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Production of Heat Shock Proteins, Cytokines, and Nitric Oxide in Toxic Stress

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Abstract—Expression of heat shock proteins Hsp27, Hsp90, and Hsp70 and production of tumor necrosis factors (TNF-α, TNF-β), interferon-γ (IFN-γ), interleukin-2, -3, -6, and nitric oxide (NO) were studied under conditions of acute and chronic intoxication of animals with lipopolysaccharides. Injection of endotoxin increased expression of heat shock proteins Hsp70 and Hsp90-α in mouse cells. Acute toxic stress also provoked a sharp increase in the production of TNF-α, TNF-β, and NO in mouse cells. The production of other cytokines (interleukins and IFN-γ) was changed insignificantly. In the model of chronic toxic stress, changes in the production of Hsp70, Hsp90, TNF, and NO were followed during 11 days after the beginning of the toxin injections. The expression of Hsp70 and Hsp90 in acute stress was significantly higher than at the final stage of the chronic exposure. The changes in the TNF and NO productions, on one hand, and the production of heat shock proteins, on the other hand, were synchronous. The findings indicate that repeated injections of increasing endotoxin doses result in a decreased ability of the body cells to respond to stress by overproduction of heat shock proteins, TNF, and NO.

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Key words: toxic stress, heat shock protein production, tumor necrosis factor, interferon-γ, interleukins, nitric oxide

Studies on the body's adaptation mechanisms to toxic agents are important not only for fundamental biology but also for clinical medicine. In the present work, toxic stress was modeled by injection of lipopolysaccharides (LPS) isolated from outer walls of gram-negative bacteria. Endotoxic shock caused by LPS is usually manifested by elevation of body temperature, disorders in the system of hypophysis–thyroid gland, dysfunctions of liver and adrenals, and can have irreversible after-effects, up to the death of the animal. Some molecular and cellular mechanisms are known which are associated with the LPS-initiated cascade of reactions based on the innate antimicrobial resistance of mammals. LPS are transferred by the LPS-binding serum protein (LBP) and bound to the CD14 and TLR4 receptors, which are present on the membranes of neutrophils, monocytes, B-lymphocytes, and macrophages [1]. The LPS-activated cells produce bioactive molecules, such as tumor necrosis factor α (TNF-α), some interleukins, proteases, and nitric oxide [2, 3]. The overproduction of proinflammatory cytokines and reactive oxygen species results in intoxication of the body leading to septic shock that accompanies the application of LPS.

There are indirect indications on involvement of heat shock proteins in the cell response to endotoxin. Thus, LPS were found to catalyze synthesis of TNF-α induced by heat shock protein Hsp70 [4]. Stimulation with LPS was recently shown to result in formations of clusters of Hsp70 and Hsp90 proteins with the TLR4 receptor, which is known to be one of two main LPS-binding receptors [5]; there are also data that LPS activate synthesis of Hsp70 [6].

Considering the bulk of recent experimental data, it was reasonable to suggest that not only Hsp70 but also

Abbreviations: FCS) fetal calf serum; Hsp) heat shock proteins; IFN-γ) interferon-γ; IL) interleukin; LPS) lipopolysaccharides; LBP) LPS-binding serum protein; TNF-α) tumor necrosis factor α.

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heat shock proteins from other families produced under the influence of other damaging factors, such as high temperature, ionizing and non-ionizing radiation, neurotoxins, and heavy metals, could be also involved in the body's defense against endotoxic shock. The protective mechanism of heat shock proteins is based on their chaperone activity, i.e., the ability to prevent degradation and polypeptide adhesion of functional proteins. Heat shock proteins are known to execute motor functions in translocation of badly folded proteins, facilitate protein folding, transfer proteins between intracellular components, promote formation of protein multimers, and all these processes are directed for "repair" of the cell protein component. Heat shock proteins were recently shown to play a key role in the immune response of the body to viral, microbial, and tumor antigens [7, 8].

In the present work the role of heat shock proteins from different families (Hsp70, Hsp27, and Hsp90), some cytokines, and nitric oxide was studied in the protective reaction of the cells and whole body against toxic stress. Models of acute and chronic stress caused by injection of LPS were used. One of the main purposes of the work was to study changes in the body's response to the toxin during long-term intoxication. Results of such investigation can be important for development of a new strategy in treatment of severe inflammation and sepsis.

