MORPHOLOGY AND PATHOMORPHOLOGY

Neutrophil Apoptosis Induction by Tick-Borne Encephalitis Virus

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Tick-borne encephalitis virus infects neutrophils and induces their apoptosis, judging from moderate increase of succinate dehydrogenase activity and a trend to anaerobic energy production in neutrophils infected with the virus (shown by an increase of lactate dehydrogenase activity).

Key Words: *apoptosis; neutrophils; enzymes; tick borne encephalitis virus*

Congenital immunity cells are involved in protection from viral diseases. Specifically, macrophages and neutrophils play the key role in the development of early anti-infection response. The presence of a sufficiently high level of neutrophils in the peripheral blood and their presence in virtually all organs suggest that they are among the first cells that react with the viruses. The immune function of neutrophils in infectious diseases is mainly associated with phagocytosis and production of cytotoxic components, including nitroxide and oxygen radical compounds [13]. After stimulation with infectious agents, these cells synthesize and release immunoregulatory proteins into extracellular environment; inhibition of this neutrophil function can partially suppress the immune response. The neutrophil functional activity can be disordered by uncontrolled apoptosis [9]. It is known that this phenomenon can be initiated by extracellular TNF-α or FAS ligand or intracellularly by, *e.g.*, high level of oxygen radicals [11]. Morphologically the neutrophil apoptosis is characterized by reduced number of cytoplasmic granules, rounding of the nucleus and condensation of its chromatin, paralleled by depression of cell functioning, particularly of anti-infection activity [13].

Despite the important role of neutrophils in the development of immune response in infectious diseases, the contribution of these cells to the pathogenesis of viral infections is little studied. Their reactions with HIV, influenza virus, and Epstein–Barr virus and neutrophil apoptosis induction by these viruses have been described [8,9,14]. Tick-borne encephalitis (TBE) is a member of the *Flaviviridae* family and an agent of a severe disease of the nervous system. In flavivirus infections the agent enters the body mainly through the site of insect bite, which causes a local inflammatory reaction. Infiltration in site of the insect bite consists mainly of poly- and mononuclear leukocytes during the first 3 days after infection; lymphocytes predominate only at weeks 2-3 of the disease [1]. Using integrin receptors consisting of glycoprotein α- and β-chain combinations, TBE adheres to blood cell surface during the early stages of infection and multiplies in monocyte/macrophages [4,12].

We studied the probability of TBE infection of neutrophils and functional activity of these cells in the course of infection.

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MATERIALS AND METHODS

Primary culture of guinea pig neutrophils was prepared by inducing intraperitoneal inflammation by injection of sterile 10% meat peptone broth (5 ml). After 18 h the peritoneal cavity of animals was washed with 10 ml cold medium 199. The resultant concentrayed leukocyte suspension was collected and its concentration was brought to 4×10^6 cell/ml and pipetted into tubes with slides (0.9 ml) and in flat-bottom 96-well microplates (100 μl/well). For adhesion, the neutrophil suspension was left in a CO_2 incubator; after 40 min the neutrophil monolayer was washed twice from free cells. The cells were infected with TBE Primorye-73 strain (Far Eastern subtype) and incubated in medium 199 with 20% fetal calf serum, 2 μM glutamine, 0.2 μM gentamicin, and 100 U/ml penicillin [10]. Virus-containing fluid from porcine embryo kidney cell culture was used in experiments. Judging from inoculation cell concentration and the titer of the virus used for infection, it contained at least 5 infectious units per neutrophil.

TBE absorption by neutrophils was monitored by indirect fluorescent antibody method. Zenon Labeling Kit Alexa Fluor 546 (Sigma) to mouse IgG1 (Sigma) served as the fluorescent serum for detection of the virus antigen. Quantitative spectrophotometry of the virus antigen in neutrophils was carried out using LSM-510 Meta laser scanning confocal microscopy system (Carl Zeiss). Labeled TBE antigen in the cells was registered at stimulation λ=543 nm (~20 μW on the sample, 100%) with isolation of the 560-615 nm spectral band corresponding to Alexa Fluor 546 emission.

