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The Metabolic Activity of Innate Immunity Cells in Experimental Infection Caused by Various Plasmid Types of Yersinia Pseudotuberculosis

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Abstract: In a comparative aspect, the functional state of inflammation effector cells in animals infected with various plasmid types of Yersinia pseudotuberculosis was studied. The metabolic activity of peritoneal exudate cells has been investigated in an experimental infection caused by four plasmid types of Y. pseudotuberculosis: type 82^+: 48^+, containing two plasmids pVM 82 and pYV; type 82^+: 48^- containing single pVM 82 plasmid; type 48^+: 82^- containing single pYV plasmid; plasmid-free type 48^-: 82^-. The parameters of enzyme activity (ATP-ase, 5'-nucleotidase, lactate dehydrogenase, myeloperoxidase) and the level of nitric oxide metabolites were determined. The variability of the metabolic activity of the cells in the inflammatory focus (peritoneal exudate containing neutrophils and macrophages) in infected animals has been established. In response to infection with Y. pseudotuberculosis strain containing two plasmids pYV and pVM82, the production of the nitric oxide metabolites, rather than the active forms of oxygen, had the primary importance in providing the bactericidal potential of phagocytes, compared to animals infected with a strain containing a single pVM82 plasmid. It was concluded that a special biological effect associated with the pVM 82 plasmid available in the Far Eastern strains of the causative agent of epidemic pseudotuberculosis (Far Eastern scarlet-like fever) was involved in the provision of predominantly nitroxide-dependent bactericidal mechanisms of innate immunity cells in this infection.

Keywords: Metabolic Activity, Innate Immunity Cells, ATPase, Lactate Dehydrogenase, Myelo Peroxidase, Nitric Oxide Metabolites, Stimulation Index (T), Variability, Plasmid Types of Yersinia Pseudotu Berculosis

1. Introduction

Currently, the causative agent of pseudotuberculosis infection, Yersinia pseudotuberculosis as the progenitor of Y. pestis, the pathogen of especially dangerous plague infection [1], attracts much attention of researchers as a model for studying the pathogenicity factors of Yersinia and the discovery of molecular mechanisms of interaction in the host-microorganism system [2]. The survival strategy of the Yersinia genus bacteria in the host organism is based on their ability to overcome the mechanisms of innate immunity [3].

Y. pseudotuberculosis has a large set of pathogenicity factors, some of which are encoded by chromosomal genes, and some are encoded by plasmid genes [4-6]. The virulence of the Yersinia genus bacteria is associated with the presence of the pYV plasmid with a molecular mass of 42-48 Mda. This plasmid codes up to 20 species of toxic Yersinia outer membrane proteins (Yop-s), their ability to type III bacterial secretion (T3SS) [7] and the stability of these bacteria to phagocytosis [8-10]. The particular interest is the little studied Y. pseudotuberculosis extrachromosomal replicon, a plasmid with a molecular weight of 82 MDa (pVM 82 plasmid), which is found only in Y. pseudotuberculosis

strains serovar I - the most common etiologic agent of epidemic pseudotuberculosis (Far Eastern scarlet-like fever, FESLF) in humans. The ability of Y. pseudotuberculosis to cause epidemic outbreaks of infection in the population is due to the simultaneous presence of two plasmids pYV and pVM 82 in the microbe, which determine the specific virulence of the strains [4]. It is assumed that the Y. pseudotuberculosis strains carrying pVM 82 plasmid have apoptosis-inducing and immunosuppressive effects [11, 12].

It is established [13] that plasmid-containing Y. pseudotuberculosis strains are more resistant to destruction by macrophages and neutrophils than bacteria of a plasmid-free strain. Bacteria that carry a combination of plasmids (pYV48: pVM 82) suppress the oxidative explosion in macrophages and neutrophils, reduce the activity of superoxide dismutase, myeloperoxidase and acid phosphatase, reduce the non-enzymatic cationic proteins and thus inhibit the bactericidal ability of phagocytes [14, 15]. The morpho-functional state and patterns of bactericidal reactions of phagocytes in their interaction with various plasmid types of Y. pseudotuberculosis have been studied fragmentarily, and the reaction of innate immunity cells determined by pVM 82 plasmid of Y. pseudotuberculosis remains the least studied. On this question for the last three years a published works were not detected. In this connection, the purpose of this work was to study in a comparative aspect of the metabolic state of innate immunity cells in animals infected with various plasmid types of Y. pseudotuberculosis.