MATERIALS AND METHODS

Animals and models of toxic stress. Male mice of the NMRI strain with body weight of 25-30 g were used. Acute toxic stress was induced by a single intraperitoneal injection of 250 μg LPS from *Escherichia coli* (026.B6 serotype; Sigma, USA) per 100 g body weight. The chronic exposure consisted of daily injections of LPS for 11 days, elevating the LPS dose every 2 days, the first dose being 25 μg and the last dose 250 μg/100 g body weight. Such a model of chronic toxic stress was used earlier [9, 10]. A gradual increase in the dose of the injected toxin is necessary for modeling a real picture of chronic inflammation when bacteria are progressively multiplying in the body.

Three groups of mice were used in all experiments: intact, injected with saline, and injected with LPS. Altogether 48 mice were used in the experiment, 12 in the acute stress model and 36 in the model of chronic stress. None of the LPS-injected animals died before the beginning of the experiments. Animals injected intraperitoneally with saline were used as control.

Culture of cells and determination of their functional activity. *Isolation of lymphocytes.* Animals were sacrificed by cervical dislocation. All subsequent procedures were performed under sterile conditions. The spleen was homogenized in a glass homogenizer, and the cells were precipitated by centrifugation at 1500 rpm for 5 min. After selective hemolysis of the erythrocytes with isotonic solution of ammonia chloride, the lymphocytes were washed thrice in large volumes of DMEM medium (Sigma), and the heterogeneous population of lymphocytes was fractionated by positive selection [11]. To do this, the lymphocyte suspension was incubated for 1 h in plastic Petri dishes (100 × 15 mm; Costar, USA) with rabbit affinity-purified antibodies to mouse IgG (200 μg/ml) preapplied onto their surface from the calculation of 10^6 cells per cm^2 of the plastic. The nonadhered cells (95% of them were T-lymphocytes) were carefully resuspended and decanted into sterile tubes. The adhered antigen-positive cells were mainly B-lymphocytes and phagocytes. The resulting cell populations enriched with T- and B-lymphocytes were washed, counted, and diluted to the concentration of $2.5 \cdot 10^6$ cell/ml in RPMI 1640 medium (Sigma) supplemented with 0.02% gentamycin, 1% L-glutamine, $5 \cdot 10^{-5}$ M β-mercaptoethanol (Sigma), and 10% fetal calf serum (FCS) (Sigma).

Isolation of macrophages. Macrophages were isolated from peritoneal exudate of the mice. The peritoneal cells were precipitated, washed thrice in DMEM medium, suspended ($1.5 \cdot 10^6$ cells/ml) in RPMI 1640 medium containing gentamycin, HEPES (Sigma), and 10% FCS, placed into 24-well plates (1 ml per well), and left for 2 h at 37°C in the presence of 5% CO_2. The supernatant fluid was carefully removed, the adhered cells were washed in RPMI 1640 medium, and the monolayer of macrophages was left for incubation in 1 ml of the medium for 24 h at 37°C in the atmosphere with 5% CO_2. After the incubation, either the supernatant fluid or cell lysates prepared by the triple freezing–thawing were used as specimens.

Determination of TNF production. Production of TNF was determined by cytotoxic effect of the specimens on target cells of the L-929 line. The L-929 cells were cultured in 96-well plates, $2 \cdot 10^4$ cells in 0.1 ml per well, in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, and 0.02% gentamycin, at 37°C in the atmosphere with 5% CO_2. Twenty-four hours later, to the resulting monolayer of the L-929 cells 1 μg/ml actinomycin D (Sigma) was added and then 0.1 ml of the macrophagal lysates. Into the control wells, only the medium was added. The plates were incubated for 24 h, stained with Crystal Violet (Sigma), and the cell survival was determined after the crystals had been dissolved in 1% SDS. The optical density was determined at 546 nm with a spectrophotometer for plates (Titertec Multiscan MCC/340; Labsystems, Finland). The specificity of the cytotoxic effect of TNF-α was tested using the reaction of neutralization with monoclonal antibodies (anti-mouse TNF-α; StressGen, Canada).

The cytotoxicity index was calculated by the formula:

$$\left(1 - \frac{\text{optical density in the experimental wells}}{\text{optical density in the control wells}}\right) \times 100.$$

In some experiments, the concentration of TNF-α was determined in parallel by enzyme immunoassay as described below. The results were fully coincident with those obtained by the cytotoxicity test. We had to use the cytotoxicity test for determination of the TNF-β production due to lack of commercial anti-mouse antibodies. TNF-α and TNF-β are known to be equally cytotoxic for target cells, and by this approach are different in their origin: TNF-α is mainly synthesized by macrophages and TNF-β is produced by T-lymphocytes.

