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Metabolic Activity of Macrophages Infected with Hantavirus, an Agent of Hemorrhagic Fever with Renal Syndrome

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Abstract—Monocytes/macrophages are thought to play an important role in pathogenesis of viral infections. These cells are involved in distribution and persistence of viruses in the organism and also influence the regulation of immune reactions. The functional and enzymatic activities of macrophages infected with an agent of hemorrhagic fever with renal syndrome were analyzed for the first time. This disease is caused by a virus of the *Hantavirus* genus, the Bunyaviridae family. Activities of ectoenzymes 5′-nucleotidase and ATPase of the plasma membrane of the hantavirus-infected macrophages decreased along with the antigen accumulation in the infected cells. The contact of phagocytes with hantavirus resulted in activation in the cells of the oxygen-dependent metabolism and NO-synthase. The NO-synthase-dependent system of the infected macrophages was activated earlier than their oxygen-dependent system. The intracellular contents of acid and alkaline phosphatases increased within the first hours after the infection. The bactericidal activity of the hantavirus-infected macrophages relatively to *Staphylococcus aureus* increased during the specific antigen accumulation in the phagocytes. Thus, the infection of macrophages with hantavirus was associated with intracellular metabolic changes.

Key words: monocytes/macrophages, hantavirus, cytoplasmic membrane enzymes, nitric oxide, reactive oxygen species

Monocytes are an essential component of the body's resistance to infecting agents. These cells render the agents harmless via phagocytosis and production of biologically active substances [1, 2]. These cells can be easily isolated from peripheral blood and peritoneal exudate, fractionated, and cultured; therefore, they are a good model for investigating general cytophysiological regularities, including enzymatic reactions of cells in various diseases.

Functions of monocytes/macrophages, in particular, their ability for synthesis of biologically active substances, are determined by their morphology. Thus, these cells have multiple intracellular membrane structures, which are mainly represented by the endoplasmic reticulum, Golgi complex, and numerous vacuoles. Macrophages are characterized by laminated plasma membranes, which increases the total area of the cell surface during its active mobility. Pino- and phagosomes are generated from invaginated regions of the plasma membrane. Thus, across the outer membrane the macrophage receives not only nutrient substances but also certain signals accepted by the membrane receptors [3, 4]. The ectoenzyme, 5′-nucleotidase, or adenosine 5′-phosphatase, bound to the outer side of the plasma membrane through the glycosyl phosphatidylinositol residue is used as a marker of the complex of enzymes bound to the outer surface of membranes [5]. According to the enzyme nomenclature (1979), 5′-nucleotidase corresponds to 5′-ribonucleotide phosphohydrolase (3.1.3.5) and catalyzes hydrolysis not only of the majority of nucleoside phosphates but also many deoxyribonucleoside 5′-phosphates as 5′-monophosphates, and is inactive (or very slightly active) to 2′- or 3′-monophosphates [6]. The rate of hydrolysis of different nucleoside phosphates in the cells varies depending on the animal species and organ [7]. Hydrolysis is believed to be especially important in the regulation of intracellular processes. Thus, stimulation of macrophages is specified by a decrease in the activity of 5′-nucleotidase [8, 9].

Adenosine triphosphatase, or ATP monophosphatase (ATPase, 3.6.1.3), of the cellular membranes in its turn is activated by Na^+ and K^+ and interacts with the corresponding substrate in the presence of Mg^{2+} [10]. This enzyme is responsible for physiological degradation

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of ATP, i.e., it hydrolyzes the energy-rich phosphate bonds with the release of energy and plays an important role in the potassium and sodium transport across the membrane (in the so-called ion pump).

The main biochemical parameters of macrophages are similar to those of other cells of humans and animals. The metabolic pathways and their regulatory mechanisms are the same. However, metabolism of macrophages has some features associated, on one hand, with the specific features of their structure and, on the other hand, with the specificity of their functions.

In varied experimental systems, phagocytosis of any foreign particle by macrophages was associated with an immediate increase in the uptake of oxygen, expenditure of glucose, and release of hydrogen peroxide and lactic acid. Such an activation of the energy metabolism was named oxidative, or respiratory, burst. At the maximal activation during this process, phagocytes generate a number of highly active unstable products of oxygen reduction: superoxide anion $O_2^{\cdot-}$, hydrogen peroxide H_2O_2, hydroxyl radical $^{\cdot}OH$, and singlet oxygen $\uparrow O_2$ ($^1O_2$). A key enzyme of the oxidative burst is the membrane NADPH oxidase (hydroxy-3-methylglutaryl-CoA reductase, 1.1.1.34), which carries the electron from reduced NADPH onto an oxygen molecule [11]. The generation of radicals can be detected using the Nitro Blue Tetrazolium reduction test (NBT-test). The NBT-test is a typical histochemical approach. But, contrasting to most histochemical reactions, the NBT-test is applied to living cells which are fixed only after the incubation with NBT as an indicator of the respiratory burst [12].