2. Material and Methods

2.1. Laboratory Animals

To reproduce the experimental infection, white male mice weighing 18-20 g (200 individuals) were infected intraperitoneally. Animals were on a standard diet in boxed rooms in compliance with all rules and international recommendations of the European Convention.

2.2. Bacteria

For the infection, strains of the four plasmid types of Y. pseudotuberculosis were obtained from the collection of Somov Research Institute of Epidemiology and Microbiology: 1) H-5015 T^+ strain (82^+: 48^+) containing two plasmids pVM 82 and pYV, LD_{50} was 3.7×10^7, the infecting dose 4×10^8; 2) H-5015 T^- strain (82^+: 48^-) containing single pVM 82 plasmid, the infecting dose $4x10^8$; 3) H-5013 T^+ strain (48^+) containing single pYV plasmid, LD_{50} was $1.4x10^8$, the infecting dose $2x10^9$; 4) H-5013 T^- plasmid-free strain (48^-), the infecting dose $2x10^9$. The infecting dose of strains causing a systemic infection was equal to 10-fold LD_{50} [16].

The material was taken on 1, 3, 5 and 7 days postinfection (pi). To obtain cells of peritoneal exudate, the abdominal cavity of the mice was washed with 5 ml of cold medium 199 containing heparin with a concentration 5 u/ml. The cell suspension was adjusted to a concentration of 2×10^6 cells / ml and transferred to flat-bottomed 96-well microplates, 100 µl per well. For adhesion, a suspension of peritoneal cells containing neutrophils and macrophages was left in a CO_2 incubator at 37°C, after 40 minutes the cell monolayer was washed twice from unadhered cells, followed by incubation for 3 days in medium 199 containing 5% fetal bovine serum, 0.004% gentamicin (KRKA), then washed twice with gentamicin and used for experiments.

2.3. Evaluation of the Functional State of Cells

The functional state of the cells was assessed by the activity of enzymes ATPase, 5'-nucleotidase (AMPase), lactate dehydrogenase (LDH), myeloperoxidase (MPO) and by the level of nitric oxide (NO) metabolites.

2.3.1. ATPase and 5'-Nucleotidase Activity

20 µl of substrate for ATPase (8 mg ATP per 1 ml Tris-HCl buffer pH 7.8 containing 87 mg NaCl, 28.7 mg KCl, 52 mg $MgCl_2$, $6H_2O$), and for 5'-nucleotidase (4 mg AMP per ml of the same buffer containing 87 mg of NaCl and 70 mg of $MgCl_2$) were added to the monolayer of cells, the samples were left for 30 and 60 minutes [17]. The reaction was stopped by the addition of 100 µl of the ascorbic and molybdic acid mixture in a 1: 1 ratio. After 20 minutes, the optical density of the substrates was measured on a Multiscan Titertek Plus spectrophotometer ("Flow lab.", Finland) at 620 nm a wave length.

2.3.2. Lactate Dehydrogenase Activity

The method of Z. Loyda in own modification [17] was used. To a fixed monolayer of cells, 100 µl of substrate - iodnitrotetrazolium violet (iodnitrotetrazolium, ICN) 2 mg / ml was added. For this, 20 mg of iodnitrotetrazolium was dissolved first in 0.5 ml of 70^0 alcohol, and then 9.5 ml of the above solution was added. The monolayer of the cells together with the substrate was incubated at 37°C for 30 minutes, after which the supernatant was removed, the monolayer of cells was washed twice with Hanks's solution and dried. The intracellular granules of Diphenazan were dissolved by adding 100 µl of isopropyl alcohol acidified with 0.04 M HCl for 20 minutes. The optical density of the substrate was determined on a spectrophotometer at 492 nm a wave length.

