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Human blood granulocyte degranulation and lysis intensity during interaction with *Yersinia pestis* in the *ex vivo* model of bacteriemia

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Abstract

Introduction. Considering the decisive role of antibacterial strategies of secretory degranulation and NETosis in the prevention of sepsis, it is of interest to study the interaction of *Yersinia pestis* with human blood granulocytes using an *ex vivo* bacteremia model to assess the effectiveness of this antibacterial strategy of the host organism in plague.

Purpose: evaluation of granulocyte degranulation and lysis in human whole blood samples in the presence of live *Y. pestis.*

Materials and methods. Bacteremia was modeled by adding *Y. pestis* EV NIIEG cells grown at 37°C or 28°C to whole blood (with heparin) at a dose of 10^8 mc/mL. Strains *Staphylococcus aureus* ATCC 6538 (209-P) and *Escherichia coli* ATCC 25922 were used in experiments with blood from the same donors as a positive control. The bactericidal effect was determined at different time points during blood incubation at 37°C (for 6 hours) using a microbiological method. Using flow cytometry, immunophenotyping of leukocytes was performed in the blood according to the Lyse/No-Wash protocol to determine the expression of the main leukocyte antigen CD45 and the secretory azurophilic degranulation marker CD63 on the surface of the granulocytes. The intensity of granulocyte lysis was assessed by the decrease in the proportion of these cells in the total leukocyte population.

Results. It has been established that live plague microbes, unlike *E. coli* and *S. aureus*, do not cause the development of azurophilic degranulation in human blood granulocytes and do not induce autolysis (NETosis) of these cells within 6 hours when bacteremia is modeled *ex vivo*.

Conclusion. Information was obtained on the ability of the plague microbe to suppress the extracellular bactericidal mechanisms of granulocytes in the blood of people not vaccinated against plague, which effectively function under conditions of bacteremia against *E. coli* and *S. aureus*. An experimental and methodological basis has been prepared for further research with blood cells from donors vaccinated against plague in order to develop new effective tests for assessing the intensity of acquired cellular anti-plague immunity in humans.

Keywords: *Yersinia pestis, Escherichia coli, Staphylococcus aureus, ex vivo bacteremia model, neutrophil, neutrophil azurophilic degranulations, NETosis, leukocyte elastase, leukocyte immunophenotyping, flow cytometry*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Russian Research Anti-Plague Institute “Microbe” (protocol No. 9, October 21, 2020).

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Оригинальное исследование
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Интенсивность дегрануляции и лизиса гранулоцитов крови человека при взаимодействии с *Yersinia pestis* на модели бактериемии *ex vivo*

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Аннотация

Введение. С учётом решающей роли антибактериальных стратегий секреторной дегрануляции и нетоза в предотвращении сепсиса, представляет интерес изучение взаимодействия *Yersinia pestis* с гранулоцитами крови человека на модели бактериемии *ex vivo* для оценки эффективности этих стратегий при чуме.

Цель работы — оценка дегрануляции и лизиса гранулоцитов в образцах цельной крови человека в присутствии живых *Y. pestis*.

Материалы и методы. Бактериемию моделировали добавлением в цельную кровь (с гепарином) клеток аттенуированного штамма *Y. pestis* EV НИИЭГ, выращенных при 37°С либо 28°С, в дозе 10^8 м.к./мл. Штаммы *Staphylococcus aureus* ATCC 6538 (209-P) и *Escherichia coli* ATCC 25922 использовали в опытах с кровью тех же доноров в качестве положительного контроля. Бактерицидный эффект определяли в различные сроки инкубации крови при 37°С (в течение 6 ч) микробиологическим методом. С помощью проточной цитометрии в крови проводили иммунофенотипирование лейкоцитов по Lyse/No-Wash протоколу для определения экспрессии на поверхности гранулоцитов основного лейкоцитарного антигена CD45 и маркера секреторной азурофильной дегрануляции CD63. Интенсивность лизиса гранулоцитов оценивали по снижению доли этих клеток в суммарной лейкоцитарной популяции.

Результаты. Установлено, что живые клетки чумного микроба, в отличие от *E. coli* и *S. aureus,* не приводят к развитию азурофильной дегрануляции в гранулоцитах крови человека и в течение 6 ч не индуцируют аутолизис (нетоз) этих клеток при моделировании бактериемии *ex vivo*.

Заключение. На модели чумной бактериемии *ex vivo* впервые получена информация, свидетельствующая о том, что в крови не привитых против чумы людей не работают механизмы внеклеточной бактерицидности гранулоцитов, эффективно функционирующие в условиях бактериемии в отношении *E. coli* и *S. aureus.* Подготовлена экспериментально-методическая основа для дальнейших исследований с клетками крови привитых против чумы доноров с целью разработки новых эффективных тестов оценки напряжённости приобретённого клеточного противочумного иммунитета.

