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Changes in the Metabolic Activity of Macrophages under the Influence of Tick-Borne Encephalitis Virus

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Abstract—The metabolic activity of macrophages infected with tick-borne encephalitis virus (TBEV) affecting the human nervous system has been studied for the first time. The penetration and reproduction of TBEV in the macrophages stimulated their oxygen metabolism, increasing the activity of NADPH-oxidase complex, as well as the mitochondrial enzymes lactate dehydrogenase, succinate dehydrogenase, and cytochrome oxidase. A wave-like change in the activity of these enzymes in the macrophages reflected the reaction of the cells to the penetration of the virus in the first period (within 3 h) and to the synthesis of the virus particles and their exit into the extracellular space in the second period (from 5 to 48 h). In the macrophages infected with TBEV, accumulation of NO metabolites was observed. In the late period of the examination (1-4 days), the activities of superoxide dismutase and lysosomal enzymes (nonspecific esterase and acid phosphatase) were detected. Thus, the early increase in the activity of the cell enzymes indicates the activation of the macrophages, and the subsequent increase in their activity corresponds to the enhanced synthetic activity of the macrophages.

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Key words: monocytes, macrophages, tick-borne encephalitis virus, enzymes, nitrogen oxide, reactive oxygen species

All biological species at different stages of evolution from unicellular organisms to mammals possess specific cells that are capable of phagocytosis, or macrophages, which are representatives of the oldest immunity system. In spite of a very high variety of macrophage populations differing in maturity degree and tissue specificity, these cells have a common origin and functions, possess similar structures, and exhibit similar metabolic processes supporting their functions [1]. In 1980, to discriminate macrophage populations, the Committee of the Reticuloendothelial Society recommended the following classification: resident macrophages, inflammatory exudate macrophages, induced macrophages, and activated macrophages. In the present study, we used macrophages of mouse peritoneal exudate without using any inducers of inflammation; therefore, the given population of the cells corresponded to the resident macrophages. After contact with virus, the macrophages were considered as activated macrophages. In the resident macrophages, the activity of most lysosomal enzymes is in a latent state, being associated with glycolipid membrane complexes [2]. In contrast to resident macrophages, activated macrophages have a larger size, higher content of protein and RNA, and exhibit high enzymatic activity.

While carrying out phagocytosis, pinocytosis, and exocytosis, macrophages constantly consume (interiorize) the plasma membrane, which is compensated by the constant synthesis of its components [3]. The degree of macrophage stimulation can be estimated by the activity of their membrane ectoenzymes (ATPase, 5′-nucleotidase, leucine aminopeptidase, D-amino-acid oxidase, serine esterase, alkaline phosphodiesterase) [4, 5]. Numerous primary lysosomes contain acid hydrolases (β-D-glucuronidase, N-acetyl-β-glucosaminidase, β-glycerophosphatase, α-mannosidase, acid phosphatase, α-naphthylesterase, esterase, RNase, arylsulfatase, lipase, β-galactosidase, naphthylamidase, cathepsins B, D, and G, and elastase) and antibacterial enzymes (lysozyme, neutral proteases, and B12-binding protein) [6]. Secondary lysosomes (phagolysosomes) are formed by the fusion of the primary lysosomes with pinocytic and phagocytic vacuoles. Many lysosomal enzymes demonstrate combined and cumulative action. For example, proteolysis is provided by seven types of proteinases and

Abbreviations: LDH) lactate dehydrogenase; NBT) nitroblue tetrazolium; SDH) succinate dehydrogenase; SOD) superoxide dismutase; TBEV) tick-borne encephalitis virus; TCA_{50}) titer of cytopathogenic virus action on 50% of the cell culture.

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peptidases. Glycoproteides are hydrolyzed by both glycosidases and proteinases; RNase and DNase can split viral nucleoproteides and take part in the synthesis of their components [7].

The interaction between the cell and the virus results in a number of complex morphological and cytophysiological changes that are specific for this interaction and are not observed under other pathological states of the cells [8]. The virus is biologically inert until the moment of its adhesion on the surface of the cells, which is provided by the integrins of the plasma membrane of macrophages, glycoproteins composed of α- and β-chains in various combinations [9, 10]. After penetrating through the plasma membrane, the virus starts functioning as the independent genetic unit, promoting metabolic processes in the host cell. The virus can use enzyme systems of the host cell without their modification, or induce the synthesis of alien enzymes. At the same time, the activity of the host enzymes can be significantly suppressed. Thus, the use of sensitive methods for the analysis of the enzyme systems of cells infected with virus can be used to detect changes connected with the activity of the virus [11]. In spite of the importance of such information, there have been few publications on this subject in recent years.

The tick-borne encephalitis virus (family Flaviviridae) is a causative agent of the dangerous disease of the nervous system and spread in many Europe countries [12]. In the literature, the role of monocytes/macrophages in the development of tick-borne encephalitis is described ambiguously. It is unclear whether macrophages function as the target cells or as the phagocytes that are capable of consuming the cells infected with the virus [13].

