Cell Microvesicles during Experimental Endotoxemia

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 142, No. 11, pp. 517-520, November, 2006
Original article submitted June 28, 2005

The dynamics of microvesicle formation in arterial blood in generalized Schwartzman phenomenon was studied. Successive (with 24-h interval) intravenous injections of endotoxin to rabbits in a dose of 1 mg/kg and 3 mg/kg caused an increase in the content of microvesicles in the blood, some of them containing ecto-5'-nucleotidase. Biphasic changes in arterial blood clotting time and erythrocyte hemolysis were observed.

Key Words: *endotoxin; microvesicles; rabbit; blood clotting; hemostasis*

The lipopolysaccharide endotoxin (LPSE) is recognized by TLR4 receptor (toll-like receptor 4) of the innate immune system. Activation of macrophagic TLR4 by endotoxin lipid A triggers biosynthesis of inflammatory mediators, activates production of co-stimulatory molecules of adaptive immune response. Lipid A stimulates the production of the blood clotting system tissue factor in mononuclear and endothelial cells [13]. This response promotes elimination of local infection. On the other hand, in systemic endotoxemia (generalized Schwartzman phenomenon is its classical model [5]) numerous inflammatory mediators and blood clotting factors promote injuries to capillary walls and are involved in the development of septic shock, associated with disseminated intravascular coagulation (DIC) and polyorgan failure [12]. The expression of tissue factor (primary physiological initiator of blood clotting in bacterial sepsis) is induced in activated monocytes and damaged endothelium within the vascular system [10]. However, not only cells, but also subcellular structures, *e.g.* microvesicles (MV) released during cell activation and apoptosis, serve as the source of tissue factor and site for assembly of the blood clotting tenase and prothrombinase complexes [7]. Observations on volunteers receiving endotoxin injections showed an increase in procoagulant activity of tissue factor associated with non-platelet MV 3-4 h after infusion, which returned to normal after 8 h [4]. However, low doses of LPSE were used in this study.

We studied the dynamics of MV formation in the arterial blood starting from the first minutes of generalized Schwartzmnan phenomenon.

MATERIALS AND METHODS

Male chinchilla rabbits (2.5-3.5 kg; n=15) received two intravenous injections of *E. coli* endotoxin (Kazan Institute of Epidemiology and Microbiology): 1 mg/kg and (after 24 h) 3 mg/kg. Controls (n=12) were injected with the same volume of 0.85% NaCl. Blood for analysis was collected from the femoral artery with Teflon cannulas. Clotting time of fresh arterial blood was evaluated in Bazaron's apparatus [3]. Plasma was separated by 15-min centrifugation of heparin-treated blood at 1500g. The number of MV was evaluated by two methods: by activity of ecto-5'-nucleotidase (CD73) [2] and by light scattering in a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest software. The blood cell-free fraction was filtered through Synpor VUFS filter with pores of ~0.2 μ. The concentration of extracellular hemoglobin was evaluated by absorption at 540 nm in blood plasma repeatedly centrifuged for 20 min at 2670g in Spekol 20 spectrophotometer microcuvettes [11]. The parameters were

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registered 5, 15, 30, 60, 120, 180, 240 min and 24 h after a single injection of LPSE and then 15, 30, and 120 min after repeated injection. Statistical significance of differences was evaluated using Student's *t* test.

RESULTS

Injection of LPSE to rabbits was associated with the development of fever reaction, body temperature rose from 38.3±0.1 to 39.4±0.3°C ($p<0.001$) over 24 h.

Total quantity of MV evaluated by flow cytometry started to increase significantly from minute 15 after LPSE injection until the end of observation (Fig. 1).

Activity of ecto-5'-nucleotidase significantly increased by the 30th minute after LPSE injection; its subsequent changes were wave-like, with a new more pronounced increase after repeated injection of LPSE (Fig. 2).

Coagulation time demonstrated biphasic changes: significant short-term acceleration of clotting by min 60 was followed by its deceleration after 2, 3, 4, and 24 h, which continued after repeated injection of LPSE (Fig. 1). In 3 experimental animals blood did not clot for more than 60 min as soon as 15 min after repeated injection of LPSE.