2.3.3. Myeloperoxidase Activity

The method of Z. Loyda in own modification [17] was used. In the wells of the plates, 100 µl of CRF (o-phenylenediamine, firm "ICN"), (4 mg per 10 ml) was added to the fixed cells, based on phosphate-citrate buffer (pH 5.0) with the addition of 500 µl of 0.33% hydrogen peroxide. The monolayer of cells was incubated at room temperature for 10 minutes, then the reaction was stopped by adding a 10% solution of sulfuric acid in 100 µl per well. The optical density was determined on a spectrophotometer at 492 nm a wave length. Blanking was carried out on a solution of CRF and 10% sulfuric acid.

2.3.4. Nitric Oxide - Nitrites (NO_{-2}) Metabolites

After incubation at 37°C, the supernatant and cells

monolayer of the infected animals were frozen and stored at -20°C. 100 µl of Griess reagent was added to the destroyed cells, which consisted of equal volumes of 0.1% N- (1-naphthyl) ethylenediamine dihydrochloride and 1% p-aminobenzidine-sulfonamide (firm "ICN"), based on 2.5% solution phosphoric acid [18]. After 10 minutes of contact, the optical density of the obtained substrates was determined on a spectrophotometer at 540 nm a wave length.

The results of the spectrophotometric analysis of enzyme activity in phagocytes were expressed as a stimulation index (T), in percent, which was calculated by the formula: T = (No-Nk): Nk x 100, where Nk is the average index of optical density of the test substrate in the cells of the uninfected animals (control); No - the average index of the optical density of the substrate in the cells of infected animals.

2.4. Statistical Analysis

Statistical processing of the data was carried out using the software packages "Microsoft Excel 2007", "Statistika 7" with the definition of the arithmetic mean (M), its error (m ±), the reliability of the differences (p) and the use of known graphical methods for expressing statistical data.

3. Result and Discussion

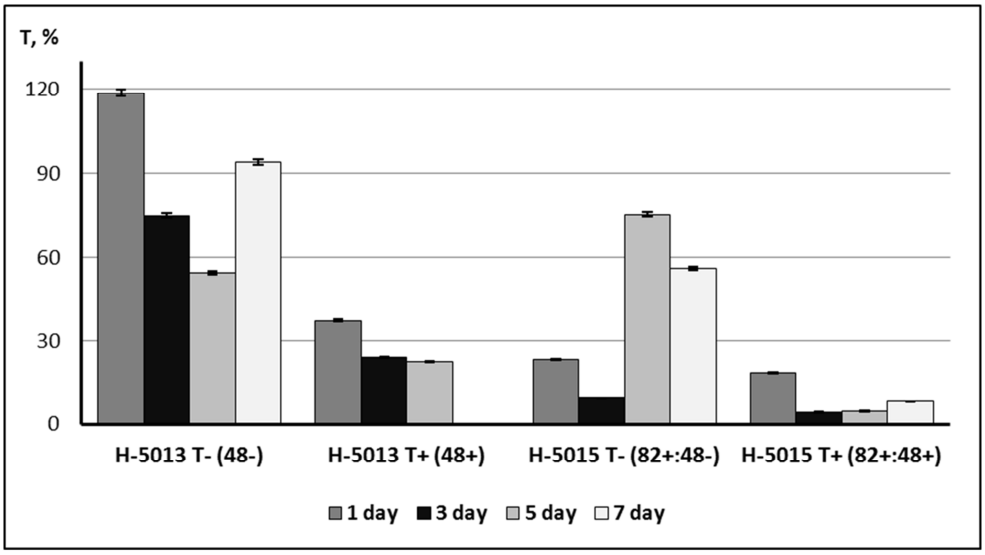
3.1. ATPase and 5'-Nucleotidase Activity

In chemotaxis, the plasma membrane of phagocytes is spatially transformed, and this process is directly dependent on the activity of ectoenzymes — ATPase and 5'-nucleotidase, whose activity is high in resting and extremely small in activated cells [19]. We established that in animals infected with Y. pseudotuberculosis, the ATPase activity in the cells of peritoneal exudate had negative values relative to control only in response to H-5015 T^+ strain (82^+: 48^+), which indicated stimulation of these cells.