Activities of ATPase and AMP (5'-nucleotidase) were measured by adding to the monolayer 20 μl substrate for ATPase (8 mg ATP/ml Tris-HCl buffer (pH 7.8) with 87 mg NaCl, 28.7 mg KCl, 52 mg $MgCl_2\times6H_2O$) and AMP (4 mg AMP/ml the same buffer with 87 mg NaCl and 70 mg $MgCl_2$). Activities of succinate and lactate dehydrogenases (SDH, LDH) were measured by Lloyd's method in our modification [3]. The reaction products were measured by the solution absorption on a Labsystem Multiscan RC spectrophotometer at wavelengths corresponding to the target substrates.

The results of spectrophotometry were expressed in the stimulation index (T) and calculated (in percent) as the proportion of the difference between the mean optical densities of solutions containing TBE-infected and intact cell reaction products to the mean optical density of solution for intact cells.

Apoptotic cells were detected by Hoechst 333258 (Sigma) staining of the cell precipitate. The preparations were examined in an LSM-510 Meta system. A total of 100 cells were examined at excitation wavelengh λ=556 nm and the percentage of apoptotic cells was evaluated as the number of cells with specific fluorescence; the result was expressed as apoptosis index (AI).

The results of experiments were statistically processed by Excel software. The significance of differences between experimental and control groups was evaluated using Student's *t* test.

RESULTS

The indirect fluorescent antibody method showed specific (mainly diffuse) fluorescence of the cytoplasm after 1-h TBE contact with neutrophils. Intracellular fluorescence (lumps) was seen after 2-h incubation, its

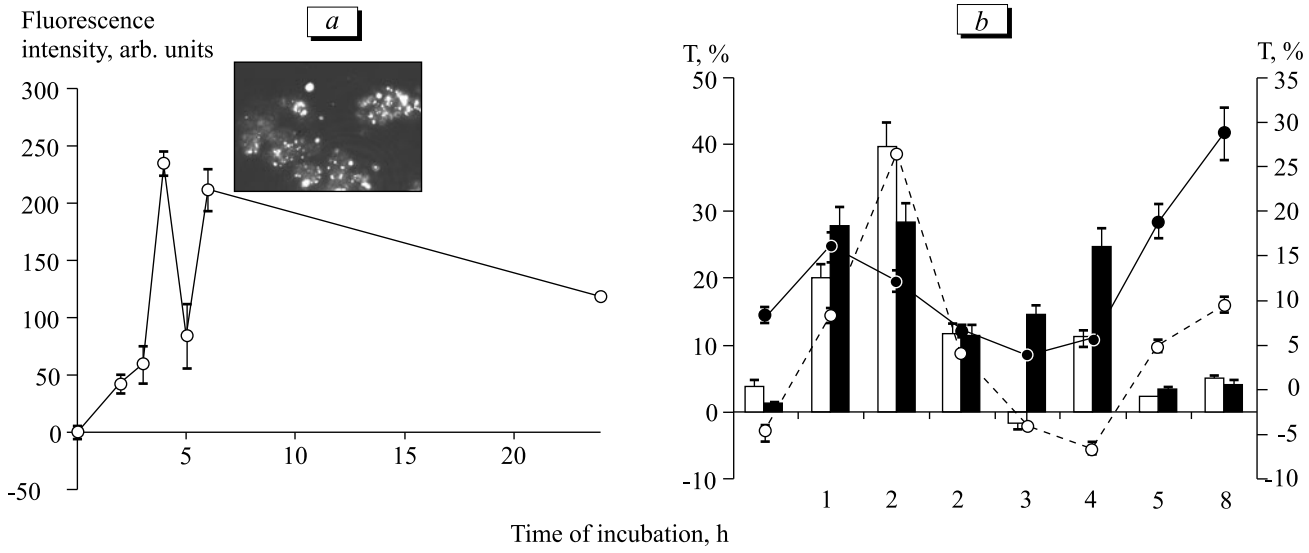

Fig. 1. Content of TBE antigen in neutrophils, laser scanning microscopy (*a*). Activities of 5'nucleotidase (AMP), ATPase (left ordinate), LDH (*1*) and SDH (*2*; right ordinate) in the cells (*b*). Light bars: AMP; dark bars: ATP. T: stimulation index, control: 0%.

