NO-Producing Activity of Macrophages Infected with Tick-Borne Encephalitis Virus

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Stimulation of NO-producing activity of macrophages infected with tick-borne encephalitis virus was demonstrated. Activity of NADPH-diaphorase, inducible NO synthase, and cytochrome oxidase, a heme-containing mitochondrial enzyme reflecting NO production by the nitrite reductase pathway was observed against the background of production of NO metabolites by cells.

Key Words: *NO metabolites; NO synthase; macrophage/monocytes; tick-borne encephalitis virus*

In macrophages stimulated by viruses and proinflammatory cytokines, NO production is detected apart from generation of reactive oxygen metabolites [9]. In macrophages, NO is produced by two pathways: during oxidation of L-arginine in the presence of NO synthase (NOS, NOS pathway) and during activation of nitrite reductase systems related to heme-containing proteins (hemoglobin, myoglobin, cytochrome oxidase (COX), *etc*.) under conditions of oxygen deficiency. [10]. These mechanisms are interrelated and are components of the NO cycle in cells [4].

Tick-borne encephalitis virus (TBEV) belongs to *Flaviviridae* family and causes dangerous nerve system disease prevalent in many European countries [1]. It was found that *Flaviviridae* stimulate adhesion and NO production of monocytes/macrophages; NO-mediated inhibitory effect on replication of these viruses was demonstrated in macrophages [6,7,12]. Experiments on mouse resident macrophages infected with Dengue virus showed that activation of oxygen-dependent enzyme system is required for competent antiviral phagocyte response. In this case, simultaneous production of reactive metabolites of oxygen and NO leads to the formation of peroxynitrite potentiating cytotoxicity of these cells against the virus [4]. In light of this, it was interesting to study NO-producing activity of monocytes/macrophages in infections caused by *Flaviviridae*.

Our aim was to study NO production in resident macrophages after infection with TBEV.

MATERIALS AND METHODS

Primary macrophage culture was obtained from the peritoneal cavity of outbred albino mice. Cell suspension (5×10^6/ml medium) in 199 medium (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalites, Russian Academy of Medical Sciences) supplemented with 5% FCS (ICN) was used in the experiments. After 40-min incubation at 37°C in mixed atmosphere with 5% CO_2, non-adherent cells were washed twice and allowed to stay for 3 days in a thermostate in a medium of the same composition. Then, the cells were infected with TBEV, strain Primorye-73, virulent for newborn albino mice. Culture medium of porcine embryo kidney cells used in experiments contained viral particles in a concentration corresponding to two cytopatho-

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genic effect titer logarithms (2 lg TCE/50). After 1-h contact, the monolayer of macrophages infected with TBEV was washed twice with medium 199 from nonadherent viral particles and the incubation was continued. Engulfment of TBEV particles by macrophages was controlled using fluorescing antibodies (indirect method).

For evaluation of intracellular content of NO metabolites (nitrites), the monolayer of TNEV-infected cells was frozen and stored at -20°C. Griess reagent (100 μl, a mixture of equal volumes of 0.1% N-(1-naphthylethylene diamine hydrochloride and 1% sulfanilamide (Sigma) on the basis of phosphoric acid [13]) was added to disintegrated macrophages. The concentration of the reaction product was measured after 10-min contact by absorption at 540 nm on a Multiscan Titertek Plus spectrophotometer (Flow lab.)

For evaluation of COX activity, fixed monolayer was incubated for 10 min with 100 μl 0.1 M acetate buffer (pH 5.5) containing 10 mg/ml $MnCl_2$, 0.33% H_2O_2, and 2 mg/ml diaminobenzidine (ICN) The reaction was stopped by adding 10% H_2SO_4. The concentration of the reaction product was measured on a spectrophotometer at 492 nm.

The results of spectrophotometry were expressed as a stimulation index calculated as the percent ratio of the difference between the mean optical densities of solutions containing reaction products of TBEV-infected and intact cells to the mean optical density of the solution of intact phagocytes.

For evaluation of activity of inducible NO synthase (iNOS), the TBEV-infected cell monolayer was treated with monoclonal antiserum against murine iNOS (1:50, Sigma) and diagnostic fluorescing antispecies antibodies against albino mouse immunoglobulins (Sigma) [2]. The preparations were examined under a luminescent microscope, 100 cells were counted in a phase contrast mode, the percent of iNOS-positive cells was determined by specific fluorescence.

Ultrastructural study of NOS activity in cells was carried out by the cytochemical method, by incubation of Ito-fixed samples in 2-[2'-benzothiazolyl]-5-styryl-3-[4'-phthalhydrazidyl] tetrazolium chloride (BSPT, Sigma) solution (substrate for NADPH-diaphorase) at 37°C for 90 min [8]. This reaction yields osmiophilic formazan. Apart from BSPT (6 mg/ml) on sodium phosphate buffer (pH 8.0), the substrate solution included 10 mg NADPH (Sigma). The control samples were incubated in the absence of NADPH. The samples were postfixed in 1% OsO_4 and embedded into epon and araldite using plane-parallel technique, ultrathin sections in the plane parallel to cell monolayer were contrasted with lead citrate and examined under a JEM-100S electron microscope (Jeol).