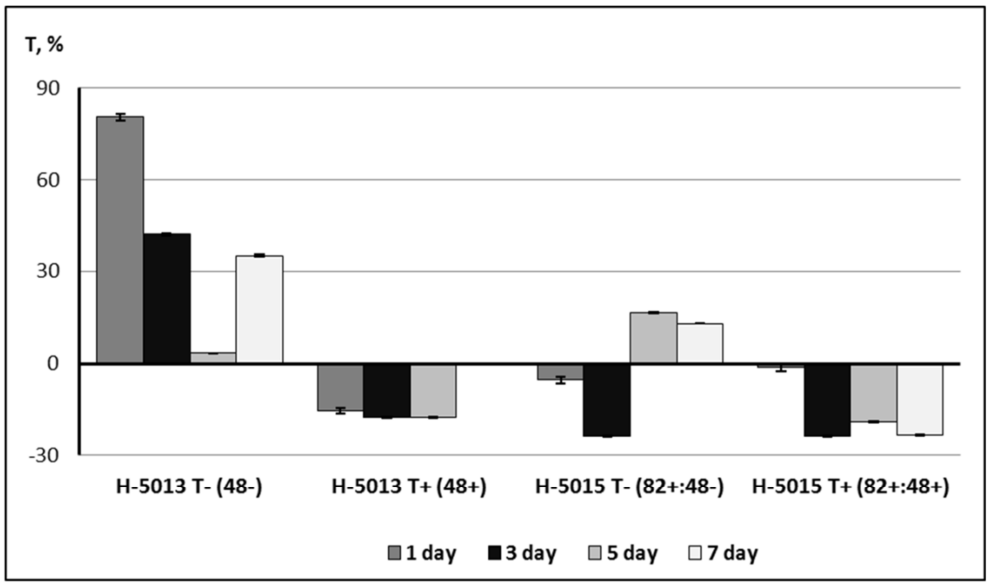
The T indicators were -3.79; -8.22; -7.59 and -3.16%, respectively, for 1, 3, 5 and 7 days pi. When infected with H-5015 T^- strain (82^+: 48^-) cell stimulation was detected only for 3 days pi (T = -5.69%), and in response to H-5013 T^+ strain (48^+), ATP-ase activity slightly decreased to 0.63% for 5 days pi. The indices of 5'-nucleotidase activity had negative values only when infected with H-5015 T^+ strain (82^+: 48^+): -2.56 and -1.48% respectively on 3 and 7 days pi. When the animals were infected with H-5013 T^- (48^-) plasmid-free strain, we have established a high activity of ATPase with a fluctuation of 18.98 to 98.10% and a simultaneous increase in the of 5'-nucleotidase activity from 29.48 to 136.53% with the maximum values for 7 days pi. Compared with the plasmid-free strain, when infected with H-5013 T^+ (48^+) strain, containing a single virulence plasmid pYV, the activity level of 5'-nucleotidase was significantly lower (22.43, 19.87 and 3.20%, respectively, by 1, 3 and 5 days pi), and positive indices of ATPase activity were approaching the control level. So, in terms of ATPase activity, the distinct stimulation of peritoneal exudate cells occurred only in response to infection with Y. pseudotuberculosis strains containing pVM 82 plasmid, especially in combination with the virulence plasmid pYV. The low level of cell stimulation, probably due to the low virulence of the infecting strains, indicated an insignificant degree of purine catabolism intension.