Determination of production of interleukins and interferon-γ. To determine the production of cytokines, T-lymphocytes were suspended in RPMI 1640 medium containing 1% L-glutamine, HEPES, 0.5% gentamycin, $5 \cdot 10^{-5}$ M β-mercaptoethanol (Sigma), and 10% FCS, stimulated by addition of 50 μg/ml phytohemagglutinin P (PGA) (DIFCO Laboratories, USA), and $1.5 \cdot 10^6$ cell/ml were incubated in 24-well plates for 72 h at 37°C in the atmosphere with 5% CO_2. After the incubation, the supernatants were stored at –20°C.

Concentration of cytokines in the supernatants of the PGA-stimulated T-cells and in the mouse blood serum was determined by enzyme immunoassay (ELISA). Rabbit polyclonal antibodies to mouse TNF-α, mouse interleukin-2 (IL-2), mouse IL-3, mouse IL-6, and mouse interferon-γ (IFN-γ) were used. All antibodies and cytokines were obtained from PeproTech (USA). Goat IgG with biotin to rabbit immunoglobulins (StressGen) were used as secondary antibodies; then the biotin-containing solution was conjugated with horseradish peroxidase (IMTEK, Russia). After incubation with the secondary antibodies, 100 μl of an ABTS green dye (Sigma) dissolved in 0.05 M citrate buffer (pH 4.0) with 0.01% H_2O_2 was applied, and after the development of a stable staining the reaction was stopped by addition of 1.5 mM NaN_3 dissolved in 50 mM citrate buffer (pH 4.0). The optical density was measured at 405 nm with the plate spectrophotometer. Every stage of ELISA was followed by multiple washing of the wells with phosphate-buffered saline (PBS) containing 0.05% Tween 20.

Determination of nitric oxide. Macrophages (10^6 cells) were placed into 1 ml of DMEM medium without Phenol Red supplemented with 1 mM sodium pyruvate, 25 mM HEPES, 2 mM L-glutamine, and 3% FCS and incubated in a 24-well plate at 37°C for 24 h in a humid atmosphere with 5% CO_2 in the presence of LPS (5 μg/ml). The production of NO was determined by the concentration of nitrites, which are the end product of metabolism of the short-living compound NO. The amount of nitrites in the supernatants was measured using the Griss reagent which contained a mixture of 0.1% solution of N-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid and 1% solution of sulfanilamide in 5% phosphoric acid at the ratio of 1 : 1. Specimens of the supernatants resulting in cultures of macrophages for 20 h were placed into a 96-well plate, 100 μl per well, the wells were supplemented with 100 μl of a freshly prepared Griss reagent, and incubated for 10 min at room temperature. The optical density was measured with a plate spectrophotometer (Titertek Multiscan MCC/340; Flow Laboratories, Finland) at 546 nm. The calibration curve was plotted using standard solutions of $NaNO_2$.

SDS-PAGE electrophoresis and immunoblotting. To prepare specimens, $50 \cdot 10^6$ lymphocytes were lysed on ice with an ultrasonic disintegrator with constant stirring for 2 min. The cell membranes were precipitated, and concentration of the cytosol total protein was determined by the Bradford's method with a commercial Bradford solution (Sigma). The protein was precipitated in acetone, solubilized, boiled for 5 min, and stored at –20°C. The final protein concentration in the specimens was 1 mg/ml, and 10 μl of the specimens was placed into each well. Commercial proteins (0.5 μg) Hsp72 and Hsp90 (StressGen) were placed as markers. Heat shock proteins in the specimens were determined by electrophoresis in 10% polyacrylamide gel, as described in [12]. The specificity of the analysis was tested by immunoblotting. The proteins were transferred from the gel onto a nitrocellulose membrane in a transblot chamber [13]. Upon blocking, the membrane was applied with monoclonal mouse antibodies to one of the heat shock proteins: Hsp70 (HSP 72, clone SPA-812, StressGen Biotechnologies, inducible form), Hsp25 (HSP 25, clone SPA-801, StressGen), and Hsp90 (HSP 90α, clone SPA-828, StressGen); then it was placed for 1 h into a biotin-containing solution of polyclonal goat antibodies to rabbit IgG (StressGen) and incubated with a complex of streptavidin with horseradish peroxidase (Sigma). The proteins were detected using an ECL system (Amersham, Sweden). Staining intensities of the bands corresponding to the marker proteins were compared with those of the bands of the purified heat stress proteins which were pretitrated before electrophoresis, and the amount of heat shock proteins in the specimens was assessed. Quantitative evaluation was performed using the Qapa program.