In addition to the oxygen-dependent system, nitrogen oxide (NO) also plays a decisive role in the microbiostatic, microbicidal, and cytostatic functions of macrophages [13, 14]. In activated macrophages, the synthesis of NO is induced via expression of the gene encoding the inducible NO synthase (iNOS) [15].

Acid and alkaline phosphatases which are mainly located in granules of macrophages and involved in digestion of a foreign material have a number of isoenzymes which can liberate phosphate from various alcohol or phenol phosphomonoethers in acidic or alkaline medium. Phosphatases are detected cytochemically based on formation of an insoluble stained precipitate in the places of the substrate hydrolysis [16]. Moreover, data on the intracellular content of phosphatases in macrophages in various diseases allow us to indirectly assess their digesting activity.

In viral infections, monocytes/macrophages are central in the immune response (the data are in the abundant literature). But not all viruses are equally sensitive to the enzyme systems of phagocytes: some of them are easily inactivated by macrophages, whereas others are resistant to them [17, 18]. Some resistant viruses are capable of active and sometimes prolonged reproduction inside the cell. In particular, hantavirus, which causes hemorrhagic fever with renal syndrome, belongs to such a type. This disease with the 1-15% lethality is common in Asia and the Far East [19, 20].

The hantavirus antigen was recently found in peripheral blood monocytes of humans and animals during the acute phase of the disease [21]. But data on the viral reproduction in these cells are contradictory. Some authors state that hantavirus is reproduced only in 10% of the cells [22], whereas other authors think that in the virus reproduces in 100% of the cells in the culture of macrophages [23]. The present work is the first to analyze changes in the functional activity of resident macrophages infected with hantavirus in correlation with their biochemical parameters.

MATERIALS AND METHODS

Primary culture of macrophages. Cells were collected from exudate by washing the peritoneal cavity of random-bred white mice with 5 ml of cold RPMI-1640 medium (Chumakov Institute of Poliomyelitis and Viral Encephalitis, Russian Academy of Medical Sciences, Moscow) containing heparin (5 U/ml). The resulting cell suspension was centrifuged at 4°C for 7 min and washed thrice from heparin. The cell concentration was adjusted to $5 \cdot 10^6$ cell/ml in 199 medium supplemented with 5% fetal calf serum (Vector, Moscow) inactivated at 50°C for 30 min, without antibiotics. The cell suspension (2 ml) of the resulting concentration was placed into two tubes with cover slips and left in a thermostat at 37°C in an atmosphere with 5% CO_2 to provide the macrophage adhesion. After 40 min, unadhered cells were washed out twice in the same medium, left in the thermostat for three days at the same atmosphere, and then used for experiments. The condition of the primary culture of macrophages was assessed by supravital observation with phase contrast.

Virus strain. Macrophages were infected with the hantavirus strain No. 60343 virulent for newborn white mice which was isolated on Vero-6 cells from lungs of infected field mice. A virus-containing cultural fluid 10^{-2} TKID/50 in 0.2 ml was used, i.e., 10 infective units per macrophage (based on the cell plating concentration and virus titer used for infecting).

The macrophage monolayer contacted with hantavirus for 15 min. Then the cells were twice washed with 199 medium from the unadhered viral particles, and the infected culture was incubated at 37°C.

Assessment of viral antigen accumulation in the cells: indirect approach with fluorescent antibodies. Cover slips with the adhered infected cells were dried in air and fixed for 5 sec in cold 96% ethanol (our modification). Such conditions for fixation were chosen in order to retain the surface antigens induced by the virus in the plasma membrane of the cells. The preparations were carefully washed with phosphate buffer, stained, and studied by an indirect

approach with fluorescent antibodies using a luminescent serum against human globulins (Gamaleya Institute of Epidemiological Medicine, Russian Academy of Medical Sciences, Moscow) as described in [24]. The preparations were viewed with a luminescence microscope, using FS, SS, and ES exciting filters and a ZhS = 18 + ZhS = 19 fastening filter with an immersion water objective. The results were based on visual assessment of color, intensity, and morphology of the fluorescent regions. With phase contrast, 100 cells were counted, among which the number of antigen-containing cells was determined and expressed in percent. The virus was titrated on the Vero-E6 cell passaged culture as described in [25].