In the present paper, data on the analysis of the enzymatic activity of resident macrophages infected with TBEV are presented for the first time.

MATERIALS AND METHODS

Primary macrophage culture. Cells of exudate were obtained from the peritoneal cavity of not bred white mice. The cell cultures (cell concentration, $5 \cdot 10^6$ cells per ml) were grown incubated in 199 medium (Chumakov GUP IPVE, Russian Academy of Medical Sciences) containing 5% bovine fetal serum (NPO Vector, Russia) without antibiotics. After 40 min of incubation in a thermostat at 37°C in a mixed atmosphere with 5% CO_2, the non-adhered cells were washed twice and left in the thermostat in the same medium for 3 days. The quality of primary macrophage cultures was evaluated by visual observation of the cells using phase-contrast microscopy.

Virus. Macrophages were infected with the virulent for newborn white mice Primorye-73 TBEV strain isolated from the brain of a person who died from tick-borne encephalitis. In the experiments, we used the virus-containing culture liquid of swine embryo kidney cells infected with the virus (2 units of the titer of cytopathogenic virus action on 50% of the cell culture; TCA_{50}).

The time of the contact of the macrophage monolayer with TBEV was 15, 30, 45, and 60 min. After 60 min of the contact, the cells were washed twice with 199 medium from the non-adhered virus particles, and the infected culture was incubated in a thermostat at 37°C during 2, 3, 4, 5, 6, 7, 9, 24, 48, and 72 h.

Evaluation of the accumulation of the virus antigen in the cells: indirect method of fluorescent antibodies. To determine the viral antigen, a cell monolayer infected by TBEV was treated with homologic immuno-ascite liquid against Primorye-73 TBEV strain and diagnostic fluorescent serum against immunoglobulins G of white mouse (Gamaleya IEM, Russian Academy of Medical Sciences) using a standard procedure [14]. The preparations were examined in a fluorescent microscope. Using the phase-contrast method, the percentage of antigen-containing cells was determined as the percentage of fluorescent cells.

The virus was accumulated in macrophages by titration on replanted swine embryo kidney cells.

NO metabolite assays (nitrite ions NO_2^-). After the incubation of the infected cells at 37°C, a monolayer of the cells and the culture liquid were frozen and stored at −20°C. The suspension of destroyed macrophages was treated with 100 μl of Griess reagent (mixture of equal volumes of 0.1% 1-naphthyl-ethylenediamine dihydrochloride and 1% *p*-aminobenzidine sulfanilamide (ICN, Belgium) in 2.5% phosphorous acid) [16]. After 10 min of incubation, the absorption of the samples was measured at 540 nm using a Multiscan Titertek Plus spectrophotometer (Flow lab, Finland). Samples containing the reagent without cells were used as controls.

Superoxide dismutase (SOD) activity assays. A monolayer of cells infected with TBEV was frozen and stored at −20°C [17]. The destroyed phagocytes were treated with 100 μl of 0.05 M calcium phosphate buffer, pH 7.8, containing 0.05 M Na_2CO_3, 0.25 mM xanthine, 0.2 mM ethylenediamine tetraacetic acid, 0.025 mM nitroblue tetrazolium (NBT), and 0.025 units of xanthine oxidase (ICN). After 60 min of incubation, the absorbance of the samples was measured at 540 nm. The absorbance of the sample in the absence of the cell suspension was taken as 100%. The activity of SOD was expressed as the amount of the enzyme that was required to suppress the xanthine oxidase activity by 50%.

NBT test. The adhered cells were treated with 100 μl of Hanks' solution without phenol red containing TBEV and 1 mg/ml of NBT (ICN) [18]. Intact macrophages after 45 min of incubation with *Staphylococcus aureus* were used as the control. After different time intervals (15, 30, 45, and 60 min and 2, 3, 4, 5, 6, and 9 h), the cell monolayer was washed three times with 199 medium and dried. Then the formazan formed in the cells was dissolved with dimethylsulfoxide heated to 83°C. The optical density of the samples was measured at 540 nm.

The activity of other enzymes was determined in plates with a monolayer of the cells fixed by formalin vapors (15 min).

The activity of ATPase and 5′-nucleotidase. A cell monolayer was treated with 20 μl of the substrates for ATPase (8 mg of ATP in 1 ml of Tris-HCl buffer, pH 7.8, containing 87 mg of NaCl, 28.7 mg of KCl, and 52 mg of $MgCl_2$ in 6 ml of H_2O) and for 5′-nucleotidase (4 mg of AMP in 1 ml of the same buffer containing 87 mg of NaCl and 70 mg of $MgCl_2$). The samples were incubated at 37°C for 30 and 60 min [19]. The reaction was stopped by the addition of 100 μl of the mixture of ascorbic and molybdenum acids (1 : 1). After 20 min, the absorption of the samples was measured at 620 nm.