Ключевые слова: *Yersinia pestis, Escherichia coli, Staphylococcus aureus, модель бактериемии ex vivo, нейтрофилы, азурофильная дегрануляция нейтрофилов, нетоз, лейкоцитарная эластаза, иммунофенотипирование лейкоцитов, проточная цитометрия*

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Introduction

Primary pneumonic plague, caused by *Yersinia pestis* and transmitted to humans through respiratory droplets from another person or domestic animal, is the most dangerous bacterial infection, in which the pathogen effectively neutralizes the protective mechanisms of the immune system and multiplies intensively in the host organism without inducing the development of an *in vivo* protective inflammatory response for a long time. The asymptomatic character of the development of the infectious process during the first 2 days after aerogenic infection with plague is the key factor determining the high epidemic danger of primary pneumonic plague [1, 2]. The pathogenesis of plague is poorly understood, especially at the stage of bacteremia [3], when *Y. pestis* cells resistant to phagocytosis, proliferating extracellularly in microcapillaries of the liver, lungs and other organs, enter *in vivo* into prolonged contact interaction with peripheral blood leukocytes, including neutrophil granulocytes (NG), responsible for the development of inflammatory reactions [4].

Nevertheless, in experiments on laboratory animals, an important feature of the pathogenetic mechanism of primary pneumonic plague has been established, which consists of the complete suppression of the inflammatory processes at the early stage of infection, such as secretory azurophilic degranulation [5], chemotaxis [6], apoptosis [7] and lysis of NG [8] by effector Yop-proteins (*Yersinia* outer proteins) synthesized by *Y. pestis* at 37°C and secreted by extracellularly multiplying plague microbes into the cytoplasm of cells of the host immune system [4, 9]. At the same time, Yop virulence proteins of *Y. pestis*, on the contrary, trigger the death of macrophages by apoptosis, which disrupts the *in vivo* functioning of the efferocytosis mechanism and inevitably leads to the generalization of the inflammatory process as a result of massive autolysis of peripheral blood NG unable to realize their bactericidal potential. Autolysis of granulocytes, rapidly triggered with a time delay in the whole organism at once, according to the hypothesis of M.T. Silva [10], leads to the release of a huge amount of leukocyte elastase (LE) molecules into the blood plasma, destroying elastin of the lungs and blood vessels, as well as cleaving cell receptors and plasma proteins responsible for the regulation of the coagulation process, which is the trigger for the unexpected and lightning fast development of sepsis in primary pneumonic plague.

To confirm (or refute) this hypothesis, a detailed study of the processes of degranulation and lysis of human and animal blood granulocytes using modern methods of cytological analysis under conditions of *ex vivo* modeling of plague bacteremia is required. As evidenced by the results of the study of COVID-19 pathogenesis associated with a pronounced imbalance in the elastase-inhibitor system [11], in the case of isolation of NG from the peripheral blood of patients, important information about the role of NETosis in immunological defense and in the development of the process of hypercoagulation in sepsis may be lost, because the procedure of centrifugation of blood cells in a density gradient does not allow differentiating from lymphocytes and monocytes the population of activated low-density NG prone to NETosis with increased expression of the marker of azurophilic degranulation CD63 on the cell surface [12].

For a long time, studies on *ex vivo* models of bacteremia were not performed, including in experiments with opportunistic bacteria [13], because it was believed that blood granulocytes realize their powerful bactericidal potential only after migration from the vascular channel to tissues, where they neutralize bacteria by phagocytosis. The situation changed after the discovery of NETosis in 2004 and the studies of V. McDonald et al. [14], who experimentally proved that autolysis of NG (NETosis), preceded and accompanied by secretory azurophilic degranulation of NG with release from LE granules, allows the organism to neutralize microbes at an early stage of bacteremia (during the first 6 h) directly in the blood stream with the help of NET (Neutrophil Extracellular Traps) DNA networks, launched by activated neutrophils into the extracellular space. Moreover, the efficiency of this previously unknown mechanism of immunological defense in terms of preventing bacterial sepsis in infections caused by *Escherichia coli* and *Staphylococcus aureus* was 4 times higher than phagocytosis.

In particularly dangerous infections, the pathogens of which are resistant to digestion in macrophages (e.g., *Y. pseudotuberculosis*), proteases and bactericidal cationic proteins released from NG during secretory degranulation and cytolysis partially or completely neutralize phagocytosis-resistant bacteria, which after such pre-treatment are rapidly absorbed and digested by macrophages [15]. In 2002, for the first time, we published the results of studies in which the flow cytofluorimetric method of assessing the intensity of degranulation was used to record differences in the ability of plague microbes grown at 28°C and 37°C to trigger *ex vivo* the process of secretory degranulation in human whole blood phagocytes. Cells grown at 37°C were characterized by a breakdown of antibacterial response by the indicator of secretory degranulation in the model of plague bacteremia [16]. Many years later, experimental data began to appear in the foreign press, which could explain the mechanism of the phenomenon of the absence of phagocyte degranulation in human blood contaminated with live plague microbes. The studies were carried out *in vivo* in mice [5,8] and *in vitro* with NG previously isolated from human blood [17, 18]. Degranulation was assessed, in contrast to our work, not using supravital staining with acridine orange (AO) dye, but by the degranulation marker CD63. Experiments in an *ex vivo* model of bacteremia were

not performed. The intensity of granulocyte lysis in human blood contaminated with plague microbes or opportunistic microorganisms had not been determined or controlled by flow cytometry before our study was conducted.