The level of plasma hemoglobin increased significantly 30 min after the first injection of LPSE, decreased by the 24th hour postinjection, more sharply increased after the second injection, and remained elevated until the end of the experiment (Fig. 2).

The size of MV was evaluated by blood filtration through 0.2-μ pore filters. Activity of ecto-5'-nucleotidase was 170.7±7.3 ncat/liter before filtration and 19.5±4.6 ncat/liter after it (11.4%), MV number determined by flow cytometry was 123,810±11,706/μl and 17,020±972/μl (13.7%), respectively (Fig. 3). Hence, 86-88% MV were >0.2 μ, which is in line with previous data [2,7].

The initial events in LPSE interactions with the cells suggest the presence of several participants. LPS-binding protein ensures transport to the myeloid cell membrane-bound CD14. Subsequent re-

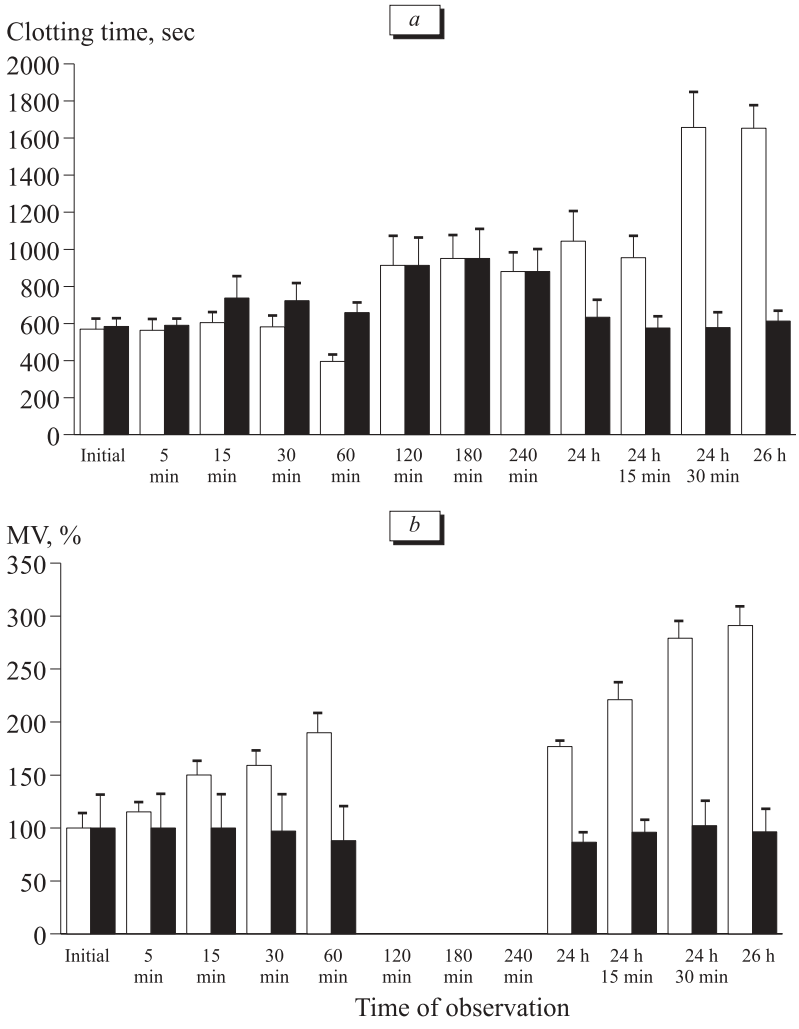

Fig. 1. Time course of blood clotting time (*a*) and microvesiculation level (*b*) as shown by flow cytometry in experimental endotoxemia. *a*) light bars: LPSE clotting time; dark bars: 0.85% NaCl clotting time; *b*) light bars: MV-LPSE; dark bars: MV-0.85% NaCl.