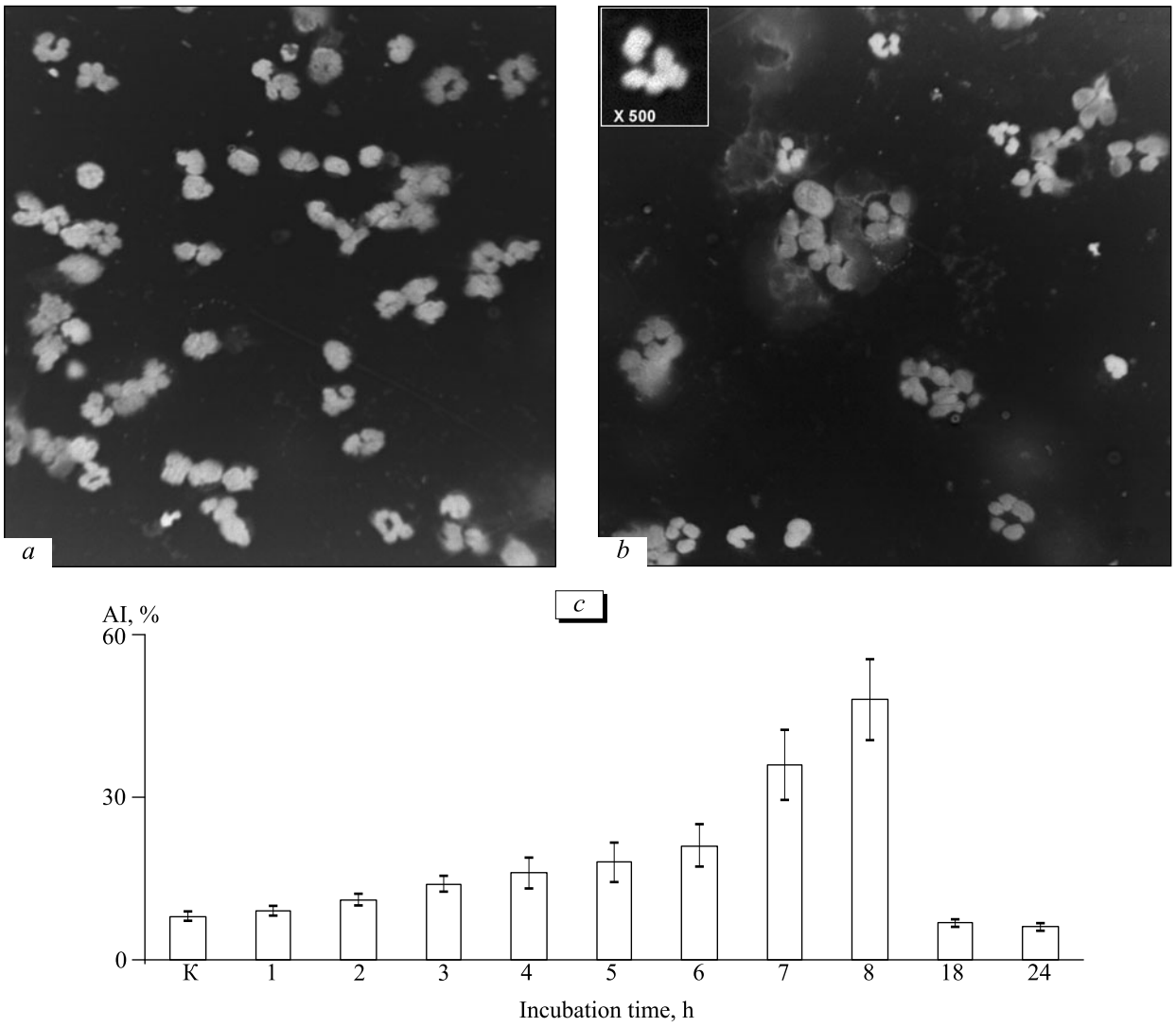

Fig. 2. Nuclear chromatin condensation in TBE- infected neutrophils. *a*) after 2-h incubation; *b*) after 8-h incubation. Hoechst 33342 staining, laser scanning microscopy, ×200; *c*) kinetics of neutrophil apoptosis induction with TBE. K: mean apoptotic index (AI) for cells incubated without TBE during the studied period.

intensity reaching 42.6±5.6 arb. units (Fig. 1, *a*), the maximum values being 234.56±15.70 arb. units after 4-h incubation, after which the fluorescence intensity decreased and then increased again until 24 h. Later (by day 2 of observation), the level of antigen-positive neutrophils was minimum because of cell degradation.