Determination of the number of stimulated cells in the culture of macrophages. Fixed preparations were stained by the Nokht–Maximov method: the cell monolayer was placed for 20 min in a dye consisting of one part 0.1% eosin and two parts of 0.1% azure II in distilled water. Then the preparations were washed in flowing water, examined with an immersion objective, and nonstimulated and stimulated cells were counted. Sprawled macrophages with pseudopodia extended on the cover slip surface were considered as intact nonstimulated cells.

To determine the activities of ATPase and 5′-nucleotidase and perform the NBT-test, flat-bottom plates were used with 50-μl wells filled with suspensions of intact and infected cells (10^6 cell/ml).

Determination of activities of ATPase and 5′-nucleotidase. The cell suspension was supplemented with 20 μl of the substrate for ATPase (8 mg ATP per ml of Tris-HCl buffer, pH 7.8, containing 870 mg NaCl, 287 mg KCl, and 52 mg $MgCl_2 \cdot 6H_2O$) and for 5′-nucleotidase (4 mg AMP per ml of the same buffer containing 870 mg NaCl and 700 mg $MgCl_2$). The samples were kept in a thermostat at 37°C for 60 and 30 min, respectively [26]. The reaction was stopped by addition of 100 μl of a mixture of ascorbic and molybdic acids (1 : 1). The results were recorded at 620 nm with a Titertek Multiscan Plus spectrophotometer (Flow Lab, Finland). Plates with the substrate solutions and the mixture of acids without cells were used as the control.

NBT-test. The plate wells with the adhered cells were supplemented with 100 μl of NBT solution (1 mg/ml) [27]. The control wells were introduced with 50 μl of Hanks solution without Phenol Red, and the experimental wells were supplemented with 50 μl of *Staphylococcus aureus* suspension (10 bacteria per phagocyte). The cell suspension was incubated for 30 min at 37°C, and then the cell layer was washed thrice with 199 medium and dried. The resulting diformazan was dissolved in dimethylsulfoxide previously heated to 83°C. The extinction was determined at 540 nm with the Titertek Multiscan Plus spectrophotometer. Both the spontaneous (control) and induced (experiment) NBT-tests were performed in triplets. The mean optical density and the stimulation index (the ratio between experimental and control parameters) were calculated for the control and experiment. The spontaneous NBT-test characterized the extinction of the control, and the induced one that of the experiment.

NOS activity was determined using our modification of the histochemical method described in [28]. The macrophage culture on cover slips was first fixed in formalin vapors for 10 min, then washed twice for 10 min in 0.15 M Tris-HCl buffer (pH 8.0) supplemented with 15% sucrose, then incubated for 1 h at 37°C in medium containing 0.5 mM NADPH, 0.5 mM NBT, and 0.3% Triton X-100 in 0.15 M Tris-HCl buffer (pH 8.0). Control preparations were placed into medium supplemented with the NOS inhibitor N^{ω}-nitro-L-arginine (10 mM). The staining intensity was from time to time monitored with a microscope. Upon staining, the preparations were washed in distilled water, dehydrated in alcohols of increasing concentration, and mounted in balm. The enzyme quantity was determined semi-quantitatively by the method proposed in [29] for determination of intracellular content of cationic proteins in neutrophils. The parameter was expressed as an average cytochemical coefficient (ACC) and calculated by the formula:

$$\mathrm{ACC} = \frac{3a + 2.5b + 2c + 1.5d + 1e + 0.5f + 0g}{100},$$

where 3, 2.5, 2, 1.5, 1, 0.5, and 0 characterize the dye incorporation into the macrophage cytoplasm: 3) the cytoplasm of granulocyte is completely filled with black granules; 2.5) the area of stained granules occupies 2/3 of the cytoplasm; 2) the area of stained granules occupies 1/3 of the cytoplasm; 1.5) the cytoplasm is filled with light-gray granules, with solitary black granules; 1) the cytoplasm is 1/2 occupied with light-gray granules; 0.5) solitary granules or a uniform diffuse gray staining of the cytoplasm; 0) no reaction to the enzyme; a, b, c, d, e, f, g) numbers of the cells with the corresponding activities; 100) total number of cells.

