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DNA Damage in Human Mononuclear Cells Induced by Bacterial Endotoxin

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Damage to nuclear DNA in human peripheral blood mononuclear cells was studied after *in vitro* treatment with bacterial endotoxin by alkaline comet assay. It was found that LPS induced DNA damage as soon as over the first 30 min of incubation, while by the 4th hour of incubation DNA damage was found in more than 95% cells. Exogenous superoxide dismutase completely protected DNA, which suggests that superoxide radical is the primary extracellular damaging agent. Polyphenol antioxidant (water-soluble lignin) and specific NADPH oxidase inhibitor (diphenyleneiodonium chloride) also produced a protective effect. Our results show that LPS-activated mononuclear cells can be used *ex vivo* as a convenient and adequate experimental system for evaluation of the efficiency of various substances in protection of lymphocyte DNA from the damaging effect of reactive oxygen species of LPS-stimulated monocytes.

Key Words: *lipopolysaccharide; DNA damage; mononuclear cells; DNA comet assay*

Bacterial endotoxin (LPS) is a classical inductor of gram-negative septic shock. After entering the blood flow, LPS binds to phagocytizing cells via the specific receptor complex. Activation of phagocytes is manifested in the synthesis of proinflammatory cytokines and reactive oxygen species (ROS) playing a role in the pathogenesis of endotoxic shock [15].

Recent studies showed that high mortality rate in sepsis is associated with extensive death of effector immune cells [9].

ROS (H_2O_2, O_2^-, and OH^-) of activated phagocytes serve as the major cause of lymphocyte death during sepsis [8].

DNA damage is a biological indicator of DNA oxidative stress (*i.e.*, cell death). Previous experiments showed that ROS of spontaneously activated monocytes cause death of NK cells [7]. Other authors reported that ROS of phorbol myristate acetate-activated mononuclear cells (MNC) have a damaging effect on lymphocyte DNA [6].

Here we studied *in vitro* the degree of LPS-induced damage to nuclear DNA in human peripheral blood MNC.

MATERIALS AND METHODS

Experiments were performed with sodium sarcosinate, Tris-(hydroxymethyl)-aminomethane, low melting point agarose, Triton X-100, LPS (serotype O127:B8), bovine liver superoxide dismutase (SOD), and diphenyleneiodonium chloride (DPI, Sigma Chem. Co.). Monocytes and lymphocytes were isolated from the peripheral venous or capillary blood of healthy nonsmoking donors by centrifugation in Ficoll-verografin [2]. The cells were suspended in

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RPMI-1640 culture medium (1×10^6 cells/ml). Cell viability in the test with trypan blue staining was not less than 99%.

Integrity of MNC genome was evaluated in the alkaline comet assay [14] with some modifications [12]. An aliquot of MNC (50-100 μl) was incubated with LPS (1 μg/ml) and/or corresponding substances (100 U/ml SOD; polyphenol antioxidant water-soluble lignin, 5 μg/ml; and DPI, 20 μM) in microcentrifuge tubes. An equivalent volume of 1% low melting point agarose (37°C) was added to the reaction mixture after incubation. This mixture was layered on the previously prepared agarose layer using a slide. Low melting point agarose (0.5%) was layered on agarose after solidification. The slides were put in a lysing and denaturing solution and subjected to electrophoresis. The samples were examined under a LUMAM I-3 fluorescence microscope (LOMO). The images were photographed with a Nikon CoolPix 995 digital camera, transferred to a computer, and processed using special image recording and analysis software (calculation algorithm for standard parameters of comets) [5]. The percentage of DNA in the comet tail was estimated [3].

Three slides were used for each experimental point, 30-40 cells per slide were photographed. The results were analyzed by Student's *t* test ($p<0.05$).

RESULTS

DNA damage was induced over the first 30 min of MNC incubation with LPS. The average percentage of tail DNA was 15.4±1.8% (5.59±1.53 *vs*. 0.99±0.15 in the control, $p<0.05$). The average percentage of tail DNA progressively increased during further incubation and reached 29.04±4.04% by the 4th hour (Fig. 1). We analyzed the histograms for cell distribution in dependence on the percentage of tail DNA. The ratio of cells with DNA damage of 15% or more was 30% after 30-min incubation. This parameter increased to 80% by the 4th hour of incubation. The ratio of normal cells decreased from 25 to 5% (Fig. 2, *a*, *b*). MNC comprise lymphocytes and monocytes. Under normal conditions, the cell ratio corresponds to 70-95 and 5-30%, respectively [1]. Hence, DNA damage occurs at least in lymphocytes.

