ca^{2+} and Mg^{2+})

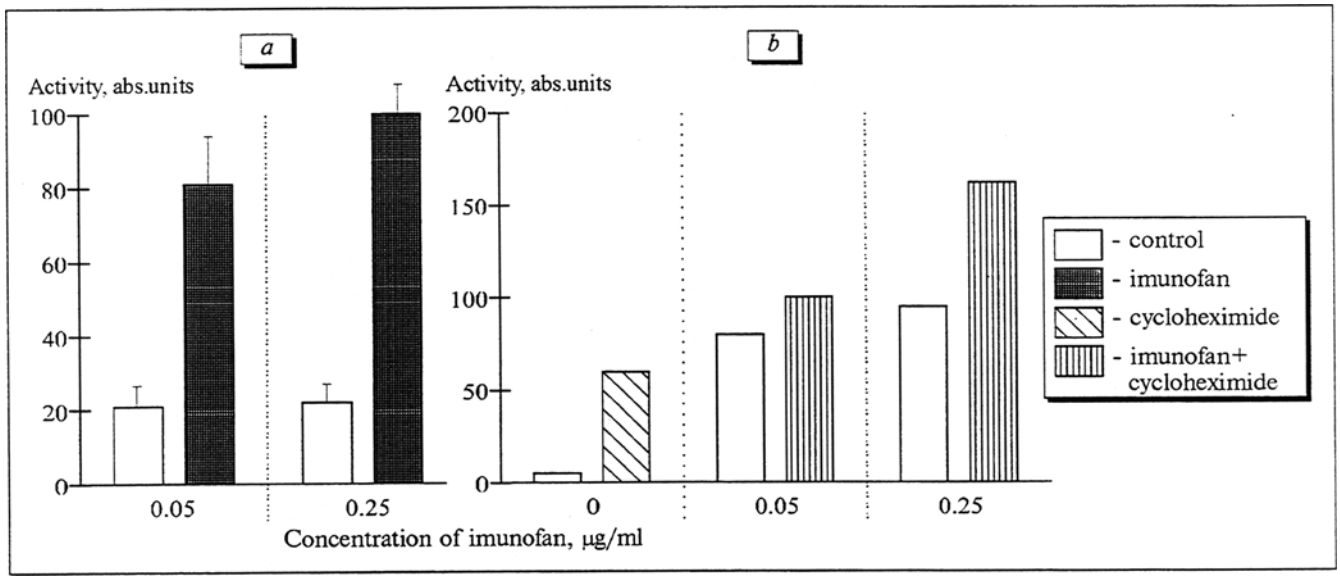

Fig. 2. Effect of imunofan (*a*) and cycloheximide (*b*) on the activity of Ca^{2+},Mg^{2+}-dependent endonuclease in human peripheral blood mononuclears. *b*) data of one of three experiments.

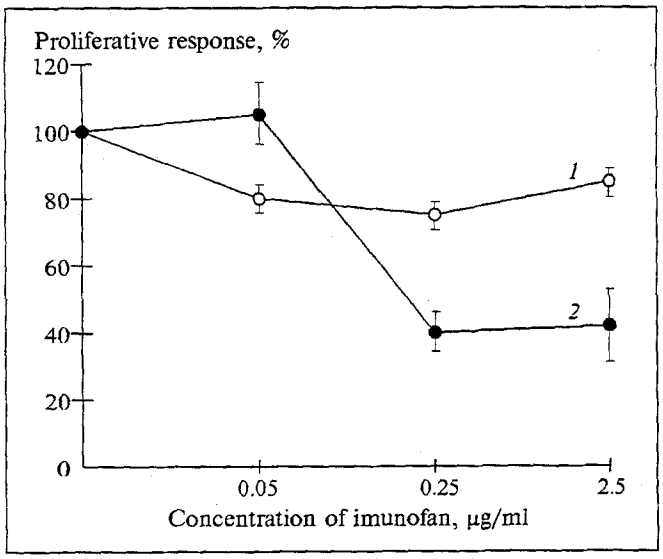

Fig. 3. Effect of imunofan on proliferation of human mononuclears. *1*) concanavalin A, 5 μg/ml; *2*) phytohemagglutinin, 5 μg/ml.

isolated from IF-treated cells the activation of endogenous nuclease and chromatin degradation were not observed. The results of electrophoretic analysis are in conformity with the data on enzyme activity: CME activity in IF-pretreated cells was 3-fold higher than in intact MN (Fig. 2, *a*).

Inhibition of protein synthesis with cycloheximide did not abolish activation of CME in IF-treated cells (Fig. 2, *b*). Moreover, cycloheximide potentiated the stimulating effect of IF (for IF concentration 0.25 μg/ml). It should be noted that inhibition of protein synthesis is able to a certain extent activate "silent" endonuclease in human MN. For instance, CME activity in cycloheximide-treated cells was 10-fold higher than in intact MN (Fig. 2, *b*).

One-hour incubation of MN with IF (0.05-1.25 μg/ml) markedly suppressed proliferative response to T-cell mitogens (phytohemagglutinin and concanavalin A). In cells treated with IF (0.25-1.25 μg/ml), proliferative response to phytohemagglutinin constitute 40% of the control level (Fig. 3).

These data suggest than intact MN are characterized by extremely low CME activity. It is evident that mechanisms of regulation of "silent" endonuclease in human MN differ from those in CTLL-2 and X-63 cell lines and mouse splenocytes exhibiting very high CME activity. Treatment with IF led to activation of an apoptotic cascade and, consequently, activation of CME. At the same time, it was demonstrated that IF considerably suppressed proliferative response of MN to T-cell mitogens. These findings as well as the data obtained in experiments with tumor cell lines [7,8] attest to antagonism between mechanisms of apoptosis and proliferation. Experiments with cycloheximide showed that IF treatment did not induce *de novo* synthesis of CME. Low CME activity in intact cells and activation of the enzyme caused by cycloheximide are probably related to the presence of a labile protein inhibitor identified in lymphocytes from patients with chronic lymphoid leukemia [2].

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