Determination of alkaline phosphatase by the Homori method. Preparations with fixed cells were incubated for 45-60 min at 37°C in incubation medium containing 1 g $CaCl_2$, 100 mg $MgCl_2$, and 300 mg sodium β-glycerophosphate in 100 ml 0.2 M Tris-maleate buffer, pH 9.2 (the solution was preliminarily kept at 37°C for 24 h). Then the preparations were washed thrice in distilled water and placed into a fresh 5% solution of ammonium sulfide [30]. The content of alkaline phosphatase was determined semi-quantitatively using ACC, as described above.

Determination of acid phosphatase. Wells of a flat-bottom 96-well plate containing plastic-adhered and washed cells (possibly, fixed) were supplemented with 50 μl of *p*-nitrophenylphosphate solution (PNPP) (ICN, USA). This solution contained 0.09 g PNPP and 0.31 g

NaCl in 37 ml of sodium citrate buffer. The reaction was performed for 30 min at 37°C and stopped by addition of 100 μl of 0.2 M NaOH per well. The result was recorded at 405 nm using the Titertek Multiscan Plus spectrophotometer [27].

Determination of intracellular bactericidal activity of leukocytes. Suspension of the *S. aureus* bacteria was washed thrice with Hanks' solution at room temperature and added to a monolayer of the primary culture of macrophages at the ratio of 10 bacteria per phagocyte. The mixture of bacteria and phagocytes was incubated for 30 min at 37°C in an atmosphere with 5% CO_2. Then the samples were washed twice with 199 medium from the noncaptured (noningesting) bacteria. Half of the samples were supplemented with 2 ml of sterile distilled water and left for 10 min to destroy the phagocytes. Then 0.02 ml of the lysate were placed into wells of an immunological plate containing 0.18 ml of 199 medium, carefully mixed, and 0.02 ml of the lysate was inoculated onto beef-extract agar (BEA) [31]. Other samples were incubated for 4 h and treated similarly. The bacteria were cultured on BEA for 48 h, the number of colonies was counted, and the average result was determined after three experiments. The data were expressed as the index of bacterial digestion (T) by the formula:

$$T = \frac{N_c - N_{ex}}{N_c} \cdot 100 ,$$

where N_c is the number of colony-forming units (CFU) after 30 min of the contact, and N_{ex} is CFU after the incubation for 4 h.

RESULTS

Using the indirect approach with immunofluorescent antibodies, a 15-min contact of hantavirus with macrophages resulted in 55.5 ± 4.6% of the cells becoming antigen-positive (Fig. 1). The cytoplasm displayed a specific diffuse fluorescence, whereas the macrophage nuclei had a nonspecific red staining (for microscopy with an exciting light filter DNA can fluoresce in red light). After incubation of the infected culture for 60 min, the fluorescence was observed of both the plasma membrane and perinuclear area of macrophages, and this fluorescence did not change with time. But the number of antigen-containing cells decreased to 29.6 ± 2.8% and remained in these limits 3 h after infecting. After 4 h of the incubation, the number of antigen-positive cells sharply increased (to 87 ± 5.7%) and by the end of observation (18 h) decreased to 18.0 ± 1.6% because of degradation of the macrophage culture. The hantavirus adhesion on phagocytes was confirmed by titration of the supernatant virus-containing fluids on the cell culture. Thus, the contact for 15, 30, and 60 min resulted in a decrease in the titer by 1.0, 2.0, and 2.5 log units, respectively, that indicated an active adsorption of the virus on the cell surface.

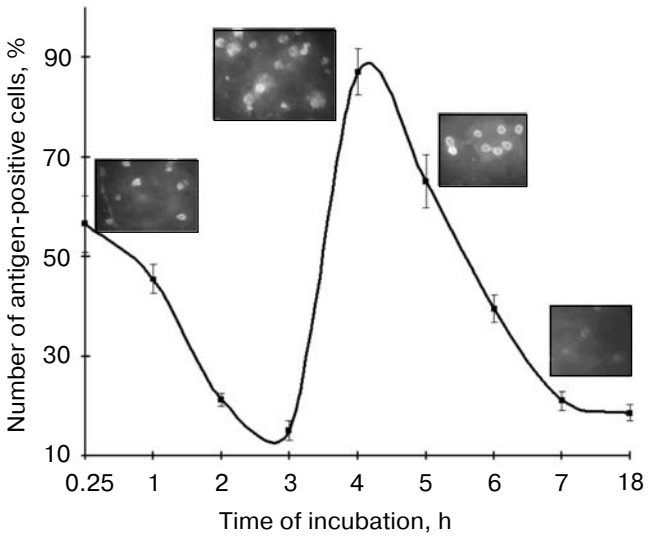

Fig. 1. Changes in the intracellular content of hantavirus specific antigen in primary culture of macrophages.