Activities of succinate and lactate dehydrogenases (SDH and LDH) were determined by the method of Lloyd [20] with some modifications. A fixed cell monolayer was treated with 100 μl of the substrates: 2 mg/ml of iodine nitrotetrazolium (ICN) for LDH and 2 mg/ml of methyl-thiazolyl-tetrazolium bromide for SDH in phosphate buffer, pH 7.2, containing 0.4% $MnCl_2$. The monolayer was incubated with the substrates at 37°C for 30 min. To dissolve the diformazan granules, the samples were incubated with 100 μl of isopropanol acidified with 0.04 M HCl for 20 min. The absorption was measured at 492 nm for LDH and at 540 nm for SDH. Samples containing the acidified isopropanol without cells were used as control.

The activity of cytochrome oxidase was determined by the method of Novikoff and Goldficher [21] with some modifications. A fixed monolayer of phagocytes was treated with 100 μl of 0.1 M acetate buffer, pH 5.5, containing 10 mg/ml of $MnCl_2$, 0.33% hydrogen peroxide, and 2 mg/ml of diaminobenzidine. After 10 min incubation at room temperature, the reaction was stopped by the addition of 10% sulfuric acid (100 μl per well). The quantity of formed product was determined by measuring the absorption at 492 nm. Samples containing the substrate solutions and 10% sulfuric acid were used as the control.

Activity of nonspecific esterase was determined by the method described in [22] with some modifications. A fixed monolayer of the cells was treated with 100 μl of 0.5% propylene glycol solution in 0.2 M phosphate buffer, pH 6.9, containing 0.25% naphthyl-AS-acetate and 80 mg of fast blue BB. Samples containing 1.5 mg/ml of NaF (inhibitor of the reaction) (ICN) were used as the control. After incubation in the presence of fresh substrate at room temperature for 60 min, the monolayer was washed twice with distilled water and stained with 0.1% nuclear fast red in 5% sulfate ammonium solution. To dissolve the intracellular products of the reaction, the samples were incubated with 100 μl of isopropanol acidified with 0.04 M HCl for 20 min. The quantity of the product formed was determined at 492 nm.

Determination of acid phosphatase activity. A fixed monolayer of cells was treated with 50 μl of *p*-nitrophenyl phosphate (ICN). To prepare the solution, 0.09 g of *p*-nitrophenyl phosphate and 0.31 g of NaCl were dissolved in 37 ml of 0.02 M sodium citrate buffer, pH 5.0. The reaction was performed at 37°C for 30 min and was stopped by the addition of 0.2 M NaOH (100 μl per well). The absorption of the samples was measured at 405 nm [18].

The results of the investigation were calculated using the equation:

$$T = [(N_0 - N_k)/N_k] \cdot 100\%,$$

where T is the index of stimulation of the phagocytes, N_k is the average absorption of the product after the interaction of the substrate with the non-stimulated cells, N_0 is the average absorption of the product after the interaction of the substrate with the stimulated cells.

RESULTS

After 60 min of the contact of TBEV with the macrophages, specific fluorescence of the cytoplasm was detected using the indirect fluorescent antibody method. The fluorescence was mainly of diffuse character, and 20 ± 0.9% of the antigen-positive cells exhibited a localized lumpy fluorescence. After 4 and 5 h of incubation, the number of such cells increased to 35 ± 1.4 and 87 ± 5.3%, respectively, and then remained constant within 24 h of observation. After 4 days of observation, the number of the antigen-positive macrophages was minimal, constituting 17.5 ± 0.8%.

The adhesion and reproduction of TBEV in the macrophages was confirmed by the titration method (Fig. 1). After 60 min of incubation, the original virus titer decreased by 2.0 log units. After 24 h, this value increased in the cell monolayer by 1.0 log, decreasing to the end of the experiment (4 days). In the supernatant, the virus titer increased to 2.0 log on the second day. These data indicate the adsorption and subsequent reproduction of TBEV in the primary macrophage culture, and then its liberation from the cells 2 days after the infection.

Microscopic investigation of the preparations stained 60 min after the contact with TBEV revealed mainly activated macrophages with large azurophilic nucleus occupying 1/3 of the cell area and numerous vacuoles on the periphery of the phagocyte cytoplasm. There were little intact lengthened forms macrophages (Fig. 2a). After 24 h of incubation, intensive vacuolization of the peripheral part of the phagocytes was observed, this indicating the beginning of degradation of the cell culture. Besides the indicated macrophages, 5.0 ± 0.38% of the cells with chemotactic activity were detected. These cells acquired a lengthened forms due to the formation of two pseudopodia on the opposite sides, and the number of such cells significantly increased 7 h after the contact of

