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Pathogenetic Role of *Yersinia pseudotuberculosis* Endotoxin in Hemostasis and Microcirculation Disturbances

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We studied the role of *Y. pseudotuberculosis* endotoxin (LPS) in the pathogenesis of hemostasis and microcirculation disorders. It was found that changes in the hemostasis system after injection of LPS had biphasic character corresponding to the stages of DIC syndrome development. Pathomorphological findings in animals with endotoxemia induced by *Y. pseudotuberculosis* LPS attested to increased permeability and destruction of the vascular endothelium in the microcirculatory bed and focal degenerative and necrotic changes in cells of the target organs (kidney, liver, and lungs) progressing with increasing the duration of the pathological process, which was determined by microcirculation disturbances and development of multiple organ failure.

Key Words: *endotoxin; LPS; pseudotuberculosis; hemostasis; microcirculation*

Undiminishing interest to yersinioses, including pseudotuberculosis, is determined by their wide spreading, difficult laboratory diagnostics, absence of pathognomic symptoms, and poorly studied pathogenetic peculiarities of these pathologies [2,4,11].

Toxins (LPS included) as pathogenic factors of *Yersinia pseudotuberculosis* play an important role in the initiation, development, and outcome of the infectious process and largely determine the main symptoms of the disease [5,6,9]. We previously studied immunogenic and immunomodulating properties of LPS from *Y. pseudotuberculosis* and demonstrated its role in the immunopathogenesis of pseudotuberculosis infection [5,6]; however, hemostasis disturbances caused by LPS remain poorly studied. Endotoxemia in pseudotuberculosis and other bacterial infections serves as a trigger factor of hemostasis disturbances [7]. Hemorrhagic rash and microhematuria are clinical symptoms of these disturbances. Sustained increase in coagulation potential of the blood is observed in 93% patients and in 50-70% patients these changes persist throughout the disease [7].

Here we studied specific features of hemostasis and microcirculation disturbances in endotoxemia induced by *Y. pseudotuberculosis* LPS.

MATERIALS AND METHODS

LPS was isolated from *Y. pseudotuberculosis* IB 598 in a Laboratory of Carbohydrate and Lipid Chemistry, Pacific Institution of Bioorganic Chemistry, Far-Eastern Division of the Russian Academy of Sciences and kindly provided by R. P. Gorshkova, Ph. D. in Chemistry.

Experiments were performed on 315 BALB/c male mice weighing 20-22 g. The mice were obtained from Stolbovaya nursery and maintained in accordance with

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European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The experiments were approved by Biomedical Ethic Committee of Research Institute of Epidemiology and Microbiology, Siberian Branch of the Russian Academy of Medical Science.

Experimental mice received intraperitoneal injection of *Y. pseudotuberculosis* LPS in a dose of 6.25± 0.50 mg/kg (LD_{100}). Control animals intraperitoneally received 0.85% physiological saline. Parameters of the hemostasis system in mice were studied 2, 4, 8, and 24 h after LPS administration. The following parameters were studied: activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen content (FG), and fibrinolytic activity (FA) in the spontaneous euglobulin lysis test. The results were processed statistically using nonparametric Dunn *Q* test at 5% critical significance level. The obtained value (*M*±*m*) is a mean of 5 tests on blood samples pooled from 10-12 animals.

For evaluation of pathomorphological changes in mouse organs, the mice were sacrificed 4, 8, 17, and 24 h after LPS injection. Specimens of the liver, kidney, and lungs were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin; 5-6-μ sections were stained with hematoxylin and eosin and by the method of Shueninov for visualization of fibrin.

RESULTS

Laboratory parameters of hemocoagulation after injection of *Y. pseudotuberculosis* LPS underwent phasic changes. After 2 h, signs of hypercoagulation were observed: coagulation time in APTT and TT tests was shorter ($p<0.05$) than in the control (initial values). After 4 h, the coagulation parameters further deceased ($p<0.05$; Table 1) and a decrease in FA was noted, which manifested in longer time of spontaneous clot lysis and increased content of FG compared to the control values ($p<0.05$; Table 1). At later terms (24 h after LPS administration), hypocoagulation phase developed. By the end of day 1, the clotting time in APTT, PT, and TT tests considerably increased compared to the control (Table 1).