On microscopy of stained preparations, we consider a sprawled macrophage with pseudopodia extended on the cover slip surface as an intact cell. After a contact with the virus for 15 min, most of phagocytes became activated and round-shaped. The maximal number of activated macrophages was achieved after the incubation for 2 and 3 h (86.5 ± 7.6 and 89.0 ± 7.2%, respectively). Later (18-24 h after the infection), the percent of activated macrophages decreased along with an increase in the number of cells with signs of degradation, such as an increased vacuolization of the cytoplasm periphery and brightening of the nucleus with its subsequent destruction.

The plasma membrane of macrophages is known to spatially transform during chemotaxis, and this directly depends on activities of its ectoenzymes 5′-nucleotidase and ATPase. To differentiate activated and resting macrophages, the intracellular contents of these enzymes were determined. Thus, the activities of 5′-nucleotidase and ATPase were high in the resting cells and very low in the activated cells [32, 33]. We found that an increase in the number of antigen-positive cells was associated with a decrease in the activity of 5′-nucleotidase (Fig. 2). The parameters were significantly different from those of the control 60 min after the infection with hantavirus (38.7 ± 2.8% as compared to the control taken as 100%). The activity of this enzyme was minimal 4 and 5 h after the infection (32.4 ± 1.7 and 31.1 ± 2.1%, respectively) and retained this level until the end of the observation.

The cell membrane ATPase is responsible for degradation of ATP because it hydrolyzes energy-rich phosphate bonds with release of energy. ATPases are tightly

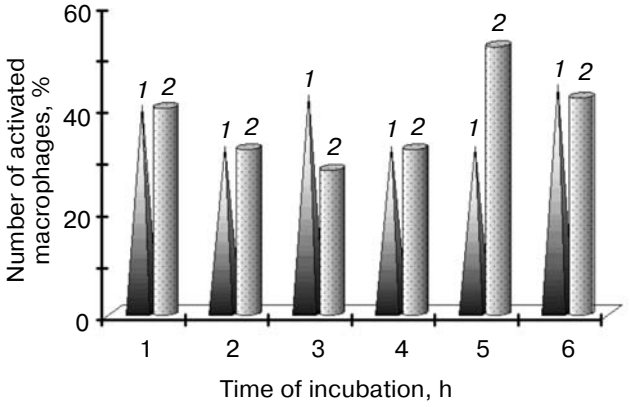

Fig. 2. Changes in the activities of 5′-nucleotidase (*1*) and ATPase (*2*) in primary culture of macrophages infected with hantavirus. Ordinates: the ratio of experimental to control parameters.

associated with the membrane structures, and their functional activities can indicate stimulation of the cell metabolism [34]. A decrease in the intracellular content of this enzyme suggests an activation of monocytes/macrophages in response to stimulation. The changes in the activity of ATPase in the primary culture of macrophages infected with hantavirus correlated with changes in the activity of 5′-nucleotidase (Fig. 2). During the observation, the activity of ATPase was lower than in the control, and the lowest activity of the enzyme was recorded 2 and 4 h after the infection (28 ± 1.6 and 32 ± 2.7%, respectively). These data suggested the pronounced stimulation of macrophages upon their infection with hantavirus.

Phagocytosis is associated with a considerable increase in the amount of oxygen consumed by phagocytizing cells. By contrast to many other cells, oxygen respiration is not an obligatory system for viability of phagocytes because they have almost no mitochondria and can function under anaerobic conditions [35]. Activation of the oxygen-dependent metabolism of phagocytes, in particular, the function of the hexose monophosphate shunt and the associated production of free oxygen radicals was assessed histochemically with NBT. We found an oxygen-dependent stimulation of macrophages infected with hantavirus (Fig. 3a). Thus, the number of NBT-positive cells increased even after 1 h of the incubation (114.3 ± 1.8%), reached the maximum in 2 h (143.0 ± 5.4%), and decreased 7 h after the infection (119.7 ± 7.8%). The number of NBT-positive cells in the control was taken as 100%.