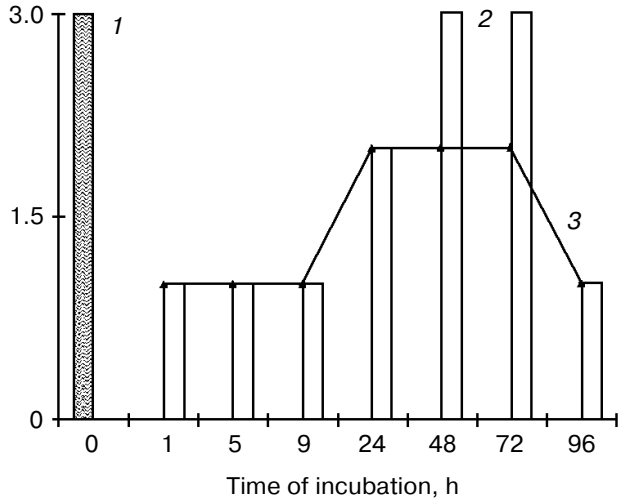

Fig. 1. Reproduction of TBEV in macrophages: *1*) quantity of introduced TBEV; *2*) TBEV in culture liquid; *3*) TBEV in cells. The ordinate indicates the virus titer (log TCA_{50} per ml).

the virus with the macrophages, constituting 25.0 ± 2.1% (Fig. 2b). Then contacting phagocytes were observed, and 24 h later, symplast-like conglomerates composed of four and more cells appeared (Fig. 2c), where the maximal specific fluorescence of the viral antigen was detected. In this period, macrophages with karyorrhexis were detected (10.0 ± 1.3%), as well as apoptotic cells (5.0 ± 0.46%) with 2-3 nuclear fragments and a reduced cytoplasm area (Fig. 2d). Further, the number of degrading cells increased, and at the end of the observation (the fourth day) the cells were destroyed.

To reveal the activated cells, investigators use the test for intracellular 5′-nucleotidase or adenosine-5′-phosphatase (EC 3.1.3.5), the enzyme bound to the external side of the plasma membrane through the glycosyl-phosphatidyl residue [23]. We determined a slight change in the activity of 5′-nucleotidase in the macrophages infected with TBEV that did not differ significantly from the value obtained for the intact macrophages during the experiment.

Adenosine triphosphatase (ATPase, EC 3.6.1.3) of cell membranes is a widespread enzyme system that is activated by potassium and sodium ions. This enzyme catalyzes hydrolysis of the high-energy phosphate bonds, and the changes in its activity reflect the activation of cell metabolism [24]. Investigation of the ATPase activity in the macrophages revealed a significant change in the intracellular content of this enzyme. The ATPase activity decreased by 13.3 ± 1.5% compared to the control value 15 min after the infection of the cells by TBEV, remaining constant for 6 h of incubation, and then increased sharply to 34.07 ± 2.5% (9 h). Then the activity decreased until the end of the experiment (the third day). Thus, the data on the ATPase activity indicate pronounced stimulation of the macrophages infected with TBEV.

In terms of biochemical characteristics, macrophages do not differ significantly from other cells. Nevertheless, the characteristic feature of their metabolism is the ability to generate various oxygen radicals under the influence of different factors. This phenomenon is called "respiratory burst" [25]. In the first step, the superoxide anion $O_2^{\cdot-}$ is formed from an oxygen molecule in the presence of the NADPH-oxidase complex as the electron donor. In 1959, it was demonstrated that this complex includes four protein components that were designated according to their molecular weights (in kD) as $p40^{PHOX}$ (PHOX, phagocyte oxidase), $p47^{PHOX}$, $p67^{PHOX}$, and $p22^{PHOX}$, and the glycoprotein $gp91^{PHOX}$ [26]. Besides the pathway when the oxygen molecule accepts electrons, reactive oxygen species in macrophages can be formed due to the hydrogen transfer from a substrate being oxidized (hydrogen donor) to another substrate (hydrogen acceptor). This reaction is catalyzed by dehydrogenases.