Isolation of serum. Blood was collected into tubes after the decapitation of the animals. The blood samples were kept for 3-5 h at 4°C, centrifuged at 2000 rpm, and supernatants were drawn.

Statistical analysis was performed using Student's *t*-test.

RESULTS

Cytokines and nitric oxide in acute stress. Figure 1 presents values of cytokine production in T-lymphocytes (A), production of cytokines and nitric oxide in macrophages (B), and also numbers of some lymphoid cell populations 6 h after injection of LPS (C). All data on acute stress are shown in white columns. Thus, the

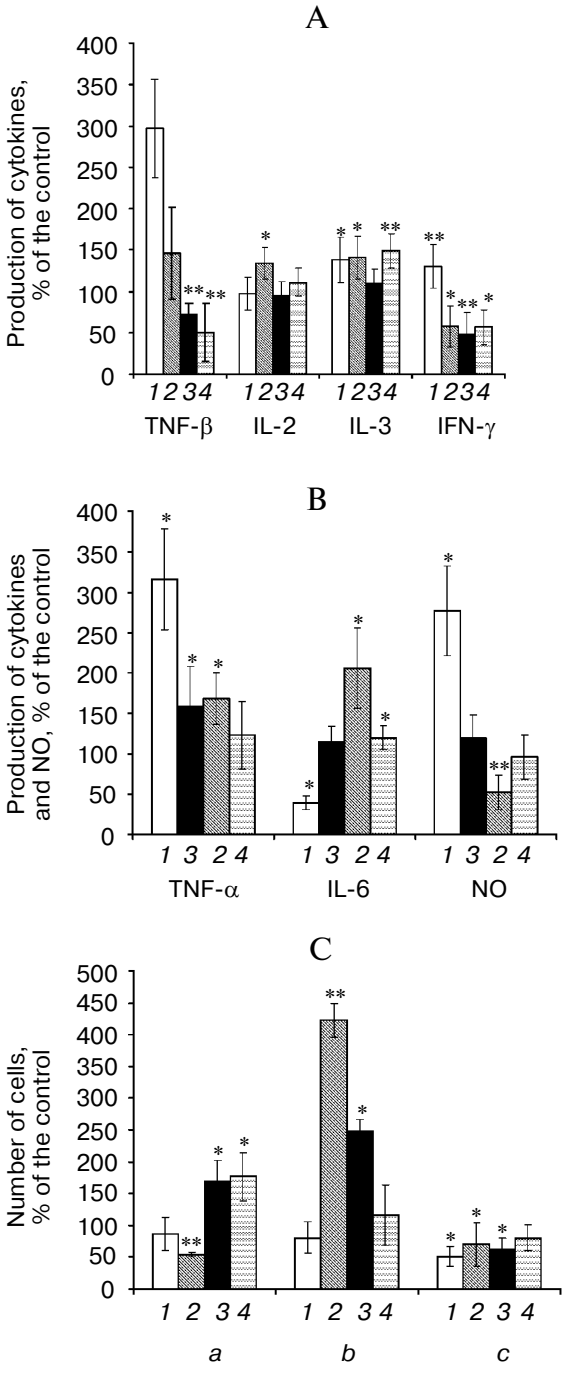

Fig. 1. Effects of acute and chronic toxic stress on production of cytokines and nitric oxide and also on the number of producer cells. The determinations were performed 6 h after the last injection of LPS. A) Production of cytokines in spleen lymphocytes; B) production of cytokines and nitric oxide in macrophages; C) cell number: *a*) spleen lymphocytes; *b*) macrophages; *c*) thymocytes. *1*) Acute stress; *2*) 4th day of chronic stress (ChS); *3*) 8th day of ChS; *4*) 11th day of ChS. Each value is the mean of three or four independent experiments; all measurements were performed in six repeats separately for each animal. The animals injected with saline were used as control. * Significant difference from the control, $p < 0.05$; ** significant difference from the control, $p < 0.01$.