The relevance of continuing our earlier studies on the *ex vivo* model of bacteremia using the marker of azurophilic degranulation CD63 and other leukocyte phenotypic markers was determined by the need to develop a cellular test for assessing the intensity of post-vaccination plague immunity in humans, based on quantitative assessment of the damage of peripheral blood neutrophils by specific antigen. In light of modern ideas, the strategy of vaccine development for emergency prophylaxis of plague and other particularly dangerous infections should take into account the ability of immunostimulating drugs to trigger the mechanism of extracellular antibody-dependent cytotoxicity (bactericidality) of NG [19], realized in the blood by interaction with antigen-antibody immune complexes through the processes of secretory azurophilic degranulation and NETosis [20]. Only when specific antibodies to the antigen previously used for immunization of animals appear in the blood, intravenous injection (or addition to the blood) of this antigen triggers in the body (or under *ex vivo* conditions) a protective IgG-mediated anaphylactic reaction associated with secretory degranulation and lysis of peripheral blood NGs [21]. The molecular mechanisms responsible for the transition of the infectious process in primary pneumonic plague from the asymptomatic to the systemic inflammation phase are poorly understood [3, 22], and one of them may be related to the *in vivo* triggering of an IgG-mediated allergic reaction.

The absence of a local protective inflammatory response in a non-immune host organism is observed not only when infected with wild virulent strains of *Y. pestis*, but also in response to non-pigmented strains with a virulence plasmid, such as the vaccine strain of *Y. pestis*, but also in response to non-pigmented strains with a virulence plasmid, which includes the vaccine strain of *Y. pestis* EV NIIEG (Pgm¬-pFra+pCad+pPst+) [1, 23]. Possessing residual virulence, such attenuated strains cause death of laboratory animals only when administered intravenously in doses of more than 10^6 mc, which is used in model experiments to study the mechanisms of immunity and virulence [23].

The aim of the present study was to evaluate degranulation and lysis of granulocytes in human whole blood samples in the presence of live *Y. pestis*.

Materials and methods

The attenuated *Y. pestis* EV NIIEG strain was used in the studies, *S. aureus* strain ATSS 6538 (209-P) and *E. coli* strain ATSS 25922 from the State Collection of Pathogenic Bacteria of the Russian Anti-Plague Institute Microbe of Rospotrebnadzor. Daily cultures of *S. aureus* and *E. coli* were grown on Hottinger's agar (pH 7.2) at 37°C. For *Y. pestis* EV cells, a two-day stationary bacterial culture grown on the same agar at 28°C (*Y.pestis*28) was obtained. An exponential 18-h culture of *Y. pestis* EV with altered antigenic properties was obtained by growing on Hottinger broth (pH 7.2) with aeration at 37°C (*Y.pestis*37) [24]. In sterile phosphate-salt buffer (pH 7.4) with 0.9% NaCl, suspensions of live bacteria with a concentration of 109 mc/ml from cultures of *E. coli*, *S. aureus*, *Y.pestis*28 and *Y.pestis*37 were prepared according to the standard turbidity sample CCA 42-28-59-85P.

The study involved 10 conditionally healthy unvaccinated against plague donors (3 men and 7 women) aged 25–55 years who gave written voluntary informed consent to participate in the study. The study protocol was approved by the Ethical Committee of the Russian Research Anti-Plague Institute Microbe (Protocol No. 9 of 21.10.2020).

Blood from volunteers was drawn into tubes with anticoagulant (heparin) and used for 1–2 h. To simulate bacteremia, 100 μL of the tested billionth bacterial suspension was added to 1 ml of blood containing on average 2 × 106 phagocytes, which corresponded to a concentration of 108 mc/mL of blood or an initial microbial load (number of bacteria : phagocyte) of 50 : 1 on average [25]. With blood from each donor, cell suspensions of *Y. pestis*28 and *Y.pestis*37 were examined simultaneously with cell suspensions of *E. coli* and/or *S. aureus*. Blood samples (1 mL each) with the tested bacteria were placed in the shaker-incubator ES-20 (BioSan) and incubated under stirring for 6 hours. Samples incubated for 6 h without bacteria with 100 μL of sterile phosphate-salt buffer per 1 mL of blood served as a control.