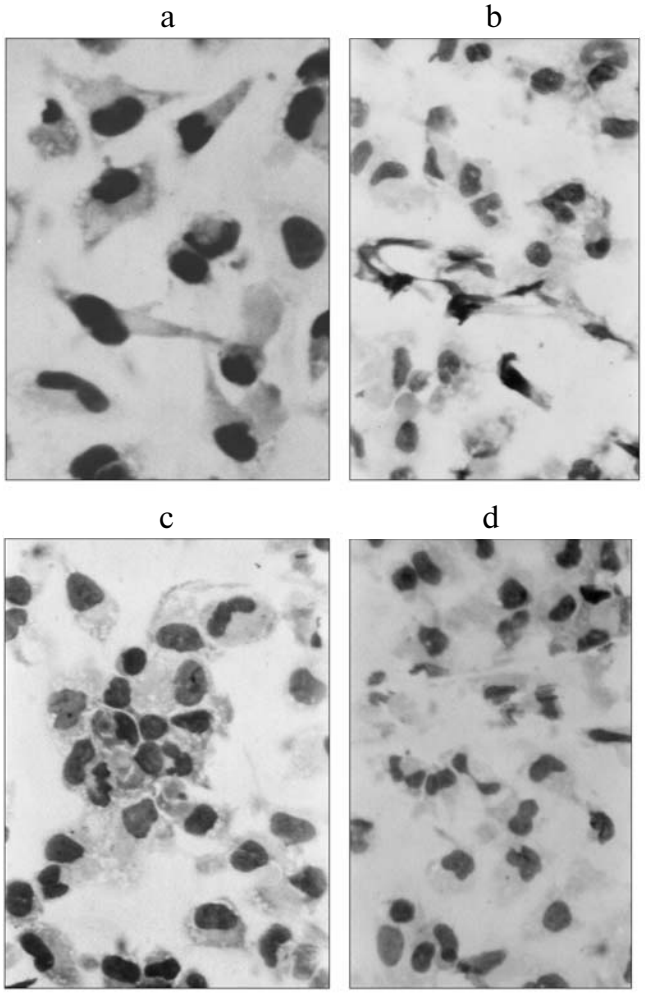

Fig. 2. Cytological investigation of resident macrophages infected with TBEV: a) intact cells; b) the chemotactic cells contacting each other (7 h); c) symplast (24 h); d) apoptotic macrophages with fragmentation of the nucleus. The cells were stained by Nocht–Maksimov (magnification, ×500).

Besides, cytochrome oxidases can also be involved in the oxidation, acting as the additional substrates of the respiratory chain. To evaluate the activity of oxygen metabolism of the resident macrophages infected with TBEV, the intracellular content of SDH, LDH, and cytochrome oxidase was determined and the total activity of the respiratory chain enzymes was evaluated in the NBT test.

Dehydrogenases catalyzing hydrogen transfer to substrates are specific towards both the hydrogen donor and the hydrogen acceptor. SDH is a flavoproteid dehydrogenase and belongs to the succinate oxidase enzyme complex that forms the membrane respiratory chain. The flavin group of this enzyme contains four iron atoms and is bound covalently to the protein. The SDH activity depends on the SH-groups of the enzyme [22]. Investigation of the intracellular content of SDH in macrophages infected with TBEV demonstrated a significant increase in the enzyme content compared to the intact cells (Fig. 3a). As seen from the figure, 15 min after the contact of the cells with the virus, the stimulation