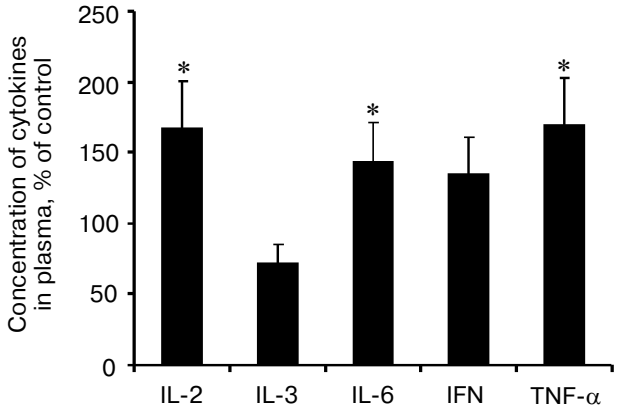

Fig. 2. Effect of acute toxic stress on cytokine concentration in mouse blood plasma. The determinations were performed 6 h after the injection of LPS. Each value is the mean of three or four independent experiments; all measurements were performed in six repeats separately for each animal. The animals injected with saline were used as the control. * Significant difference from the control, $p < 0.05$.

intraperitoneal injection of a high dose of LPS induced a dramatic increase in the production of TNF (TNF-α in macrophages and TNF-β in T-lymphocytes) and nitric oxide (Fig. 1, A and B). Under these conditions, the IL-3 production increased and there was a tendency for increase in the production of IFN-γ in the spleen lymphocytes of the mice, which had received a single injection of LPS (250 μg/100 g body weight). The production of IL-2 was not significantly changed, whereas the production of IL-6 was sharply decreased. Note that the blood concentration of nearly all cytokines under study, except IL-3, was increased (Fig. 2). The most pronounced accumulation in the blood was shown for TNF-α and IL-2.

The progress of acute endotoxic stress during its early stage was associated with a twofold decrease in the number of thymus lymphocytes and a slight (statistically insignificant) decrease in the number of peritoneal macrophages and spleen lymphocytes (Fig. 1C). Thus, acute endotoxic stress accelerated involution of the thymus, which is a very sensitive organ to various damaging factors, such as starvation, infection, trauma, ionizing radiation, etc. However, the cell numbers in other lymphoid organs, spleen lymphocytes and macrophages of peritoneal exudate, were nearly unchanged. On this background, the cells manifested a high functional activity, suggesting adaptive character of the response to acute toxic exposure.

Cytokines and nitric oxide in chronic stress. Figure 1 also presents parameters of immune status of the animals under conditions of chronic exposure to the toxin. The determinations were performed at three stages of the intoxication development: on the 4th, 8th, and 11th days.

The production of TNF-α in macrophages was increased about 1.5-fold on the 4th and 8th days, and on the 11th day its production was decreased nearly to the control level (Fig. 1B). Still more demonstrative were changes in the production of TNF-β in spleen lymphocytes in the course of chronic intoxication. Thus, the TNF-β production was significantly higher than in the control on the 4th day of the intoxication, but on the 8th and especially on the 11th day the synthesis of TNF-β was noticeably suppressed (Fig. 1A).