Immunophenotyping of leukocytes in the studied blood samples for flow cytofluorimetric analysis was performed according to the Lyse/No-Wash protocol [26] using labeled mouse monoclonal antibodies to human leukocyte antigens CD45-FITC and CD63-PE (Bekman Coulter). Granulocytes were identified by the degree of their intracellular granularity (side light scattering intensity) and by the expression of total leukocyte antigen (CD45). The relative content of cells positive for the expression of the azurophilic degranulation surface marker CD63 was determined in the granulocyte gate [5, 17]. The results were taken into account for each experimental and control blood sample in dynamics: after 0, 1, 2, 4, 6 h of incubation.

The total number of live bacteria in blood (in plasma and within active phagocytes) was determined by a microbiological method based on osmotic lysis of blood cells in distilled water [27]. To 1 mL of H_2O, 10 μL of blood was added. After 30 s, serial 10-fold dilutions of plasma containing bacteria and blood cell lysis products were prepared in phosphate-salt buffer from water-diluted plasma containing bacteria and

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blood cell lysis products for seeding on dishes with Hottinger's agar (pH 7.2). For each dilution, the number of colony forming units (CFU) of *Staphylococcus aureus* or *Escherichia coli* were counted after one day of growth at 37°C, and *Y. pestis* cells were counted on the 3rd day of growth at 28°C. In relation to the number of CFU after 0 h of incubation, taken as 100%, the survival rate of bacteria of each species in blood (in %) after 1, 2 and 6 h of incubation was estimated to comparatively characterize the development of bactericidal effect under *ex vivo* conditions [25].

The intensity of granulocyte lysis was quantified *ex vivo* by flow cytometry by a decrease in the proportion of these cells in whole peripheral blood samples, as well as by an increase in the relative content of cellular debris in the blood [25].

To assess the light scattering and immunofluorescence intensity of CD-marker-labeled blood leukocytes, a DakoCytomation (Dako) flow cytometer with Summit v.4.3 Built 2445 software was used.

The obtained experimental data were statistically processed using the standard Microsoft Office Excel 2016 software package, Statistica 10.0 (StatSoft Inc.), presenting the results in the form of median (Me) and quartile deviations [Q_1; Q_3] with calculation of the reliability of differences in the studied groups using the Mann–Whitney U-criterion. A value of $p < 0.05$ was considered significant.

Results

When *Y.pestis*37 was added to blood for 6 h, granulocytes lacked changes related to the state of cytoplasmic granules, as well as lysis of these cells under conditions of *ex vivo* modeling of plague bacteremia, while in the blood of the same donors opportunistic bacteria induced intensive degranulation and inevitable lysis of the overwhelming majority of granulocytes in the total population of peripheral blood leukocytes by 6 h of incubation. Antibacterial response of granulocytes according to the studied indicators of degranulation and cytolysis took place in the case of *ex vivo* modeling of bacteremia by *Y.pestis*28 cells. However, in comparison with the reaction to *E. coli* or *S. aureus*, this response was significantly less intense (**Table**).

The registered differences in the studied parameters are clearly illustrated by the example of *Y.pestis*37 and *E. coli* characteristic cytograms presented in **Fig. 1**, where granulocytes are localized by the degree of intracellular granularity (intensity of lateral light scattering) and density of CD45 expression in the ellipse-shaped region R3 at their automatic differentiation from lymphocytes and monocytes in control blood samples. In the presence of *E. coli*, the proportion of granulocytes (cells in the R3 region) decreased after 6 h in the total leukocytic population relative to the control index by 10 times – from 47.1% to 4.8%, and in the presence of *Y.pestis*37 actually did not change, remaining close to the control (43.8%). In blood contaminated with *E. coli*, the proportion of granulocytes decreased sharply during this period due to massive lysis of dead phagocytes, significantly increasing the relative amount of signals from cellular debris registered outside the R1 region. On the cytogram of the experimental blood sample with *Y.pestis*37 cells, the proportion of debris

The results of granulocyte azurophilic degranulation and lysis intensity estimation in an *ex vivo* modeling of bacteremia by live *E. coli, S. aureus* and *Y.pestis*37 in depending of blood incubation time at 37°C, Me [Q_1; Q_3]