Pathomorphological study of organs from control mice revealed no pathological changes. Microscopic structure of the liver, kidneys, and lungs was normal.

In the liver of experimental group mice, plethora of primarily central veins (terminal hepatic venules) and portal tract vessels with local destructive changes in the vascular walls was observed 4 h after LPS administration (Fig. 1, *a*). Erythrostasis with admixtures of polymorphonuclear leukocytes was found in vascular lumens; some vessels contained aggregates of erythrocytes and fibrin fibers with plasma separation and formation of homogeneous eosinophilic mass. Small solitary necrotic foci primarily centrolobular were seen in the liver parenchyma near blood vessels, sometimes they looked like typical pseudotuberculous foci with karyorrhexis in the central zone. In the kidney, microcirculatory disturbances in the form of plethora and vascular wall destruction, small diapedesis hemorrhages, as well as plethora and serous imbibition of renal glomeruli (some of them were collapsed) were observed 4 h after LPS injection (Fig. 1, *b*). In the lungs, microvessels and most alveoli at the initial stages of LPS-induced pathological process were filled with eosinophilic mass, leukocytes and fibrin fibers were also seen in some vessels and in the perivascular space (Fig. 1, *e*).

At later terms (after 8-17 h), the pathomorphological picture in the liver, kidneys, and lungs was characterized by augmentation of hemocirculatory disturbances, fibrinoid swelling of the vascular walls, and appearance of destructive and inflammatory changes in the parenchyma of the studied organs, primarily in the perivascular space.

TABLE 1. Dynamics of Hemostasis Parameters in Mice Receiving *Y. pseudotuberculosis* LPS

Parameter	Control (initial values)	Time after LPS injection, h			
		2	4	8	24
APTT, sec	47.4±2.6	30.2±2.8*	25.0±2.0*	44.1±3.4	54.5±3.4*
PT, sec	16.8±2.6	13.4±1.1*	11.6±0.9*	17.7±1.8	18.1±0.8
TT, sec	18.6±0.6	12.3±0.7*	12.8±0.4*	16.1±1.4	20.3±1.6
FA, min	310±32	510±31*	533±28*	260±35	270±23
FG, g/liter	4.10±0.25	5.5±0.4*	6.1±0.4*	3.2±0.3	3.1±0.3

Note. *$p<0.05$ compared to the control.