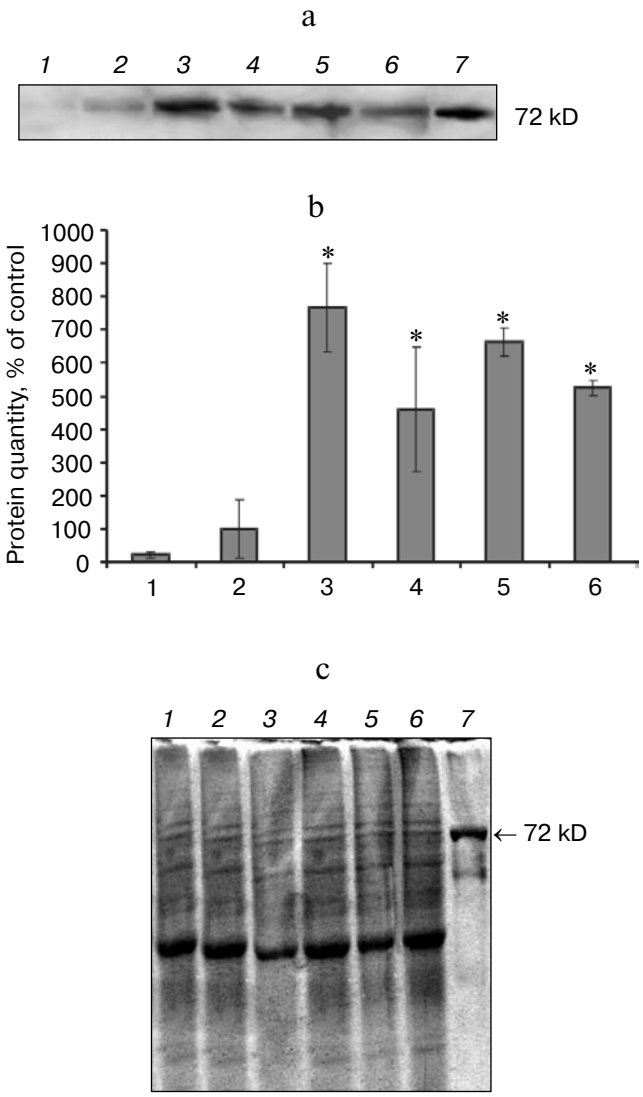

Fig. 3. Hsp70 expression in mouse spleen lymphocytes upon single and chronic injections of LPS. The determinations were performed 6 h after the last injection of LPS. The animals injected with saline were used as control. a) A membrane after immunoblotting with antibodies to Hsp70; b) results of quantifying Hsp70 in the specimens (in arbitrary units) using the Qapa program; c) Coomassie-stained gel. *1*) Intact animals; *2*) control; *3*) acute stress; *4*) 4th day of chronic stress (ChS); *5*) 8th day of ChS; *6*) 11th day of ChS; *7*) recombinant protein Hsp72. * Significant difference from the control, $p < 0.05$ (b).

The NO production in macrophages was significantly suppressed on the 8th day, but no significant changes in the NO production were observed on the other stages of chronic stress (Fig. 1B). The productions of three interleukins under study (IL-2, IL-3, IL-6) in T-lymphocytes changed non-monotonically depending on the accumulated dose of the injected polysaccharides. The IL-2 production was increased on the 4th day, the production of IL-3 was increased on the 4th and 11th days, and that of IL-6 was increased on the 8th day after the beginning of LPS injections. The production of IFN-γ was suppressed during the whole period of LPS injections (Fig. 1A).

It should be noted that the number of producer cells (macrophages and spleen lymphocytes) mainly remained increased during the toxic stress development, except the decrease in the number of splenocytes on the 4th day after the beginning of the injections (Fig. 1C). Obviously, the sharp increase in the number of peritoneal exudate cells was induced by a focal inflammation because of the intraperitoneal injection of LPS, but the increase in the number of spleen lymphocytes was associated with a systemic response of the body to the endotoxin. In any case, an inverse dependence was recorded between the number of cell-producers and their functional activity: the increase in the lymphocyte pool in the spleen during the toxic stress development was accompanied by the decrease in their ability for producing TNF-β and IFN-γ. On the other hand, the secretion of IL-3 and IL-6 by these cells was increased. The cell number in the thymus in the course of the chronic toxic stress development was decreased, similarly to the case of acute toxic stress.

Thus, a difference in the body's response to acute and chronic toxic stress has been found. The response of the body to acute intoxication is expressed by an adaptive increase in the functional activity of the cells producing cytokines and reactive oxygen species, and this activation occurs alongside with a slight decrease in the number of producing cells. Another tendency was recorded when the animal was subjected to the long-term intoxication with the increasing dose of LPS. At first, the production of all cytokines, especially TNF-α and TNF-β, was increasing, although not to the extent recorded in acute stress. This was associated with a sharp increase in the number of cell-producers, peritoneal macrophages. Prolongation of toxic exposure of mice resulted in a noticeable increase in the number of spleen lymphocytes, which produced TNF-β, but the production of this cytokine was twofold decreased. Thus, the compensatory increase in the number of lymphoid cells in the course of development of severe intoxication occurred concurrently with a decrease in the functional activity of these cells that was manifested by suppression of their ability to produce TNF.

Heat shock proteins in acute and chronic toxic stress. Studies on production of three heat shock proteins,

Hsp27, Hsp70, and Hsp90, revealed expression of the Hsp70 protein in both acute and chronic injection of LPS (Fig. 3). The expression of the inducible form of this protein was especially high upon the single injection of the high dose of LPS (acute stress). In the model of chronic toxic stress, changes in the Hsp70 expression were followed during 11 days after the beginning of the toxin injections. The inducible Hsp70 protein was expressed during the whole period of observation, but on the 11th day the Hsp70 expression was slightly decreased as compared to its level measured after the single injection of LPS. Immunoblotting with mouse monoclonal antibodies to the Hsp27 protein failed to detect expression of this protein in both acute and chronic toxic stress (data not shown). As to Hsp90, its expression was recorded in acute stress and also on the 4th, 8th, and 11th days of the chronic stress development (Fig. 4). It is interesting, that on the 11th day the Hsp90 expression was markedly lower than on the 8th day of development of the LPS-caused chronic intoxication. Note that antibodies to the inducible form of Hsp90 were used in the experiments; nevertheless, this protein was always present in the controls. Our findings indicate that Hsp90 is involved in the cell response to LPS-caused toxic stress, and during the development of chronic toxic stress the production of Hsp90 in the cells is decreasing.