Parameter	Blood sample	Duration of incubation, min			
		60	120	240	360
Granulocyte lysis intensity, %	Control	5,2 [3,8; 7,4]	9,1 [8,6; 11,8]	12,2 [10,5; 13,7]	14,6 [12,1; 16,8]
	S. aureus	27 [25,4; 29,6]*	56,0 [51,2; 59,7]*	78 [73,6; 82,5]*	82,0 [76,8; 85,2]*
	E. coli	22,3 [20,7; 24,5]*	38,8 [37,3; 40,5]*	63,6 [56,5; 70,4]*	78,6 [73,4; 84,7]*
	*Y.pestis*28	7,3 [6,2; 9,1]	10,4 [8,7; 13,5]	20,6 [18,6; 21,5]*	19,0 [17,5; 20,8]*
	*Y.pestis*37	6,7 [4,3; 8,6]	8,0 [6,2; 9,5]	9,3 [6,7; 12,8]	10,6 [6,8; 13,0]
Share of granulocytes with $CD63^+$ phenotype, %	Control	10 [9,2; 13,8]	14 [11,4; 16,3]	17 [15,5; 19,7]	18 [16,3; 21,4]
	S. aureus	35 [34,5; 35,7]*	70 [67,0; 72,4]*	83 [74,5; 93,0]*	79 [68,4; 90,2]*
	E. coli	28,3 [25,6; 32,4]*	50,3 [44,6; 55,3]*	70,6 [61,0; 75,7]*	80[72,2; 88,2]*
	*Y.pestis*28	19,7 [17,8; 23,5]*	26,0 [21,1; 30,2]*	32,3 [28,4; 36,6]*	31,2 [22,2; 38,7]*
	*Y.pestis*37	14,2 [11,9; 18,3]	13,4 [10,6; 17,3]	15,1 [13,2; 18,6]	20,8 [16,5; 22,7]

Note. *$p < 0.05$ compared with control.

after 6 h of incubation was, on the contrary, lower than in the control without bacteria.

In contrast to opportunistic bacteria, *Y.pestis*37 cells did not induce *ex vivo* increased expression on the granulocyte surface of the lysosomal protein CD63 (tetraspanin), which is a marker of the development of secretory azurophilic degranulation (**Fig. 2**). In blood samples contaminated with live *E. coli* and *S. aureus* cells, the process of CD63 secretion from granules to the granulocyte surface began after one hour, significantly intensified from the 2nd hour of incubation and preceded the lysis of activated granulocytes under *ex vivo* conditions (Table).

Against the background of the absence of antibacterial response of granulocytes according to the studied indicators of degranulation and leukocytolysis, increased survival of *Y.pestis*37 in human whole blood samples in comparison with *Y.pestis*28 was registered by the microbiological method. The plague microbe grown at the temperature of the host organism began to multiply intensively after 6 h under conditions of *ex vivo* modeling of bacteremia. In the blood of the same donors, opportunistic bacteria were quickly killed under the influence of bactericidal effect of active phagocytes. The survival rate of *E. coli* and *S. aureus* decreased by at least 80% by 6 h from the moment of bacteremia modeling (**Fig. 3**).

Discussion

When conducting the present study by flow cytometry, experimental data were obtained for the first time indicating that the Lyse/No Wash procedure for immunophenotyping of blood leukocytes using fluorochrome-labeled CD markers, which excludes cell losses and the effect of centrifugation on cells [26], allows rapid assessment in human whole blood samples of both the intensity of the secretory degranulation process and granulocyte autolysis triggered by an infectious agent *ex vivo* at the initial stage of bacterial modeling. Because of experiments with *E. coli* and *S. aureus*, new information has been obtained that confirms the importance of rapid development of degranulation and granulocyte autolysis processes in blood for the killing of these bacteria under conditions of bacteremia, as previously established in animal experiments [14], using human cell models. The inability of *Y.pestis*37 cells multiplying intensively in the blood to trigger functional activation of granulocytes by secretory degranulation and cytolysis is important for understanding the causes of asymptomatic development of the infectious process in primary pneumonic plague [10, 22]. The results of microbiological studies obtained simultaneously with the data of cytofluorimetric analysis in an *ex vivo* model may help to explain why plague bacteremia always inevitably leads to sepsis unlike staphylococcal bacteremia.