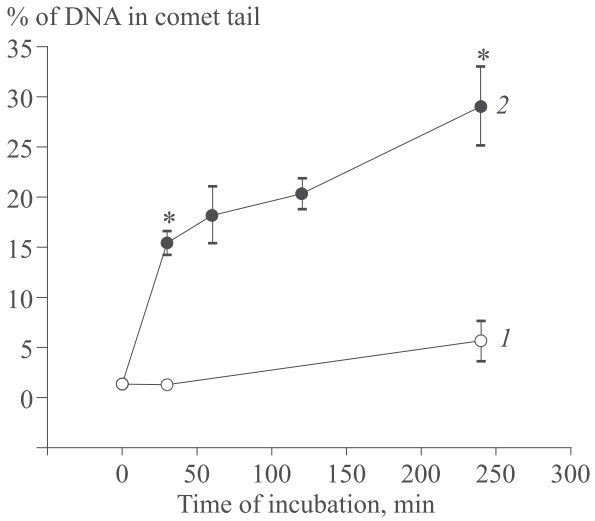

Fig. 1. Dependence of DNA damage in venous blood MNC on the time of incubation with LPS. Control (*1*); in the presence of LPS (n=3, *2*). *$p<0.05$ compared to the control.

The formation of a LPS—CD14—TLR4 complex is one of the mechanisms for ROS synthesis in monocytes induced by stimulation of these cells with LSP. The complex induces self-assembly of

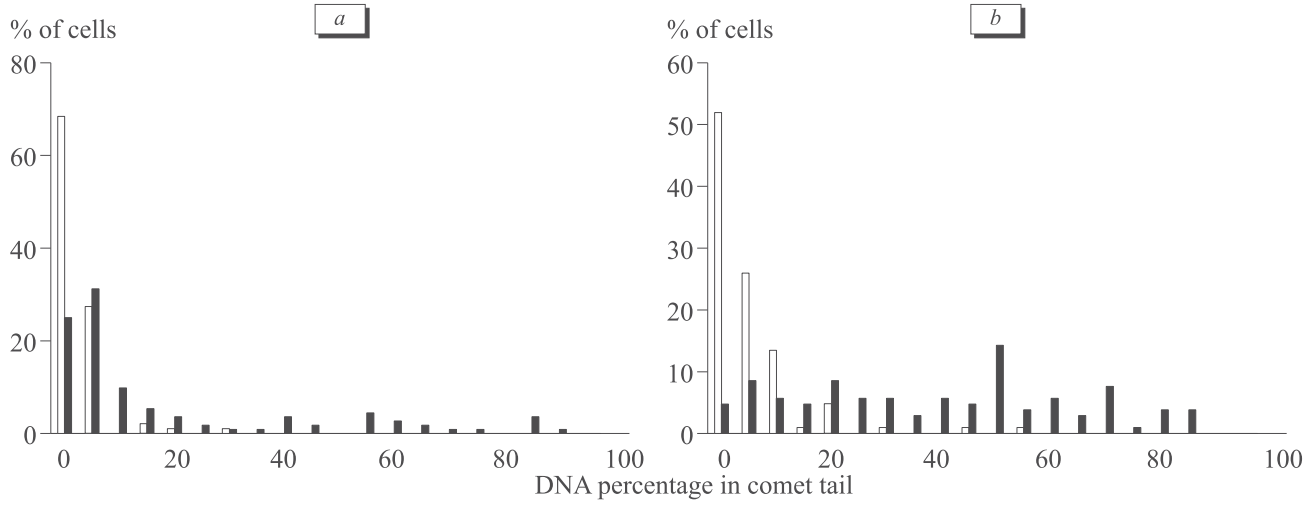

Fig. 2. Histogram for cell distribution in dependence on DNA percentage in the comet tail after incubation for 30 min (*a*) and 4 h (*b*). Venous blood MNC (light bars, control; dark bars, in the presence of LPS).

NADPH oxidase from membrane and cytosolic components. This enzyme catalyzes reduction of molecular oxygen with the formation of superoxide anion radicals (O_2^-) [11]. For evaluation of the role of exogenous ROS (*e.g.*, O_2^-) in DNA damage, MNC were incubated with LPS in the presence of a specific NADPH oxidase inhibitor DPI [10], SOD (enzyme antioxidant catalyzing O_2^- dismutation with the formation of H_2O_2 and molecular oxygen), and natural polyphenol antioxidant (water-soluble highly polymeric compound obtained by partial degradation and carboxylation of lignin) [13]. SOD and polyphenols cannot permeate cell membrane because of high molecular weight and charge. These reagents produce a strong protective effect: DPI, by 80% (1.96±0.38, $p<0.05$); polyphenols, by not less than 70% (2.34±0.53, $p<0.05$); and SOD, by 98% (1.07±0.25, $p<0.05$) compared to the control (0.99±0.15).

Thus, our experiments demonstrated LPS-induced DNA damage in MNC and effective protective effect of SOD, which indicates that exogenous superoxide radical serves as the primary damaging agent. Other authors reported that SOD exhibited no protective activity [6,7]. However, catalase that degrades H_2O_2 with the formation of water and molecular oxygen has a strong protective effect. These data show that H_2O_2 is a primary damaging agent. This discrepancy is probably associated with the fact that we used another inductor for monocyte stimulation (LPS). Experiments with human MNC showed that bacterial endotoxin induces DNA damage at least in lymphocytes. The exogenous superoxide anion radical synthesized by monocyte NADPH oxidase serves as a primary damaging agent. Our study with antioxidants showed that the mixture of LPS-activated MNC can be used *ex vivo* as a convenient and adequate experimental system for evaluation of the effectiveness of various substances in protection of lymphocyte DNA from the damaging effect of ROS of LPS-stimulated monocytes.

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