RESULTS

Infection of resident macrophages with TBEV was associated with elevation of intracellular content of NO metabolites, nitrites (Fig. 1). The increase in the content of NO metabolites was gradual and significant compared to that in control cells ($p<0.05$) starting from the first minutes after infection. This parameter peaked after 24-h incubation (53.4±3.2%; $p<0.05$), while by the end of observation (day 3) it was 31.03±2.80% ($p<0.05$).

COX is a mitochondrial respiratory chain enzyme and takes part in aerobic oxidation. NO molecule binds to cytohemin, prosthetic of the enzyme. In this case, interaction of superoxide anion and NO yields peroxynitrite, a potent oxidizer that can inhibit various cell enzymes and impair mitochondrial integrity. Measurement of intracellular content of this enzyme helps to evaluate indirectly cell capacity to NO production by the nitrite reductase pathway [14].

COX activity in macrophages increased after 1-h contact of cells with TBEV; this parameter decreased by the 3rd hour, increased again after 6 h ($p<0.05$), and then decreased after 24-h incubation of TBEV-infected macrophages (Fig. 1). Maximum activity of the enzyme was observed after 48 h.

Evaluation of iNOS activity in TBEV-infected macrophages showed that the number of cells with specific fluorescence was maximum after 1-h incubation (75.0±6.5%; Fig. 2, *b*), after 5 h this parameter decreased to a level observed in intact cells (5.0±0.6%), after 7 h it increased to 25.0±1.6%

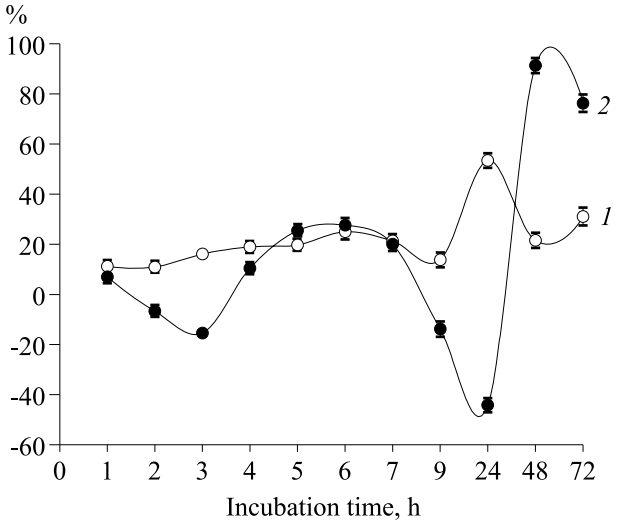

Fig. 1. Intracellular content of NO metabolites (*1*) and activity of COX (*2*) in TBEV-infected macrophages.