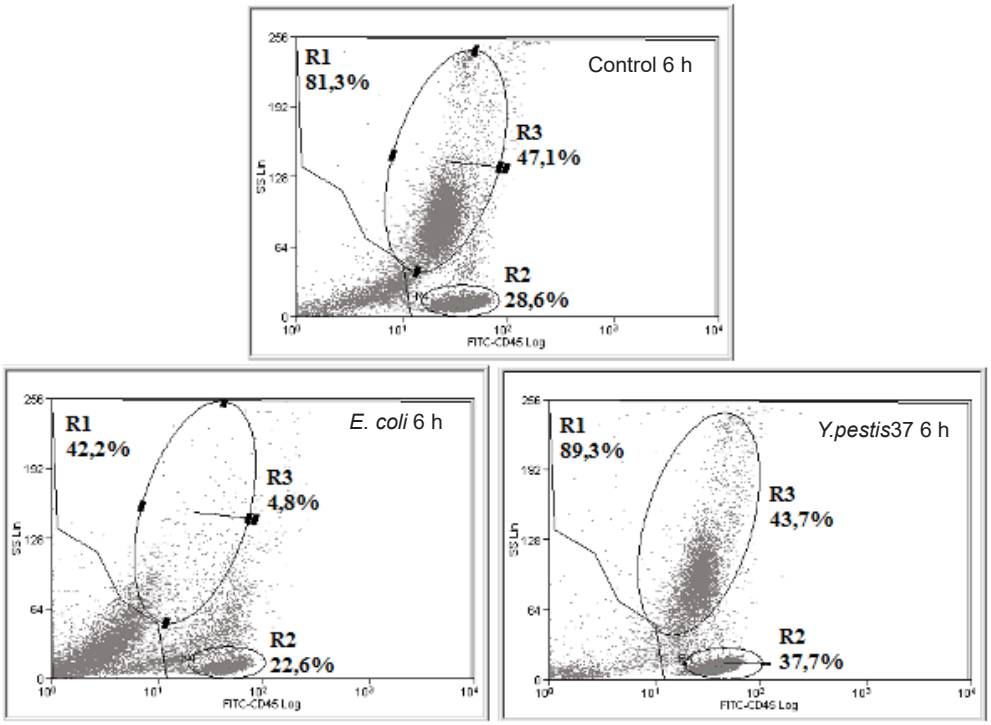

Fig. 1. Cytograms of the blood leukocyte distribution according to the degree of intracellular granularity and the cell surface common leukocyte antigen CD45 expression density at 6 h after the addition of *Y. pestis* and *E. coli* to the blood of the same donor.

Regions R3 and R2 correspond to granulocytes and lymphocytes. Monocytes are localized in the area between the R2 and R3 regions. All intact undamaged leukocytes were counted by the cytometer in the R1 region, beyond which signals from cellular debris, products of the breakdown of leukocytes with a low level of leukocyte antigen expression, accumulated. The proportion of cells in each region is expressed as a percentage of the total number of registered leukocytes. The proportion of debris corresponds to (100 – R1)%.

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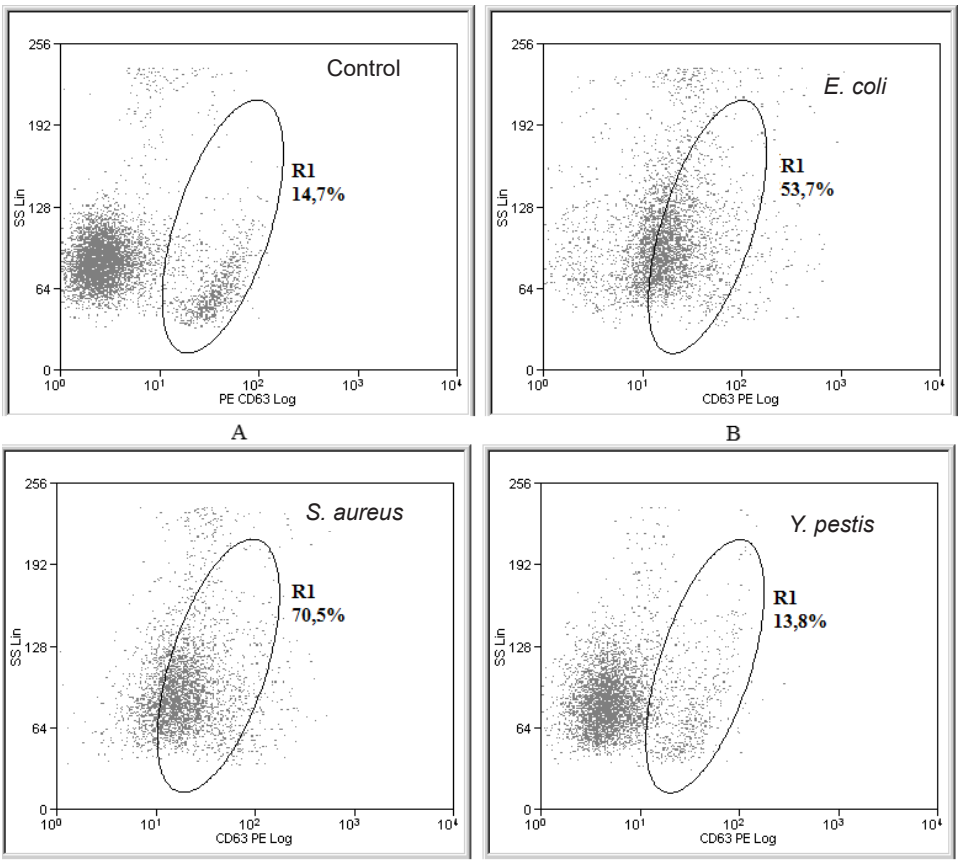

Fig. 2. Relative content of activated cells with CD63⁺ phenotype in the granulocyte gate after 2 h of *ex vivo* interaction with living cells of *Y.pestis*37, *E. coli* and *S. aureus*.