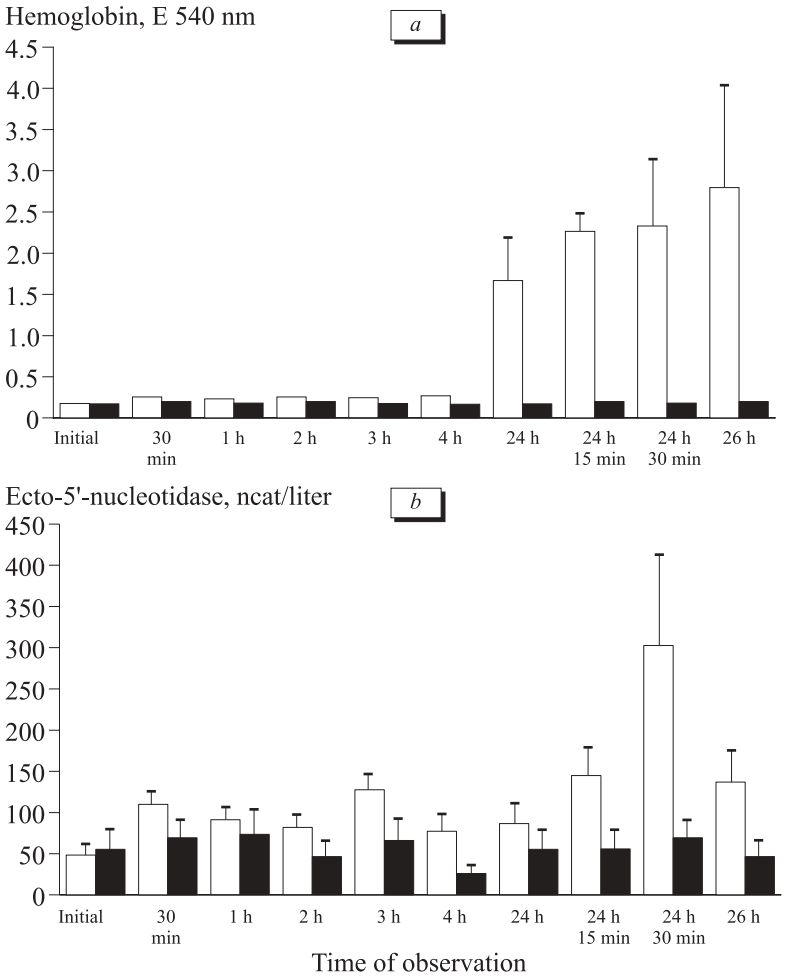

Fig. 2. Dynamics of plasma hemoglobin (*a*) and activity of ecto-5'-nucleotidase (*b*) in experimental endotoxemia. Light bars: endotoxin; dark bars: 0.85% NaCl.

cognition of lipid A or CD14-lipid A-TLR4 complex is the earliest known event in signal transduction leading to the production of cytokines and tissue factor [13]. The detected increase in the number of MV 15 min after injection of LPSE indicates rapid initiation of Ca^{2+}-dependent remodeling of cell membranes, resulting in expression of procoagulant phosphatidylserine and MV release. In addition, the signal from TLR4 triggers the alternative pathway of complement activation. Significant increase in plasma hemoglobin level observed starting from the 30th minute of endotoxemia and then over the entire experiment indicates permanent hemolysis. The induction period of complement-dependent hemolysis is about 5 min [1]. Hence, we cannot rule out participation of complement in stimulation of MV formation, because C5b-9 cytotoxic complex is known as one of the most potent factors of microvesiculation [8].

The population of MV is heterogeneous by its lipid and protein composition, which is explained by functional characteristics of the cells from which they originate. In our experiments, this heterogeneity manifested in different dynamics of the total number of MV (according to flow cytometry results) and of MV population containing ecto-5'-nucleotidase. Due to the glycosyl-phosphatidylinositol "anchor", ecto-5'-nucleotidase is located near cholesterol- and glycolipid-rich membrane subdomains, so called lipid rafts [6]. According to our data, MV populations containing ecto-5'-nucleotidase are mobilized for a shorter period after injection of LPSE (Fig. 2). Some MV seems to be carriers of lipid rafts (membrane mirodomains involved in assembly of specialized proteins, including the blood clotting tissue factor) [9].

The increase in MV level after the first injection of LPSE was paralleled by acceleration of blood clotting, while further increase in their number after repeated LPSE injection corresponded to inhibition of clotting, presumably as a result of DIC.