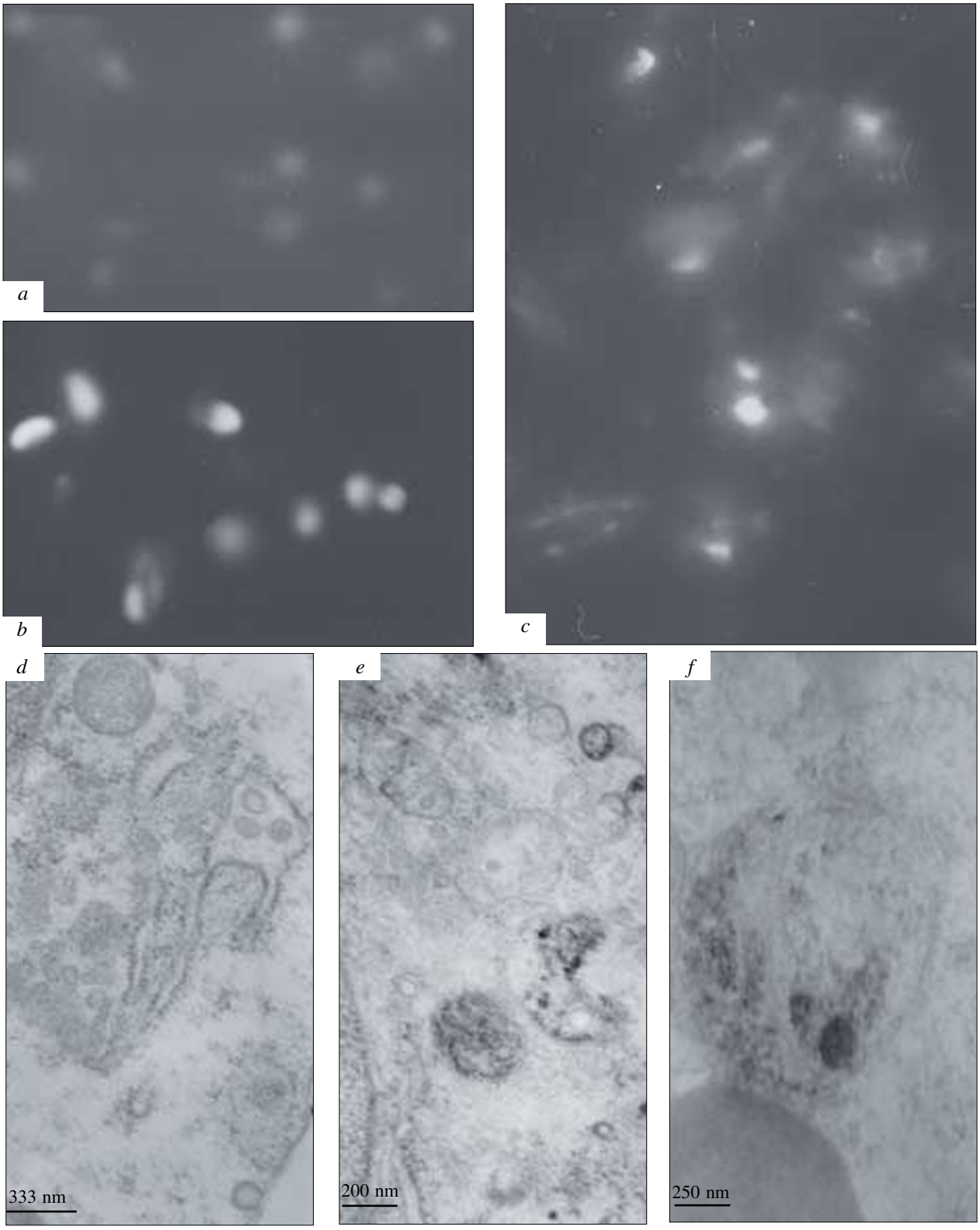

Fig. 2. Activity of iNOS and NADPH-diaphorase in TBEV-infected macrophages. *a*) intact cells; *b*) iNOS-positive macrophages 1 h after TBEV infection; *c*) the same after 7 h; *d*) absence of reaction to NADPH-diaphorase in TBEV-infected phagocytes (arrow shows viral particles) in medium without NADPH; *e*) vesicles in macrophage cytoplasm with positive reaction to NADPH-diaphorase 10 min after infection; *f*) the same after 2 h. *a-c*) indirect immunofluorescent method, *1500; *d-f*) electronograms.

(Fig. 2, *c*), and decreased again by the end of observation. It should be emphasized that the changes in iNOS and COX activities in TBEV-infected macrophages were oppositely directed. COX activity decreased with increasing the percent of iNOS-positive cells, the cell content of NO metabolites remained elevated (Fig. 1; 2, *b, c*).

Ultrastructural study showed that NADPH-diaphorase was located primarily in cytoplasmic vesicles of TBEV-infected macrophages (Fig. 2, *d-f*). The number of macrophages with these granules attained ~25% as soon as 10 min after TBEV infection, 15% after 2 h, and remained at this level to the end of observation. In parallel samples incubated without NADPH, no electron-dense granules with positive reaction to NADPH-diaphorase were found. It should be noted that positive reaction to NADPH-diaphorase was observed in 7% intact cells not infected with TBEV. These findings confirm the data on activation of monocyte/macrophage enzyme systems in response to adhesion [4].

Cells of the monocyte lineage infected with viruses can produce NO, which in turn modulates the synthesis of IFN-γ and TNF-α. It was demonstrated that the production of NO metabolites considerably decreased in murine macrophages infected with TBEV [11]. Addition of IFN-γ to infected macrophage culture increased NO-producing activity of cells, while its combination with TNF-α inhibited it. Nevertheless, infection of monocyte/macrophage populations with other *Flaviviridae* viruses (West Nile, Dengue, and Japanese encephalitis viruses) increased production of NO metabolites in cells [7,12]. Apart from *in vitro* increase in the content of NO metabolites released by blood monocyes from patients infected with Dengue virus, expression of iNOS was found in these cells [6] and the necessity of activation of oxygen-dependent enzyme systems for competent antiviral response of macrophages was shown.

Using various methods we demonstrated increased NO production in TBEV-infected macrophages. Activities of NADPH-diaphorase and iNOS were detected against the background of active production of NO metabolites by cells throughout the observation period. The parameters of activities of these enzymes in TBEV-infected cells in phagocytes not always coincided with the dynamics of COX activity. We believe that in this case NO can be produced by the nitrite-reductase pathway, which is seen from increased activity of mitochondrial COX in TBEV-infected macrophages. At the same time, at the initial stages of infection macrophages produce NO metabolites by the nitroxide synthase pathway with participation of NOS.

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