In addition to reactive oxygen species, among oxygen-dependent products nitric oxide radicals are now given special attention. In macrophages, the production of nanomolar concentrations of nitric oxide is associated with the presence of cytokine-inducible NOS (iNOS). And the production of reactive forms of NO in macrophages correlates with such classic characteristics of activation as the increased production of reactive oxygen species and bactericidity [36, 37]. We found histochemically that the intracellular activity of iNOS was increased after 30 min of the phagocyte contact with hantavirus. Then the activity of the enzyme decreased by 2 h of the incubation and sharply increased later. Thus, 30 min after the infection ACC was 1.92 ± 0.5, in 2 h it was 0.66 ± 0.07, with the maximum in 6 h (2.84 ± 0.15). In the control sample ACC was 1.16 ± 0.08. Thus, the NO-dependent system of macrophages infected with hantavirus was activated earlier than the oxygen-dependent system. And the activity of the NO-dependent system of phagocytes was in the opposite phase to the activity of the oxygen-dependent system.

Hydrolytic lysosomal enzymes of macrophages, acid and alkaline phosphatases, are involved in the intracellular digestion of bacteria. Acid phosphatase-positive granules are found in most nucleus-containing blood cells, and the reaction is more pronounced in leukocytes than in lymphocytes. The positive reaction in macrophages is mainly manifested by small granules of irregular shape, which are considered to be tertiary granules.

The activities of acid and alkaline phosphatases in macrophages were found to increase within the first hour (from 15 min to 1 h) after the infection with hantavirus (Fig. 3b). Thus, ACC values for acid phosphatase were from 0.68 ± 0.05 to 0.88 ± 0.08 (15 min and 4 h after the

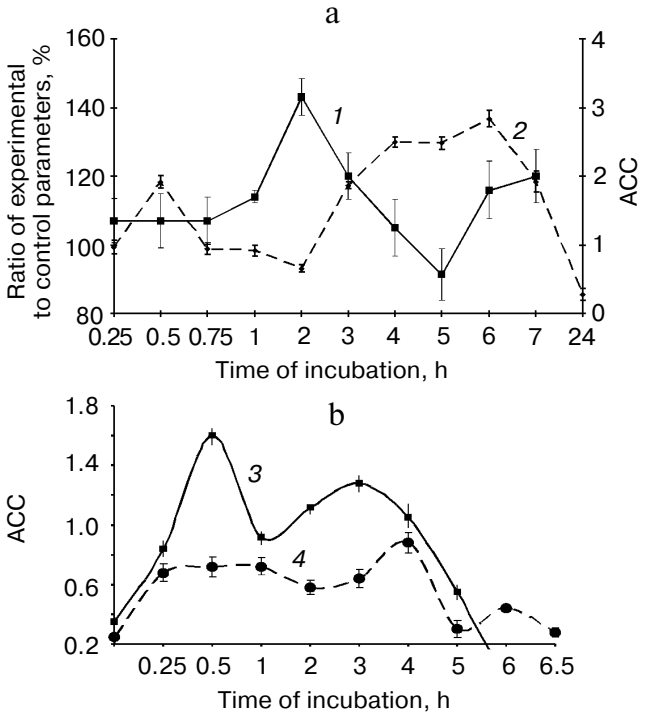

Fig. 3. Enzyme activities in macrophages infected with hantavirus. a) The activity of NADPH oxidase ((*1*), the NBT-test) and NOS (*2*); b) the intracellular content of acid (*3*) and alkaline (*4*) phosphatase.

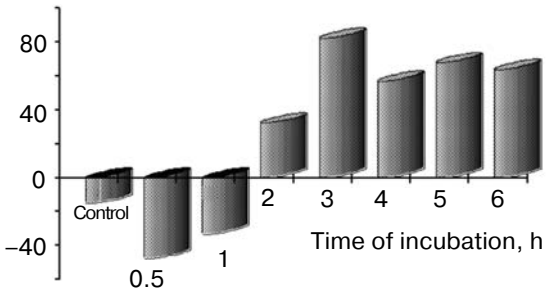

Fig. 4. Bactericidal activity of macrophages (*T*, %) relatively to *Staphylococcus aureus*. The primary cell culture was pre-infected with hantavirus.

infection, respectively); ACC values for alkaline phosphatase were from 1.6 ± 0.06 to 1.05 ± 0.12 (30 min and 4 h after the infection, respectively). In the intact cells, ACC values for acid and alkaline phosphatases were 0.25 ± 0.015 and 0.35 ± 0.026, respectively. On increasing the incubation time of macrophages with the virus (to 5-6 h), the activity of these enzymes decreased. Seven hours after the infection, the activities of acid and alkaline phosphatase were 0.28 ± 0.017 and 0.01 ± 0.07, respectively.