CD63⁺ granulocytes are localized in the R1 region of cytograms; control is the blood after 2 h without bacteria. The proportion of cells in R1 region is expressed as a percentage of the total number of registered granulocytes.

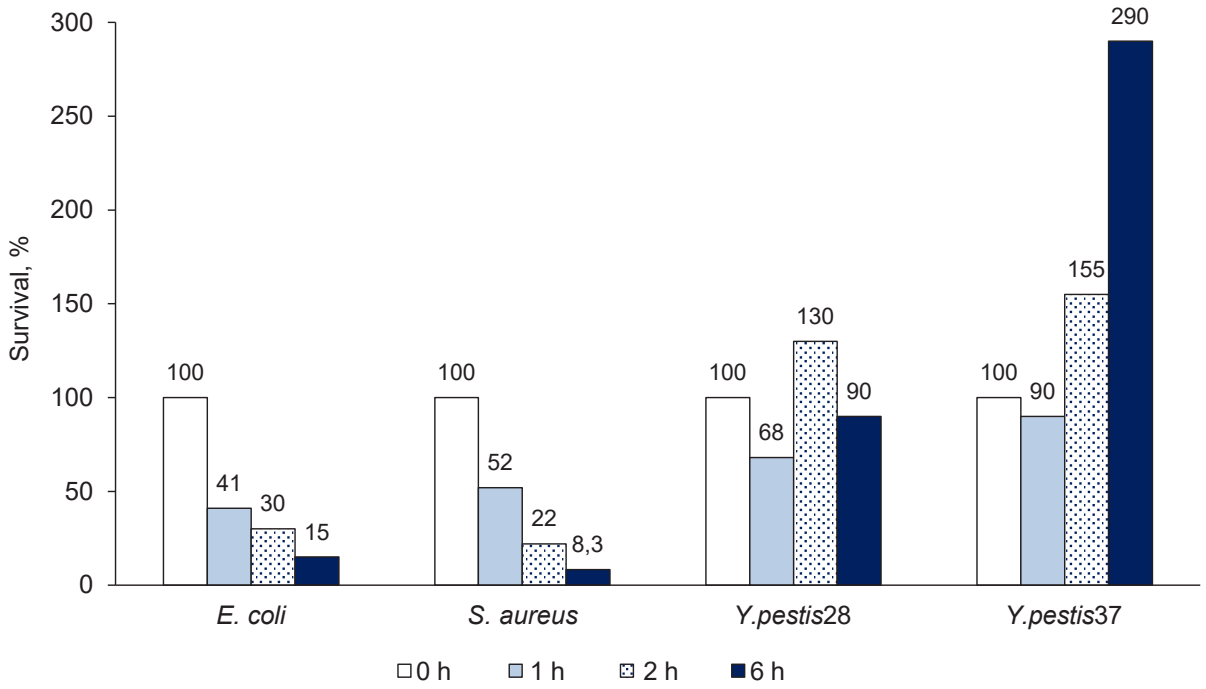

Fig. 3. Increased survival of *Y.pestis*37 in human blood compared to *E. coli, S. aureus* and *Y.pestis*28 in an *ex vivo* bacteremia model.

*Y.pestis*37 cells proliferating in human blood at 37°C did not trigger the secretion of lysosomal protein tetraspanin (CD63) from the primary granules of granulocytes to the cell surface, which is consistent with the results of studies by K.R. Eichelberger et al. conducted *in vivo* on laboratory animals [5] and *in vitro* on the model of NG previously isolated from human blood [17]. In this regard, the fact that CD63 protein is involved in the process of secretion from the same granules of LE [28], which is a generally recognized biochemical marker of the inflammatory process [29] and a key protein of innate antibacterial defense, responsible for rapid selective cleavage of virulence factors of *Yersinia* spp. and other *Enterobacteriaceae* in the focus of infectious inflammation, may be of great importance [30].

To kill bacteria in blood plasma and other biological fluids, this protease forms antibacterial peptides with broad-spectrum antibiotic properties from inactive lysosomal precursor proteins during degranulation [31]. By cleaving histones in the nuclei of activated neutrophils, LE triggers and regulates, together with myeloperoxidase, the process of decondensation of nuclear chromatin during NETosis, in which the autolysis of NG is accompanied by the release into the blood and tissues of the body of DNA nets with the products of NG decay adsorbed in them, which have pronounced bactericidal properties (LE and myeloperoxidase, histones, antibacterial peptides, etc.). [32].