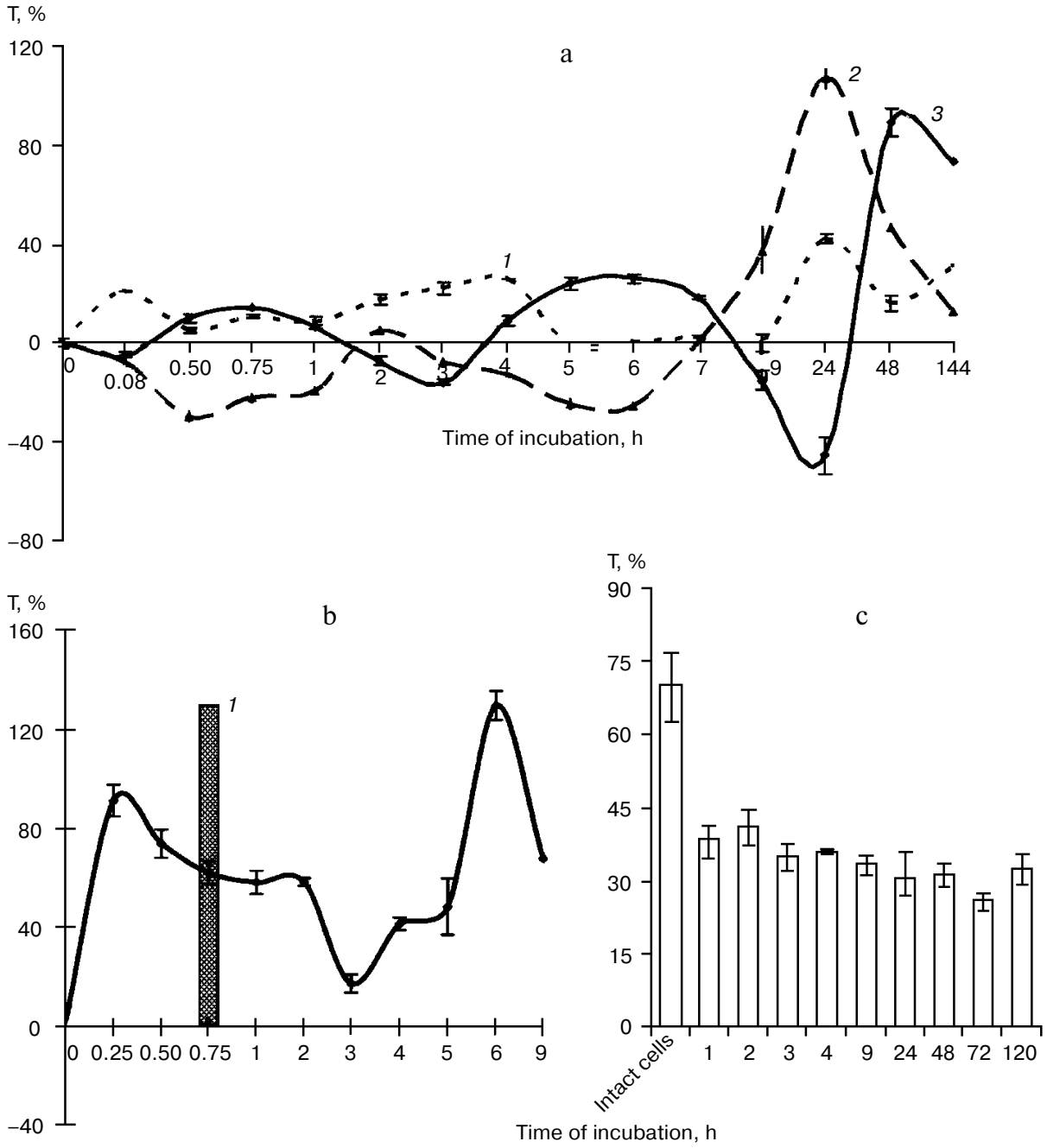

Fig. 3. Activity of the enzymes of oxygen metabolism in macrophages infected with TBEV: a) intracellular content of SDH (*1*), LDH (*2*), and cytochrome oxidase (*3*); b) NTB test: macrophages after 45 min of incubation with *S. aureus* (*1*); c) intracellular content of SOD.

index for SDH was 21.7 ± 1.9% and remained virtually constant during 4 h of incubation (26.9 ± 1.4%). Then the index decreased to the level of intact cells, and then increased during the observation time (48 h).

LDH is a coenzyme-dependent dehydrogenase and catalyzes the transfer of a reduced equivalent (hydrogen) from lactate to NAD^+ or from NADPH to pyruvate. Usually, NAD^+ is the coenzyme of LDH, but NADPH also can function as the coenzyme. LDH acts on the last step of hydrolysis that occurs under anaerobic conditions and results in the reduction of pyruvate yielding lactate and NAD^+. Most of the enzyme in the cell is weakly bound to the cell structure and localized in the cytoplasm, a smaller part being attached firmly to mitochondrial membranes [20]. Histochemical analysis can result in the enzyme diffusion into the incubation media. The spectrophotometric analysis used in the present work provided more precise determination of LDH activity in the macrophages. The activity of LDH in macrophages infected with TBEV increased significantly (39.4 ± 2.6%, Fig. 3a) after 7 h of incubation. The maximal value of the stimulation index for this enzyme was observed 24 h after the infection (109.1 ± 7.4%). Thus, in contrast to SDH, a significant increase in the intracellular content of LDH in the macrophages was observed at the later period of the infection.

Cytochrome oxidase and SDH are the main components of the normal aerobic oxidative system of the tissue cells that are also known as the succinate-dehydrogenase complex, where SDH is the first component and cytochrome oxidase is the second. Cytochromes are subdivided into three groups according to their chemical structure and spectrum: cytochromes *a*, *b*, and *c*. Oxidized cytochrome oxidase is reduced by cytochrome *c* catalyzing the transfer of four electrons to the oxygen molecule. Thus, cytochrome oxidase is a representative of the third group of oxidases. The cytochrome oxidase activity in the macrophages reflects the level of oxidative metabolism [27]. It was shown that the activity of this enzyme in the macrophages increased 30 min after the contact with TBEV (10.6 ± 0.9%, Fig. 3a). A decrease in the cytochrome oxidase activity was observed during the period from 1 to 4 h after the infection. After 6 h, the activity increased and reached 27.6 ± 1.8%, and then decreased again. It is necessary to emphasize the opposite direction in the changes in the activities of cytochrome oxidase and SDH in the macrophages infected with TBEV (increase in the activity of one enzyme while decreasing the activity of the second).

Among the known methods for determination of the oxygen-dependent activity of phagocytes, the cytochemical test for reduction of nitroblue tetrazolium (NBT-test) is the simplest and the most reproducible method. The NBT-test allows evaluation of the total activity of NADPH-dependent enzymes of the respiratory chain. The activated macrophages consume tetrazolium and their enzymes convert it into insoluble diformazan. The reduction of NBT into diformazan is attributed mainly to the powerful redox agent superoxide anion [28]. Significant increase in the NBT-test index (91.6 ± 6.5%) was observed in macrophages 15 min after contact with TBEV (Fig. 3b). During 3 h of the experiment, the index decreased to 17.3 ± 0.9%, and then increased again, reaching its maximal value after 6 h of incubation (130 ± 11.6%). This value exceeded slightly the value obtained for macrophages treated with the bacterium *Staphylococcus aureus* as the stimulating agent (129.8 ± 7.8%).

The level of oxygen radicals in the cells is regulated by the highly specific antioxidant enzyme superoxide dismutase (SOD, EC 1.15.1.11) with molecular weight of 31 kD [29]. The scavenging of the oxygen radicals by SOD proceeds in the presence of O_2^- at pH > 4.8 [30]. It is known that in an inflammation area, 80% of H_2O_2 is generated by phagocytes due to this reaction [31]. The activity of SOD can be evaluated as the amount of enzyme required for 50% inhibition of the superoxide-anion formation in the presence of xanthine oxidase and NBT. An increase in SOD activity in macrophages infected with TBEV was detected after 1 h of incubation (41.6 ± 3.4%, Fig. 3c). During the time of observation, the activity remained constant, exceeding significantly the value for the intact macrophages. The SOD activity was maximal after 72 h of incubation and constituted 26.4 ± 1.8%.