The bactericidal activity of macrophages in the monolayer of the cells pre-infected with hantavirus relatively to *S. aureus* was increasing during the accumulation of the antigen (Fig. 4). At the moments of virus adsorption (15 min) and its penetration into phagocytes (60 min), the digesting activity of the phagocytes was decreased. After 4 h of the cell incubation with phagocytized bacteria, the number of *S. aureus* CFU increased, which suggested the proliferation (reproduction) of the bacteria. The bactericidity index was negative (–48.1 ± 2.7 and –33.3 ± 0.07) after 30 and 60 min incubation, respectively, of the hantavirus-infected cells. After 2 h of incubation and until the end of observation (7 h), the absorbing activity of macrophages increased along with increase in their bactericidal activity. This was indicated by the increase in the bactericidity index to positive values that suggested intracellular digestion of the microorganisms by the macrophages. Thus, the bactericidity index was 32.35 ± 1.7 and 82.3 ± 4.75 after 2 and 4 h of the incubation and remained in these limits until the end of the observation.

Thus, we have found wave-like changes in the enzyme activity in macrophages infected with hantavirus. The initial increase in the enzymatic activity of the cells was followed by its decrease. And the bactericidal activity of the phagocytes increased concurrently with an increase in the intracellular content of the hantavirus antigen.

DISCUSSION

It was shown by immunofluorescence that infection of the primary culture of macrophages with hantavirus was associated with location of the antigen on the plasma membrane and inside the phagocytes, mainly in the perinuclear zone of the cytoplasm. This indicated the adhesion of hantavirus to the outer surface of the plasma membrane and its ability for penetration into the macrophages.

Hantavirus is known to reproduce in endothelial and macrophagal cells of humans and animals [38, 39], and endothelial cells are the main targets [40]. We have shown for the first time that even 15 min after the infection of the primary culture of mouse macrophages 55.5% of the cells contain the antigen, and the decrease in the infecting titer of the virus-containing supernatant fluid during the time from 15 to 60 min of the contact suggested an active adsorption of the virus on the macrophage surface. This was confirmed by electron microscopy [41]. In further studies, we chose the contact between the virus and macrophages for 15 min. Then we removed the virus-containing fluid, and only intracellular transformation of hantavirus can be followed. Thus, we believe that the detected decrease in the number of antigen-containing cells 1-4 h after the infection was associated with transformation of viral particles which were on the stage of intracellular uncoating. But at this moment it was difficult to detect the antigen presence by the indirect approach with fluorescent antibodies because proteins of the hantavirus supercapsid were destructured.

The most pronounced distinction of resident macrophages from those stimulated during inflammation is the decreased activity of the plasma membrane enzyme 5′-nucleotidase [42]. We found similar changes in the activities of the ectoenzymes 5′-nucleotidase and ATPase in the macrophages infected with hantavirus. Thus, the minimum activities of these enzymes correlated with the greatest number of the antigen-positive cells and indicated an active stimulation of the cells. In particular, ATPase regulating the transcription of genetic information can characterize the stage of the cell cycle [43]. And 5′-nucleotidase is also of some importance in assimilation of nucleotides for the energy supply for realizing the genetic information of the cell. Thus, similar changes in the activities of the plasma membrane ectoenzymes represented changes in the cell metabolism when it was stimulated by hantavirus. The coincidence of the minimal activities of the ectoenzymes with the period of the greatest accumulation of the antigen in the macrophages suggested an increase in the synthetic activity of the infected cells with respect to nucleotides, which seemed to be associated with the reproduction of hantavirus RNA in the cytoplasm.