3.2. Lactate Dehydrogenase Activity

At the first stage, to form from the oxygen molecule the superoxide anion of oxygen O_2, succinates and lactates may be the electron donor in addition to the NADP-H oxidase complex. Succinate dehydrogenase and lactate dehydrogenase participate in the reaction of their transformation, which are activated at the last stage of glycolysis [20]. The decrease in LDH activity below the control level for pathogenic exposures can be regarded as a decrease in the energy potential of leukocytes, which reflects the presence of an adaptive response of the organism to external, including bacterial, factors.



(a)



(b)

Figure 1. *Activity of enzymes of oxygen-dependent system in cells of peritoneal exudate in animals infected with different Yersinia pseudotuberculosis plasmid types: a) lactate dehydrogenase; b) myeloperoxidase. The stimulation index T,%.*

In animals intraperitoneally infected with Y. pseudotuberculosis, in the cells of peritoneal exudate, the highest level of LDH activity was revealed in response to the H-5013 T^- (48^-) plasmid-free strain with a cyclic increase of the stimulation index: 118.5; 74.6; 54.4 and 94.1%, respectively, on 1, 3, 5 and 7 days pi (Figure 1a). When infected with H-5013 T^+ strain (48^+) containing a single pYV plasmid the LDH activity was 37.1; 24.0 and 22.3% on 1, 3 and 5 days pi (follow-up period). When infected with H-5015 T^- (82^+: 48^-) strain, the enzyme activity on 1 day pi increased to 23.2%, then decreased to 9.2% on 3 day and increased to 75.1% again on 5 day pi, but until the end of the observation period remained at a high level - up to 56.1% compared with the control. The level of LDH activity in macrophages was minimal in response to infection with the H-5015 T^+ (82^+: 48^+) two-plasmid strain: 18.1; 4.2; 4.6%, respectively on 1, 3 and 5 days pi.

3.3. Myeloperoxidase Activity

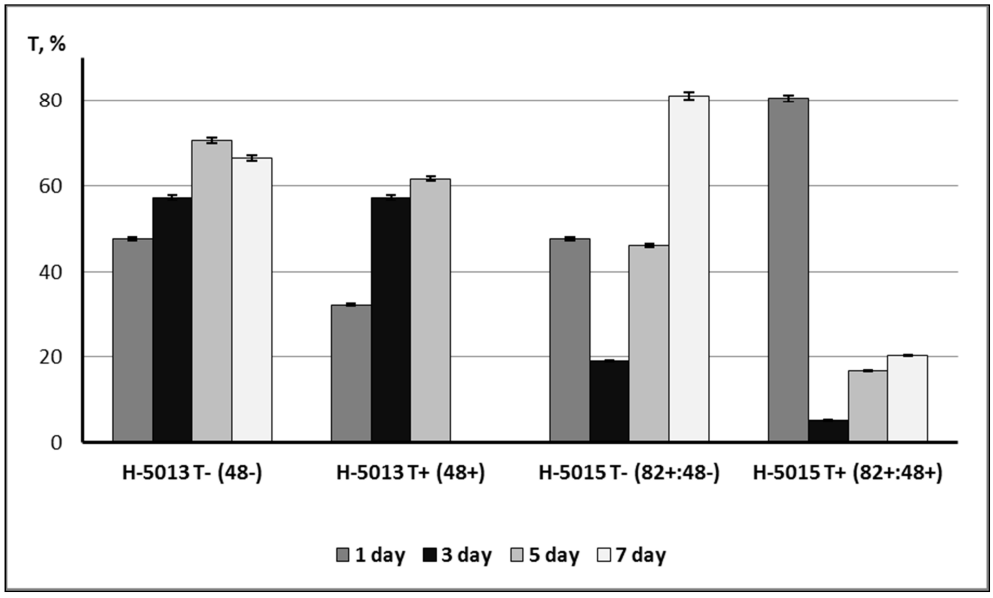
Myeloperoxidase, present in the azurophilic granules of polymorphonuclear leukocytes, enters the phagolysosome when the cell is activated. This enzyme, by converting the superoxide anion radical into hypochloric acid, protects the cell from an excessive number of reactive oxygen intermediates.