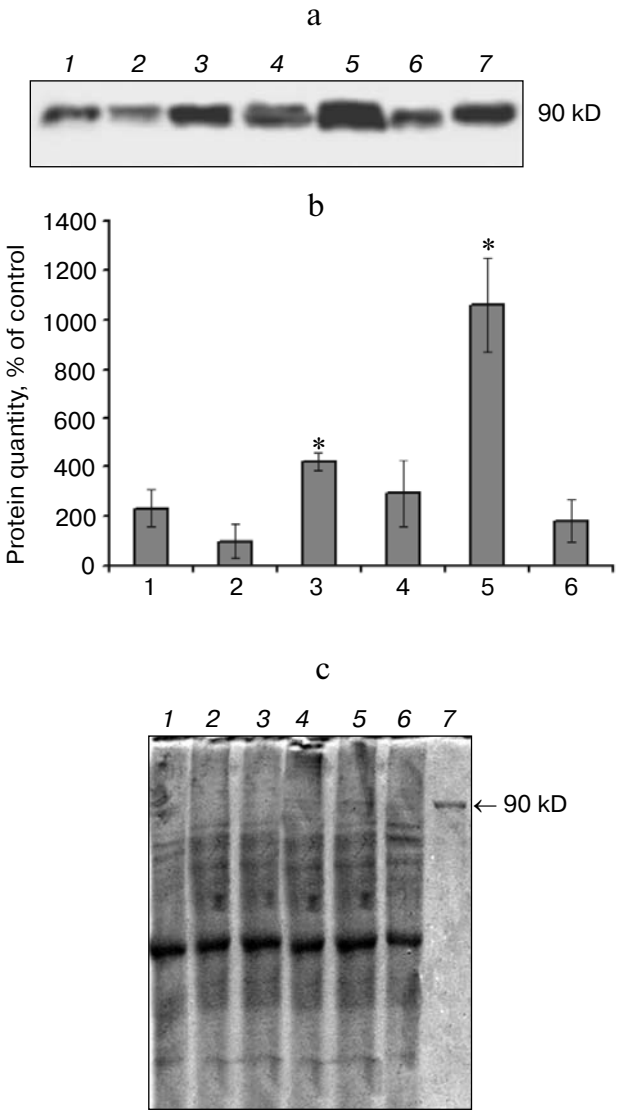

Fig. 4. Hsp90 expression in mouse spleen lymphocytes upon single and chronic injections of LPS. a) Membrane after immunoblotting with antibodies to Hsp90-α; b) results of quantifying Hsp90 in the specimens using the Qapa program; c) Coomassie-stained gel. *7*) Recombinant protein Hsp90-α. Other notations as in Fig. 3. * Significant difference from control, $p < 0.05$ (b).

DISCUSSION

Although the LPS model of bacterial infection is under intensive study by many researchers, the present work has revealed some unknown regularities in the cell response to acute and chronic exposure of the whole body to endotoxin. Among all immunocompetent cell products under determination, TNF and nitric oxide (their production increased nearly threefold) occurred to be main messengers involved in the response to acute toxic stress. The production of interleukins (IL-2, IL-6) and IFN-γ was nearly unchanged; only the level of IL-3 was slightly increased. As expected, acute stress induced a noticeable accumulation of cytokines in blood, and this suggested a systemic inflammation in the mice injected with the high dose of LPS.

Thus, results of the present work obtained on the model of acute stress do not contradict the earlier findings. In fact, LPS was earlier shown to be a powerful stimulant of macrophagal activity: a single injection of endotoxin, as well as the macrophage *in vitro* culture in the presence of LPS significantly increased TNF-α secretion [2, 4].

As differentiated from acute stress, the long-term injection of LPS was associated with suppression of the NO production, and the increase in the TNF production was less than after the single injection of the high dose of LPS (Fig. 1). Upon further injections with elevating doses of LPS, the animals' cells lost the ability of hyperproducing TNF, and it was especially pronounced in the case of TNF-β production in spleen cells.

We have found that in chronic toxic stress the IFN-γ production in spleen lymphocytes is suppressed. IFN-γ is known to enhance the toxicity of LPS: non-lethal doses of LPS become lethal when applied together with IFN-γ. The synergy of LPS and IFN-γ effects is due to the priming effect of IFN-γ on the cells via activation of NF-κB synthesis and increase in the production of TNF-α and NO [14]. Just this is the cause of formation of a very

strong response to LPS in the presence of IFN-γ, which leads to the tissue injury and sepsis. It is reasonable to suggest that a mechanism should exist to limit the biological effect of LPS (or any other bacterial material). Regulation of the IFN-γ level, the production of which was decreased in the presence of high concentration of LPS, is likely to be a link of such a mechanism, as shown in our experiments.