The activities of acid and alkaline phosphatases changed similarly. Hydrolytic lysosomal enzymes in macrophages are mainly inactive. Stimulation of the cells induces synthesis of active forms of the enzymes at the cost of the substrate binding to the coenzyme [44]. The observed increase in the activities of acid and alkaline phosphatases on the initial stage of the macrophage infection with hantavirus suggested the cell stimulation in

response to the virus penetration. It seemed that metabolic changes in the phagocytes were associated with the intracellular changes and stimulation of the lysosomes, as the activities of these enzymes are known to increase in diseases [45, 46]. The subsequent decrease in the activities of these enzymes seemed to be associated with cytopathic changes under the influence of reproductive activity of hantavirus. We think that such changes (the initial increase followed by the decrease) in the activities of the lysosomal enzymes in the hantavirus-infected macrophages precede the development of cytopathic changes because there are data on the involvement of these enzymes in destruction of cellular components [47].

We found that during the accumulation of the virus antigen in the cells and maximums of the enzymatic activities the bactericidal activity of the phagocytes to *S. aureus* increased. Thus, hantavirus stimulated the functional activity of the phagocytes that corresponded to enhancement of the total synthetic activity associated with the viral reproduction.

The activities of the oxygen-dependent (NBT-test) and NO-dependent systems of the infected macrophages changed differently. Thus, an increased reaction in the NBT-test was associated with a decrease in the NOS activity in the phagocytes. The gradual increase and the following decrease in the activity of the oxygen-dependent system by the end of observation was caused by activation of the plasma membrane of macrophages in response to penetration of hantavirus, and this correlated with the activities of the plasma membrane ectoenzymes 5′-nucleotidase and ATPase. This became clear because the enzyme NADPH oxidase is located in the plasma membrane and membrane phagosomes produced from its invaginations. And the plasma membrane is structurally or functionally conjugated with receptors, which recognize external signals and are activated upon binding the ligands or on the background of general rearrangement of the membrane [48]. The activity of the plasma membrane of the hantavirus-infected macrophages was confirmed by electron microscopy. Thus, hantavirus, which is an "enveloped" virus, after the adsorption on the phagocyte surface penetrates into the cell via the fusion of its spike glycoproteins with the plasma membrane [41]. Concurrently, the oxygen-containing enzymatic system is activated. Thus, although we failed to detect ultrastucturral invaginated regions and endosomes on the plasma membrane surface, the determination of enzymatic activities revealed the stimulation of the resident macrophages within 1 h after the penetration of hantavirus.

It is known that iNOS is colocated with the membrane structures of macrophages, and its normal concentration is very low. Cytokines and some other biologically active substances induce a high level of activity of this enzyme [49]. The activation of iNOS is associated with an increase in the endogenous production of NO, which is an ingredient of aggressive substances used in the defense of the organism against penetrating pathogenic organisms [15]. Thus, endogenous NO is involved in the limitation of replication of human immunodeficiency and Japanese encephalitis viruses in human macrophages [50, 51]. The penetration of hantavirus into phagocytes was accompanied by activation of iNOS (within 60 min after the infection). Then the enzyme activity decreased and increased later, until the end of the observation (18 h). We think that the coincidence of the iNOS activity peak with the period of the hantavirus intracellular replication indicates the realization of protective functions of macrophages in response to infection.

Before the penetration into the cell, extracellular virion is biologically inert. It remains inert until the viral genome begins function intracellularly as an independent unit. Later the virus can cause the cell death, because during intracellular replication it induces synthesis of virus-specific proteins, which can more or less inhibit the cell metabolism. We have shown that hantavirus penetrates into the macrophage and initially does not damage it. Then during the activation of its genome in the cytoplasm of macrophage, the virus can change metabolism of the host cell. The infection of the macrophage is autonomous, as the viral genome is replicated independently of the cell genome. This is an acute infection [41], because the reproduction of hantavirus in macrophages is associated with changes in the cell metabolism and appearance of virus-specific and virus-induced formations and also some pathological intracellular changes. According to the classification of A. D. Al'stein [52], this is a productive infection because a complete virus is produced. We are dealing with a lytic form of productive infection because during the hantavirus reproduction in macrophages structural viral components are accumulated and virus-specific proteins are detected which can suppress the cell metabolism. This is indicated by the decreased amount of the phagocyte intracellular elements by the end of observation.

We think that data about the enzymatic activity of the infected macrophages allows us to reveal their metabolic changes during the virus reproduction in them. Because components of a viral particle are synthesized with involvement of enzymes of the host cell, significant changes in its enzymatic activity may be considered as evidence of the cell interaction with the virus. Highly sensitive methods for determination of the enzyme activities and finding of correlations between their changes and specific features of virus interaction with cells seem to be important for detection of viruses and differentiation of types of their cytopathogenic effect.

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