In animals, infected with various plasmid type of Y. pseudotuberculosis, a marked increase in MPO activity in peritoneal exudate cells was detected only in response to the introduction of the H-5013 T^- (48^-) plasmid-free strain to 80.1; 41.9; 3.3 and 34.9%, respectively, on 1, 3, 5 and 7 days pi compared with the control (Figure 1b). Such dynamics of the MPO activity level, with the highest index after 1 day pi, can be explained by the influx of neutrophils containing MPO into the inflammatory focus in the early stages of infection. The number of neutrophils in peritoneal exudate on 1 and 3 days pi with a plasmid-free strain was 64% and 25%, respectively. A slight increase in MPO activity above the control level was noted when infected with H-5015 T^- (82^+: 48^-) strain containing a single pVM 82 plasmid: -5.2; -23.7; 16.1 and 12.8%, respectively, on 1, 3, 5 and 7 days pi. When the animals were infected with Y. pseudotuberculosis H-5013 T^+ (48^+) and H-5015 T^+ (82^+: 48^+) strains containing the virulence plasmid pYV, the MPO activity had negative values throughout the observation period: in response to the H-5013 T^+ (48^+) strain -15,%; -17, on 1, 3-5 days pi and for H-5015 T^+ (82^+: 48^+) strain, respectively -1.3; -23.7 and -19.1%, i.e. below the control level. This indicated a violation of the protective function of phagocytes in response to infection with these plasmid type of Y. pseudotuberculosis. However, it should be noted that when infected with H-5015 T^- (82^+: 48^-) strain containing a single pVM 82 plasmid, positive values of MPO were found on 5 and 7 days pi: 16.1 and 12.8%, respectively, indicating on the protective reaction of cells from an excessive number of reactive oxygen intermediaries in this period.

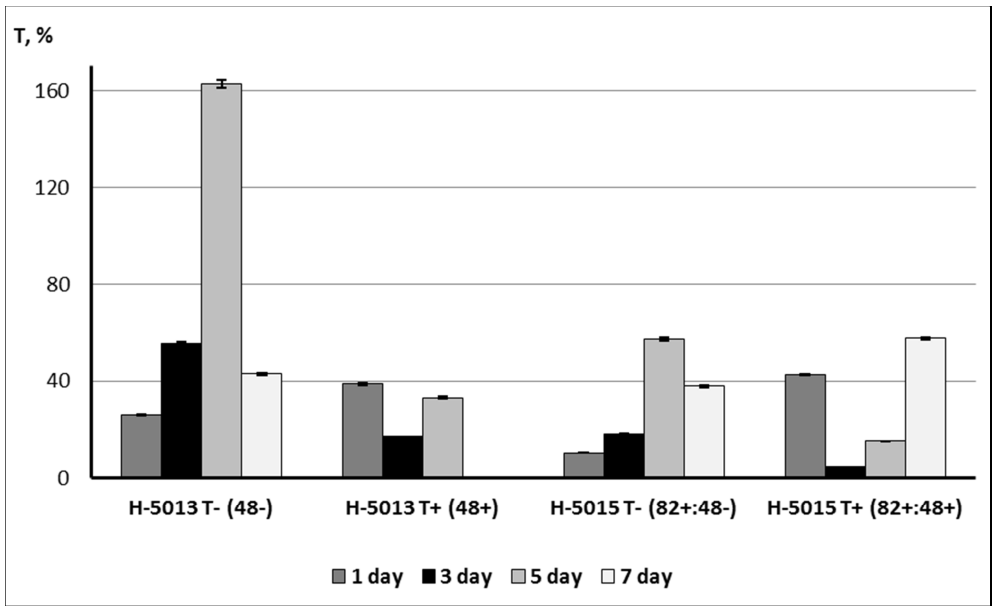
3.4. Production of Nitric Oxide Metabolites