The information presented in the table about the intensity of azurophilic degranulation *ex vivo* by CD63 marker is confirmed by the results of our earlier studies, in which phagocyte degranulation was assessed by supravital staining of cells with AO dye [16] and the viability of granulocytes in blood with *Y. pestis* and *S. aureus* cells was monitored by flow cytometry by identifying and counting the number of dead diploid cells with reduced (less than 2C) DNA content [24, 33]. It is known that blood granulocytes accumulate AO dye in much larger amounts than lymphocytes and monocytes in the form of its positively charged form AOH^+, which is formed and accumulated in the granules of living cells in the process of continuous pumping of H^+ protons through intact membranes of lysosomes [34]. The level of AO accumulation in azurophilic granules depends on the amount of LE molecules in them, and in the process of degranulation, LE is released from the granules into the extracellular space together with AO molecules [35]. In blood contaminated with live *S. aureus* cells, granulocytes lost the initially intense red fluorescence of their granules between 3 and 4 h of incubation. The preservation of intense fluorescence of granulocytes in the analysis of blood samples contaminated with live cells of *Y.pestis*37 indicated the absence of degranulation in phagocytes at the early stage of *ex vivo* modeling of plague bacteremia, the stability of azurophilic granule membranes in peripheral blood granulocytes retaining their viability during 6 h of incubation [16].

In a study by S.C. Dudte et al., in which the degranulation of human NG isolated from blood was evaluated *in vitro* in interaction with *Y. pestis* and *S. aureus* both by CD63 expression and LE expression on the cell surface, only live *Staphylococcus aureus* induced *in vitro* intensive degranulation [18]. Our data obtained in experiments with attenuated strain of *Y. pestis* EV NIIEG on the *ex vivo* model of bacteremia agree with the conclusions of S.C. Dudte et al. However, the model we used was more informative because it allowed us to additionally assess the intensity of leukocytolysis in blood by flow cytometry.

The lower content of cellular debris registered by us in blood with cells of *Y. pestis* cells after 6 h of incubation, compared to control blood samples without bacteria, can be explained by the ability of *Y.pestis*37 to suppress spontaneous apoptosis and lysis of granulocytic cells at the early stage of infection, as known from animal experiments [7, 8, 10].

The reason for the intensive multiplication and rapid spread of *Y. pestis* in the host organism is explained by a change in the structure of its lipopolysaccharide (LPS) when the temperature rises from 28°C to 37°C. It is believed that *Y. pestis* does not induce a protective inflammatory response in the organism at LPS due to the inability of its structurally altered LPS to stimulate TLR4 cells of innate immunity [36]. This explanation does not contradict the results of our studies, since LPS of Gram-negative bacteria is a powerful inducer of secretory degranulation of NG with the release of CD63 and LE on the cell surface and in the extracellular space [37]. However, the proteolytic effect of LE released from phagocyte granules, which determines the ability of this receptor to respond to the presence of LPS *in vivo*, was found to be necessary for TLR4 activation [22]. It is known that tolerance to high doses of endotoxin is formed in mice when the organism is exposed to LPS for a long time (with repeated intravenous administration of low doses over several days). Neutrophils of tolerant animals more effectively neutralized pathogenic bacteria due to the *in vivo* inclusion of an additional mechanism of antibacterial defense (NETosis), which did not function in the organism of intact animals [38]. According to our data, a live plague vaccine had a similar effect on mice, forming intense post-vaccination anti-plague immunity in animals of this species [39].

In the present study, we do not present experimental data on the identification and determination of NG content in blood by their specific phenotypic marker CD16 (Fc_γRIIIb receptor) in order not to complicate its description. Since the proportion of NG in the total population of blood granulocytes in the samples studied by us was more than 90% [25] and corresponded to clinically established normal values of this index [12], the possibility to call the total population of granulocytes

NG when discussing the obtained experimental data is allowed in our study.

The flow cytometry method is known to differentiate IgG-dependent anaphylaxis associated with activation of the secretory function of peripheral blood neutrophils from IgE-dependent anaphylactic reaction that develops as a result of stimulation of mast cells and basophils [40]. It is possible that when functionally active IgG-antibodies to specific antigens of *Y. pestis* appear in the blood, they will activate the mechanism of extracellular antibody-dependent cytotoxicity of NG, which can be registered by flow cytometry on the *ex vivo* model according to the intensity of degranulation and lysis of peripheral blood granulocytes. Such analysis may be more sensitive and informative than in cases when an alternative skin test indicator of neutrophil damage is subjectively assessed in practice in human whole blood samples using a long and laborious method of microscopic analysis.

Conclusion

The study of degranulation processes and lysis of granulocytes in human whole blood samples infected with live *Y. pestis*, helped obtain new information using CD markers and flow cytometry, indicating that the mechanisms of extracellular granulocyte bactericidal activity, which function effectively against *E. coli* and *S. aureus*, do not work at the early stage of bacteremia in the blood of people unvaccinated against plague. In *ex vivo* modeling of bacteremia using the attenuated strain of *Y. pestis* EV NIIEG has created the necessary experimental and methodological basis for further study of the processes of degranulation and lysis of NG in blood samples of people inoculated against plague, for the development of new, more effective tests to assess the intensity of acquired cellular anti-plague immunity.

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