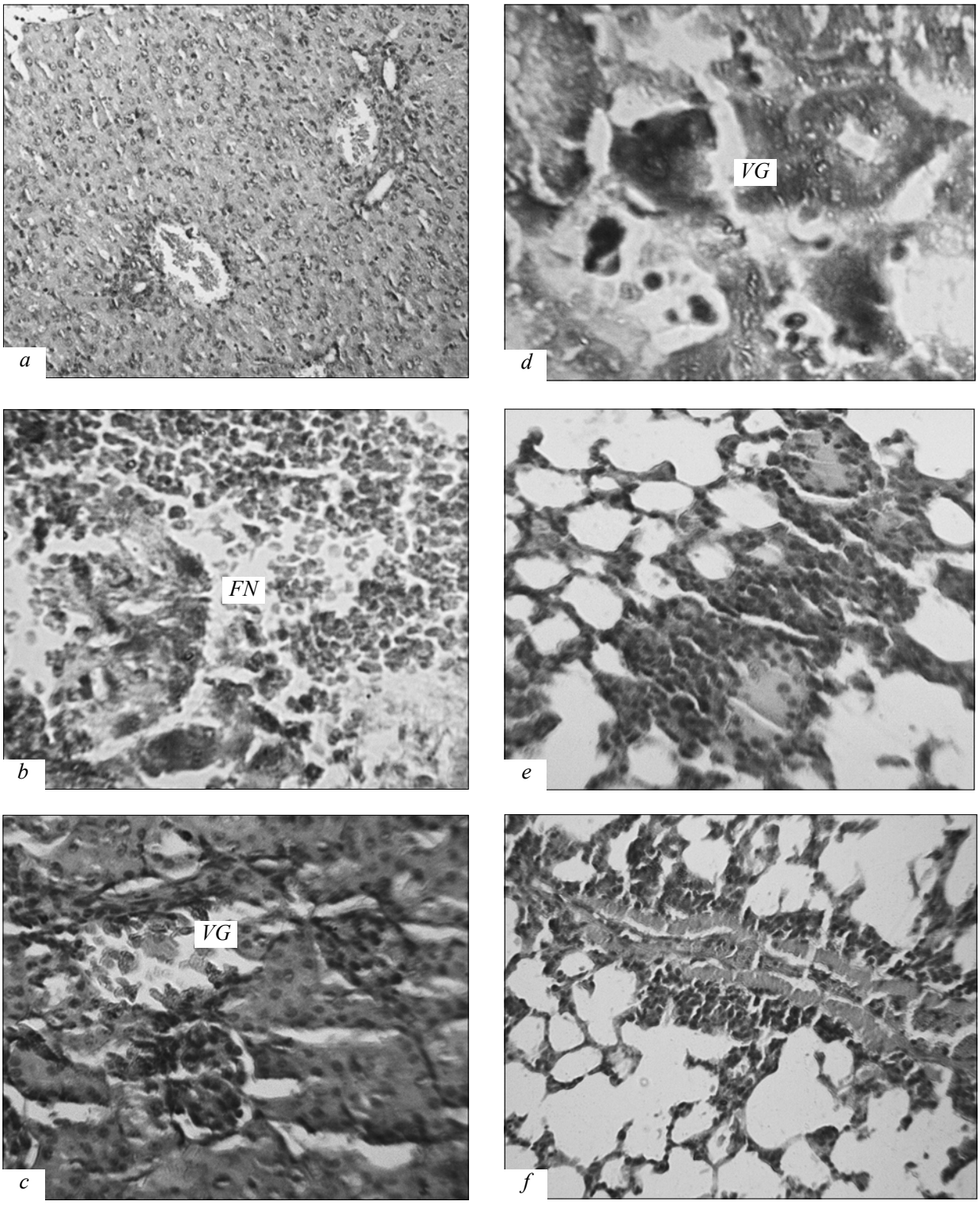

Fig. 1. Pathohistological changes in target organs of mice receiving *Y. pseudotuberculosis* LPS. Liver: *a*) pronounced plethora of portal tract vessels with swelling and local destruction of its wall, 4 h. Hematoxylin and eosin staining, ×100. *b*) focus of fibrinoid necrosis (FN) of a portal tract vessel with perifocal inflammatory reaction, 24 h. Shueninov staining, ×200. Kidney: *c*) destructive and inflammatory changes in the cortical matter, necrosis of epithelium in proximal convoluted tubules, 4 h. Hematoxylin and eosin staining, ×200; *d*) fibrinoid necrosis of vascular glomeruli (VG), 24 h. Shueninov staining, ×200. Lung: *e*) eosinophilic mass, leukocyte aggregates in the vascular lumen, 4 h. Hematoxylin and eosin staining, ×100; *d*) fibrinoid necrosis of vascular wall, perivascular polymorphocellular infiltration, 24 h. Hematoxylin and eosin staining, ×200.

Twenty-four hours after LPS administration, necrotic foci with central fine detritus typical of pseudotuberculosis, numerous small granulomas without central zone karyorrhexis, fibrinoid necrosis of the walls of portal tract vessels, and perivascular inflammatory reaction were found in the liver against the background of vascular changes (Fig. 1, *b*). In the kidneys, signs of necrotic nephrosis with predominant involvement of proximal and distal convoluted tubules in combination with destructive changes in renal glomeruli still persisted. In the cortex, round fibrin-positive conglomerations formed as a result of fibrinoid necrosis of renal glomerular capillaries were found (Fig. 1, *d*). In the lungs, pronounced necrotic changes in the walls of microvessels filled with fibrin-positive conglomerations and perivascular polymorphocellular infiltration were revealed (Fig. 1, *f*).