Recently, while studying the cytostatic function of macrophages, special attention has been paid to the nitroxide radicals [32]. The dynamics of the accumulation of NO metabolites in macrophages infected with TBEV differed from that observed for enzymes of oxygen metabolism (Fig. 4a). A gradual increase in the content of NO metabolites was observed from the very beginning of the infection to the end of the observation. The maximal value of the index was observed after 48 h of incubation (53.4 ± 3.2%).

Thus, infection by TBEV activates oxygen metabolism of macrophages. The activity values of the investigated enzymes exhibited wave-like changes, this being in contrast to the gradual accumulation of NO metabolites. The first increase in the enzyme activity is probably connected with the cell response to the introduction of the virus, and the second is due to the virus reproduction in the macrophages. This becomes obvious when considering the data on the enzyme activity together with the data on the titration of the virus-containing liquids in cell cultures. The pronounced activation of the enzymes of oxygen metabolism in the macrophages correlated with the accumulation of the virus particles in the cells within a day after the infection.

Nonspecific esterases and acid phosphatases are lysosomal enzymes of the macrophages providing proteolytic and digesting functions. Nonspecific esterases take part in hydrolysis of simple esters, N-free alcohols, and

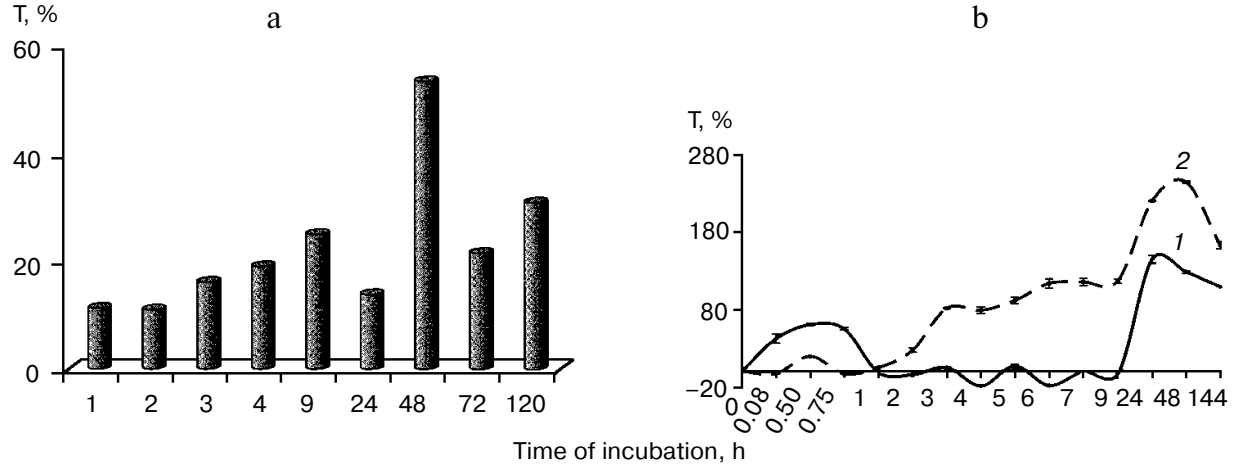

Fig. 4. Intracellular content of NO metabolites (a) and activity of lysosomal enzymes (b)—nonspecific esterase (*1*) and acid phosphatase (*2*)—in macrophages infected with TBEV.

organic acids, and acid phosphatases are capable of liberating the phosphate group from many alcohol and phenol phosphomonoesters. Changes in the activity of these enzymes reflect the physiological stimulation of monocytes/macrophages [22]. A significant increase in the intracellular content of these enzymes was detected in macrophages infected with TBEV (Fig. 4b). The dynamics of the activity of the lysosomal enzymes exhibits two phases: the first phase of the activity was detected during the period from 15 to 60 min of incubation, and the second phase was from 2 h to the end of the observation (for acid phosphatase) and from 9 h to the end of the observation (for nonspecific esterase). The maximal values for the enzyme activities constituted 221.9 ± 11.5% for acid phosphatase and 145.8 ± 9.7% for nonspecific esterase after 24 h of incubation of the infected cells.

DISCUSSION

According to the data described in [13], the response of macrophages to infection by Langat virus (a member of TBEV complex) results in their ability to phagocyte the cells containing the adhered virus particles. We have demonstrated that TBEV is able to adhere from the virus-containing liquid to the surface of the resident macrophages, penetrate into the cells, and reproduce without the involvement of any other factors. Similar data on selective adsorption to monocytes/macrophages were obtained while studying the interaction of these cells with Dengue virus, which belongs to the family Flaviviridae, as does TBEV [33, 34]. Morphological examination of the cell culture infected with TBEV revealed the features of the cytopathic action of this virus on the macrophages. The infected cells exhibited a granular cytoplasm, enhanced eosinophilicity of the cytoplasm, which characterizes the increase in the RNA and protein content, and karyopyknosis in some cells. Apoptotic macrophages were also observed.