The main component of purine metabolism playing an important role in perception of external signals by the cells is 5'nucleotidase (AMP). It is located mainly on the cellular outer membrane and regulates the cAMP level, providing signal transmission from the plasmalemma into the cell. We found elevation of AMP activity in the neutrophils during the period of 1 to 4 h after TBE infection (Fig. 1, *b*). Activity of this enzyme was 39.4±2.9 and 11.1±1.1%, respectively, in comparison with the control taken as 0%. The cell membrane adenosine triphosphatase (ATPase, EC 3.6.1.3) activated by sodium and potassium ions is one of the most prevalent enzyme systems. This enzyme catalyzes hydrolysis of energy-rich phosphate bonds, and changes in its functional activity reflect the cell metabolism stimulation process [2]. Studies of ATPase activity in neutrophils showed significant changes in the intracellular level of this enzyme. Activity of ATPase increased after 1-4-h incubation of TBE-infected cells to 28.0±2.9%, after which it dropped to 3.6±0.5% (5 h). Hence, studies of ATPase and AMP activities showed pronounced stimulation of neutrophils infected with TBE.

A characteristic feature of the neutrophil metabolism is their capacity to immediately generate oxygen radicals under the effects of various factors. Enzymes (dehydrogenases) are involved in the formation of reactive oxygen species, specifically, in hydrogen trans-

fer from the substrate subjected to oxidation (hydrogen donor) to the other substrate (hydrogen acceptor). Succinate dehydrogenase (SDH; acceptor), oxidoreductase (EC 1.3.99), catalyzes succinate oxidation into fumarate in Krebs cycle [2]. Activity of SDH was significantly elevated in TBE-infected neutrophils in comparison with intact cells (Fig. 1, *b*). The stimulation index for SDH reached 16.1±1.9% after 1-h incubation with the virus, then reduced to 5.56±0.80% (4 h) and increased again by the end of observation, reaching 28.9±2.9% (8 h). A similar time course was observed for LDH activity (L-lactate NAD-oxidoreductase; EC 1.1.1.27) in TBE-infected neutrophils (Fig. 1, *b*). This enzyme catalyzes the final stage of glycolysis: inverse reaction of pyruvate reduction to lactate.

A drastic clear-cut condensation of nuclear chromatin, presenting as homogenous mass with even green fluorescence (Fig. 2, *a*) was an early manifestation of programmed cell death. Apoptosis can be arrested by inhibitors at this stage, and therefore, this stage of cell death is regarded as pre-apoptosis [15]. The intermediate stage of apoptotic cell death is associated with nucleus shrinkage. At the final stage of apoptosis the cell nucleus disintegrates into discrete fragments, 3-5 or more (Fig. 2, *b*). Nuclear Hoechst 33342 stain (used in our study) binds to damaged cellular DNA sites during the final stage of apoptosis.

We found an increase in the counts of apoptotic neutrophils infected with TBE. The number of apoptotic neutrophils increased with prolongation of observation, reaching the maximum 8 h postinfection (48.0±7.3%). Reduction of apoptosis index after 18-24 h of incubation can be explained by cell degradation.

Neutrophils are cells with predominantly aerobic metabolism [5]. In these cells SDH is essential for metabolism, as increase of its activity is not paralleled by lactate accumulation, and glucose is completely oxidized through the tricarbonic acid cycle with release of an appreciable amount of energy [6]. An increase of the energy potential of the cell in this case is a manifestation of the neutrophilic adaptive reaction to TBE. Increase of LDH activity, in turn, is associated with changes in glycolysis intensity, its biological significance consisting in the formation of energy-rich phosphoric compounds, this indicating more intense energy supply to neutrophils due to anaerobic glycolysis involvement [2]. This oxidation pathway can be regarded as a compensatory mechanism of the neutrophil adaptive reaction to TBE. Hence, a moderate increase of SDH activity and a trend to anaerobic energy production, glycolysis activation (LDH) in TBE-infected neutrophils, indicate stability of the cellular energy homeostasis. This eventually leads to neutrophil death mainly by apoptosis, because necrosis is associated with bioenergetic hypoxia of the cells under conditions of complete inhibition of the respiratory chain enzymes [7].

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