Our findings are essential for understanding of the role of *Y. pseudotuberculosis* endotoxin (LPS) in hemostasis disturbances, an important aspect of the pathogenesis of pseudotuberculosis infection.

At early terms after administration of LPS in the lethal dose to experimental animals we observed the initial stage of DIC-syndrome with symptoms of hypercoagulation, which was seen from increased blood clotting parameters; these changes peaked 3-4 h after LPS administration. Activation of blood clotting after injection of *Escherichia coli* LPS was reported by other researchers [13-15]. Increased time of clot lysis at the initial stages of endotoxemia also attests to fibrinolysis depression and is considered to be a sign of prethrombosis, which also reflects hypercoagulation or promotes its development [1]. This can be determined by excessive activation of anticoagulation mechanisms responsible for (apart from blockade of procoagulant factors) inhibition of the fibrinolysis system. Analysis of the state of fibrinolytic system revealed a trend to its activation 8 h after LPS injection. Activation of proteolytic systems, *e.g.* fibrinolytic system, is typical of endotoxemia [10,12]. No enhanced FG utilization typical of the initial state of DIC-syndrome was observed 2-4 h after LPS administration. Moreover, the level of FG increased by the 4th hour; similar dynamics was reported by other researchers at early stages after endotoxin administration under experimental conditions [3,13]. The increase in the content of FG, an acute phase protein, can be explained by the fact that its utilization did not increase during the initial period after LPS administration, but after 8-24 h its level decreased due to enhanced consumption of coagulation factors.

The parameters of hemostasis system underwent phasic changes after administration of *Y. pseudotuberculosis* LPS, which was confirmed by pathomorphological analysis of the target organs. For instance, during the first few hours after LPS injection, morphological signs of hypercoagulation (the first stage of DIC syndrome) were intravascular aggregation of platelets and erythrocytes, development of sludge syndrome (irreversible erythrocyte aggregation), and formation of fibrin fibers.

The picture observed after 8 h can be characterized as the second transitional stage of DIC-syndrome with increasing consumption coagulopathy and thrombocytopenia, which manifested in lengthening of the blood clotting time, decrease in the fibrinogen level, and corresponding pathomorphological changes in the target organs (swelling and desquamation of vascular endothelium, plasmatic imbibition, fibrinoid swelling, and fibrinoid necrosis of the vascular walls). After 24 h, lengthening of blood clotting time compared to that during the initial period of endotoxemia and a decrease in fibrinogen level were noted, which corresponded to hypocoagulation stage of DIC syndrome. Pathomorphological analysis at this term revealed primarily microcirculatory disorders in the form of blood cell aggregation and sludge with plasma separation and microthromboses leading to degenerative and necrotic changes in cells of parenchymatous organs. The final stage of DIC-syndrome is the stage of outcomes and complications. This stage is characterized by the development of multiple organ pathology, most commonly shock lung, liver, or kidney [8]. By the end of the study, multiple necrotic foci appeared in organs of experimental animals receiving lethal dose of LPS, which led to their death.

Thus, administration of *Y. pseudotuberculosis* LPS to mice leads to the development of hemostasis disturbances corresponding to the stage of DIC-syndrome. Pathomorphological manifestations of LPS-induced endotoxemia were erythrocyte sludge in microvessels, hemocirculatory disturbances, microthrombosis, increased permeability and destruction of vascular endothelium, and degenerative and necrotic changes in cells of parenchymatous organs, which progressed with increasing the duration of the pathological process. These changes were determined by microcirculatory disorders and development of secondary circulation-related changes in organs, but primary disturbances caused by direct toxic effects of LPS can also play a role.

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