Hence, LPSE induced vesiculation of vascular cells as early as 15 min after injection; this process continued and augmented over the course of generalized Schwartzman's phenomenon. The resultant MV is a component in the pathogenesis of the developing DIC syndrome. Some MV carry lipid rafts (membrane microdomains involved in the assembly

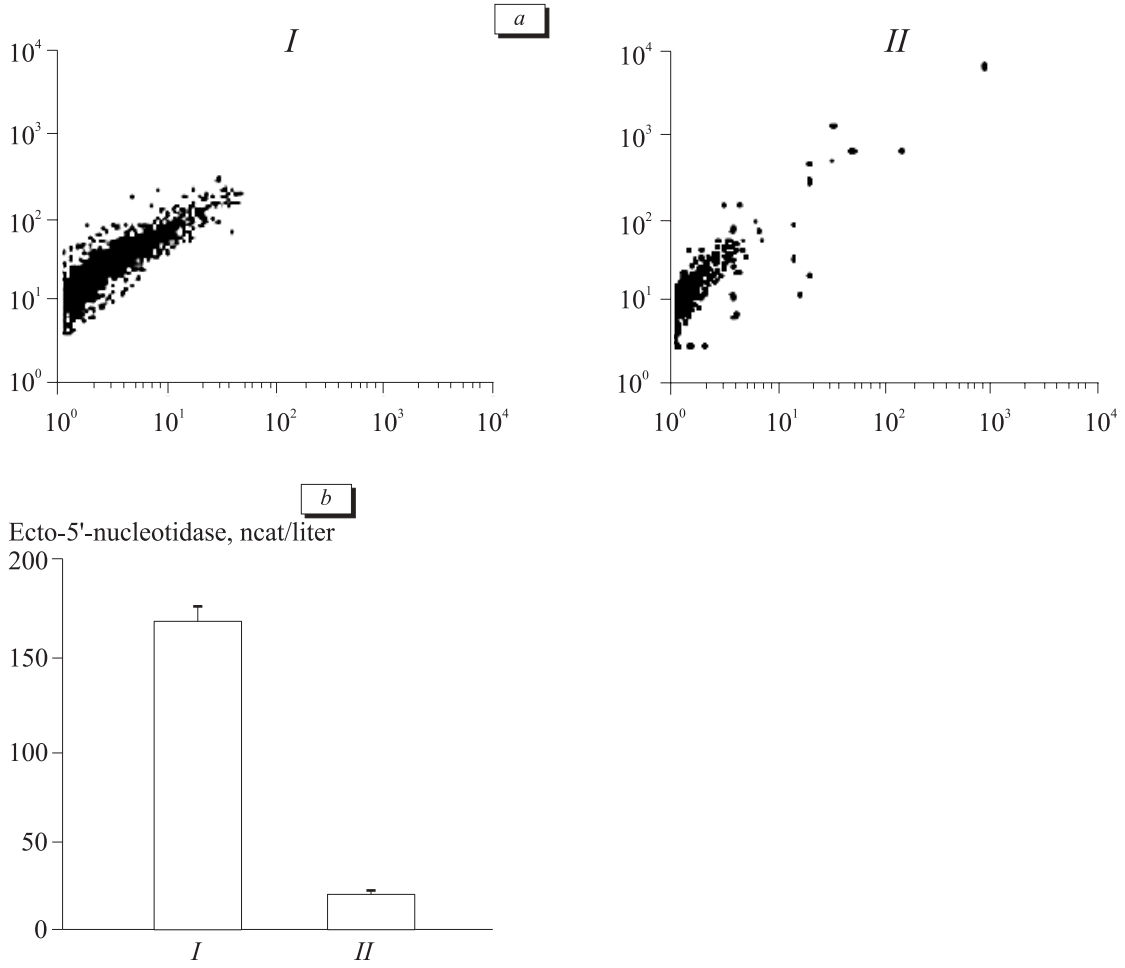

Fig. 3. Evaluation of MV size by filtration. *a*) flow cytometry of blood plasma before (*I*) and after filtration (*II*); *b*) level of ecto-5'-nucleotidase activity.

of specialized proteins, according to our data, ecto-5'-nucleotidase).

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