Recent studies have shown that in the phagocytes stimulated by bacteria and proinflammatory cytokines, along with the production of active metabolites of oxygen, the formation of nitric oxide (NO) occurs, the value of which has been proven in the pathogenesis of various infections [21, 22]. The results of our experiments indicated the accumulation of NO in the cells of peritoneal exudate and its excretion into the extracellular space in animals infected with various plasmid types of Y. pseudotuberculosis (Figure 2a, b). The level of NO metabolites in peritoneal exudate cells in response to infection with the H-5013 T^- (48^-) plasmid-free

strain reached 47.6; 57.2; 70.5 and 66.4% and extracellularly 25.5; 55.5; 162.5 and 42.8%, respectively, on 1, 3, 5 and 7 days pi. In the infection caused by H-5015 T^- (82^+: 48^-) strain, containing a single pVM82 plasmid, the NO level was intracellularly to 47.6; 19.0% 46.0 and 80.9%, extracellularly 10.3; 17.7% 8, 57, 3 and 37.9%, respectively, on 1, 3, 5 and 7 days pi. In the infection caused by Y. pseudotuberculosis strains containing the pYV virulence plasmid, a more pronounced nitroxide-forming activity was established, the level of which was higher when infected with the H-5015 T^+ (82^+: 48^+) two-plasmid strain than the H-5013 T^+ (48^+) strain containing only the pYV virulence plasmid. In the first case ((H-5015 T^+ (82^+: 48^+) strain)), the NO metabolites level was intracellularly to 80, 53; 5.1 and 16.8%, extracellularly 42, 6; 4.3 and 14.9% on 1, 3 and 5 days pi. When infected with H-5013 T^+ (48^+) strain with a single pYV plasmid, the indices were intracellularly to 32.1; 57.2 and 61.7%, extracellularly 38.7; 16.7 and 33.1% on 1, 3 and 5 days pi.



(a)



(b)

Figure 2. *Nitric oxide metabolites in peritoneal exudate of animals infected with different Yersinia pseudotuberculosis plasmid types: a) intracellular level; b) extracellular level. The stimulation index T,%.*

The obtained results testify about the features of the response of effector inflammatory cells upon infection cause by various plasmid types of Y. pseudotuberculosis. According to the activity index of the plasmalemma ectoenzyme ATPase, the stimulating effect on the cells of peritoneal exudate (neutrophils, macrophages) was provided only by Y. pseudotuberculosis strains containing the pVM 82 plasmid, especially in combination with the pYV virulence

plasmid. When infected with the Y. pseudotuberculosis plasmid-free strain and strain having a single pYV virulence plasmid, no stimulation of these cells was detected. The Y. pseudotuberculosis plasmid-free strain had an activating effect on the production of lactate dehydrogenase and myeloperoxidase, and also caused a significant increase in the level of nitric oxide metabolites with the release of it into the extracellular space. This indicated the realization of the bactericidal potential of these cells in infected animals. Compared with the plasmid-free strain, when infected with Y. pseudotuberculosis strain containing only the pYV virulence plasmid, the LDH activity was much less pronounced, the MPO activity was negative, and the intracellular accumulation of NO revealed a weak excretion of it into the extracellular space, which allowed to assume the violation of the protective reaction of innate immunity cells.

4. Conclusion

Based on the presented results, a conclusion was made on the variability of the metabolic activity of innate immunity cells when infected with different plasmid types of Y. pseudotuberculosis. In response to infection with the plasmid type of Y. pseudotuberculosis containing two plasmids pYV and pVM 82, in providing the bactericidal potential of phagocytes a primary importance had a production of nitric oxide metabolites, compared with the plasmid type containing a single pVM 82 plasmid. This indicates the participation of a special biological effect associated with pVM 82 plasmid, contained in the Far Eastern strains of the causative agent of epidemic pseudotuberculosis (Far Eastern scarlet-like fever), in providing mainly nitroxide-dependent mechanisms of bactericidal activity of innate immunity cells at this infection.

Abbreviations: AMPase: adenosine monophosphatase; ATPase: adenosine triphosphatase; MDa: megadalton; MPO: myeloperoxidase; LDH: lactate dehydrogenase; NO: nitric oxide; pi: postinfection

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