Our study is the first to reveal the involvement of heat shock protein Hsp90 in the body's response to acute and chronic exposure to endotoxin. Note that as differentiated from the inducible form of the Hsp70, which we did not detect in the absence of LPS, Hsp90 was also expressed under normal conditions. These findings are in agreement with the data on an important role of Hsp90 in the cell under stress conditions or in the absence of stress. This protein is known to be an inalienable part of the cytosol, constitute about 1-2% of the total soluble protein under normal conditions, and increase under stress conditions [15]. Hsp90 protein has been earlier shown to be fundamentally different from other molecular chaperones because it has a high affinity only to particular substrates. Thus, the majority of Hsp90 substrates are proteins of the signaling transduction system, e.g., steroid hormone receptors and kinases of the signaling cascade [16]. The increase in Hsp90 production upon loading with LPS suggests a possible involvement of some signaling proteins in pathogenesis of endotoxic stress.

Among three heat shock proteins studied, only two of them, Hsp70 and Hsp90, were expressed under conditions of acute and chronic intoxication caused by injection of LPS. Studies on the development of toxic stress caused by repeated injections of a powerful inducer of macrophage secretory activity have shown that under such conditions production of heat stress proteins, TNF, and nitric oxide begin to gradually die out. These changes in the TNF and NO synthesis, on one hand, and in the Hsp70 and Hsp90 production, on the other hand, were synchronous to a large extent.

The results of the present work are consistent with data of other authors. Thus, Hsp70 synthesis is known to be associated with nitric oxide synthesis in thermal stress [17]. The interrelation between Hsp70 and nitric oxide was confirmed by the increase in NO production previously to the Hsp70 expression caused by heat shock, and by the complete suppression with an NO synthase inhibitor of the NO production and significant decrease in the Hsp70 expression under conditions of heat shock [18, 19]. Obviously, such regulatory interrelationships between the enzyme system regulating the production of reactive nitrogen and the expression of heat shock proteins occur not only in heat shock but also in acute toxic stress induced by LPS, as shown in the present work. However, such an interrelation was not found in chronic toxic stress; on the contrary, the NO production was sharply suppressed on the 8th day of chronic stress when the Hsp70 expression was elevated. Moreover, the correlation between changes in Hsp70 expression and TNF production found by us in toxic stress is consistent with earlier findings. Thus, exogenous Hsp70 protein has been shown to stimulate the production of TNF-α and some interleukins, and this regulation could be realized either through binding to the CD14 receptor or via a CD14-independent pathway [20]. The proof of Hsp70 affinity for CD14 receptor expressed on monocytes allowed the authors to conclude that the extracellular Hsp70 protein manifesting itself as a stimulant of the cell activity may be assigned to cytokines.

Injection of LPS did not induce expression of Hsp27 in either acute or chronic exposure. This protein seems to be expressed only on elevation of temperature. In fact, Hsp27 is shown to play a protective role just under conditions of thermal stress because it inhibits *in vitro* aggregation and heat inactivation of some proteins. Moreover, *in vivo* studies revealed a specific pathway of regulation directed to protect polymerization of the actin thread under conditions of heat shock [21].

Results of the present work once more confirm that molecular reactions of the cell to stress exposures are based on universal and rather conservative mechanisms, which form the T-cell response to various types of external damaging signals. This is in agreement with the concept that the function of heat shock proteins is not limited only to their chaperone activity. The finding of Hsp70 secretion by living cells of the body is also very important [22, 23]. In these works the mechanism of protein export with involvement of the cell signaling systems was determined. Since the secreted protein has been shown to be captured by other cells and become endogenous for them, the use of heat shock proteins for treatment of various diseases is very promising. There are already many works in this line, e.g., the use of hyperthermia. Thus, hyperthermia accompanied by hyperexpression of Hsp70 displayed a noticeable protective effect in the LPS-induced liver cirrhosis in rats [24] and in pulmonary inflammation also induced by LPS injection [25].

Since the involvement of signal transduction proteins in the export of heat shock protein is proved, it is necessary to pay special attention to expression in endotoxic shock not only of Hsp70 but also of Hsp90, which has signaling proteins as substrates. Considering this circumstance, further studies on mechanisms of Hsp90 involvement in the development of toxic stress are necessary, as well as possibilities of using heat shock proteins to correct consequences of the body's intoxication.

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