The introduction of TBEV did not change significantly the activity of 5′-nucleotidase. This fact suggests no spatial displacement of the cytoplasmic membrane under the influence of TBEV. Nevertheless, the activation of oxygen metabolism in the cells was revealed after the infection by the virus. Under physiological conditions, in contrast to other cells of the organism, superoxide-anion is not produced in macrophages or produced in small amounts [35]. NADPH oxidases and oxidases of D-amino acids on the internal surface of the membranes also virtually do not function. After the infection with TBEV, the activity of the primary NADPH oxidase complex (plasma membranes) increased according to the results of the NBT-test. The activities of the mitochondrial enzymes of the third level (LDH, SDH, and cytochrome oxidase) also increased. These data indicate the stimulating effect of TBEV on the macrophages. It is known that redox molecules, active metabolites of oxygen, take part in the transmission of external signals arising on the plasma membrane. Further intracellular transfer of the signal also can be connected with the activation of the redox molecules of certain cytoplasm elements and transcriptional factors [36]. Our results have revealed an instant reaction of the macrophages in response to the introduction of TBEV, since within the first 15 min of the infection a sharp increase in the activity of the enzymes involved in the generation of superoxide radicals was detected. In our opinion, the observed recurrence in the dynamics of the activity of the investigated enzymes in the macrophages infected with TBEV reflect the cell reaction to the penetration of the virus into the cell in the first period (within 3 h), and to the synthesis and liberation of the virus particles into the extracellular space in the second period (5-48 h). It should be noted that in the second period the activity of ATPase increased, this like-

ly correlating with the increase in total synthetic activity of the cells. There are some data indicating that under certain conditions these enzymes are capable of displacing iron ions from protein complexes, generating the radical $OH^{\cdot}$. This explains the enhanced activity of ATPases in the period of active reproduction of TBEV in the macrophages (9 h). The subsequent (after 48 h) decrease in the activity of the indicated enzymes of oxygen metabolism can be accounted for by the beginning of degradation of the cell culture under the influence of TBEV.

In our opinion, during the infection of an organism by TBEV, the role of macrophages in the pathogenesis of the disease is determined rather by the selective adsorption of the virus on these cells that can serve as the target for its reproduction than by their ability to serve as the professional phagocytes. Monocytes are now considered to be of special significance in viral infections, which is determined by their ability to produce nitric oxide in the stimulated state. Nitric oxide affects synthesis of γ-interferon and tumor necrosis factor α [37, 38]. There are two mechanisms of NO generation: 1) the nitroxide synthase mechanism, or NADPH-dependent synthesis of NO and L-citrulline from oxidized L-arginine catalyzed by the enzyme nitroxide synthase, and 2) nitrite reductase mechanism, acting under oxygen deficiency. These mechanisms are interrelated and are the components of the NO cycle [39]. In macrophages infected with TBEV, an active generation of NO metabolites was detected. The dynamics of the generation of NO metabolites in the first period of the infection (15 min to 4 h) correlated with the changes in the activity of the enzymes of oxygen metabolism. It should be noted that there was no two-phase dependence in the case of NO generation, in contrast to the changes in the activity of the enzymes of oxygen metabolism. Presumably, NO can be produced in these cells also through the nitrite reductase mechanism, which requires additional investigation. This is also supported by the data on the increase in activity of mitochondrial cytochrome oxidase in the macrophages infected with TBEV. It is known that this enzyme is involved in generation of NO using the nitrite reductase mechanism [40].

It is necessary to mention the enzymes defending the macrophages from the excess of peroxide products. Although the cell culture infected with TBEV exhibited characteristic features of cell degradation in the late period of the observation (1-4 days), the activity of SOD (the enzyme scavenging excessive amounts of reactive oxygen species) and lysosomal enzymes (nonspecific esterase and acid phosphatase) were detected. It is known that the most of the hydrolytic lysosomal enzymes exist in the inactive form, and are activated by the binding of the coenzyme to the substrate after the stimulation of the cell [41]. Presumably, the highest activity of these enzymes in the late period of the infection coinciding with the period of TBEV reproduction points to the defensive function of the macrophages. This period is characterized by intensive synthesis of different protein and nucleic components of TBEV in the cytoplasm of the infected cells. These viral components can serve as the specific stimulus for the activation of macrophage enzyme systems.

The complex evaluation of the metabolic activity of the macrophages infected with TBEV suggests that the period of activation of the viral genome in the cytoplasm of the host cell is accompanied by the first increase in the activity of the cell enzymes, and the subsequent period of transcription of the viral RNA and synthesis of its component coincides with the highest enzymatic activity of the macrophages. This conclusion is based on the fact of the significant shift in the enzymatic activity of the cells, as well as on the revealed connection between the dynamics of the enzymatic activity and the periods of specific interaction of the virus with the phagocytes. Thus, complex evaluation of enzymatic changes in macrophages is a highly sensitive method of the indication of the reproductive activity of the virus in the cell cytoplasm allowing differentiation of the types of its cytopathogenic action.

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