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Spontaneous and Stimulated Production of Cytokines by Blood Cells *Ex Vivo* as a Biomarker of Initially High or Low Hypoxia Resistance in Rats

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Spontaneous and stimulated production of cytokines by peripheral blood cells obtained from the caudal vein of male Wistar rats was assessed before testing their resistance to oxygen deficiency in a decompression chamber. To study the spontaneous production of cytokines, heparinized blood cells were incubated in a culture medium (24 h, 5% CO_2, 37°C) and the content of proinflammatory cytokines IL-6 and TNFα and anti-inflammatory IL-10 in the culture medium was assessed by ELISA. To stimulate cytokine production, blood cells were incubated for 24 h with LPS, phytohemagglutinin, and concanavalin A at final concentrations of 2, 4, and 4 μg, respectively. Two weeks after blood sampling, individual resistance of the animals to hypoxia in a decompression chamber was determined. In animals with low resistance to hypoxia, the levels of spontaneous production of all three cytokines were significantly higher, while after stimulation, the level of IL-1β increased by more than 2 times. The animals with spontaneous production of IL-10>50 pg/ml, IL-6>10 pg/ml, and TNFα>10 pg/ml, as well as with the increase in IL-1β production by more than 2 times upon stimulation were classified as low-resistant. At IL-10<15 pg/ml, IL-6<9 pg/ml, and TNFα<7 pg/ml, as well as the absence of the increase in IL-1β production upon stimulation, they were classified as high-resistant. The identified features of spontaneous and stimulated production of cytokines can be used as non-invasive biomarkers to determine the resistance to hypoxia without exposure to sublethal hypoxia in a decompression chamber.

Key Words: *cytokines; biomarkers; resistance to hypoxia; rats*

It is known that there are differences in the resistance of organisms to hypoxia [1-3]. High-resistant (HR) and low-resistant (LR) to hypoxia animals differ in the level of oxidative stress, activity of antioxidant defense enzymes, and other parameters, including the content of hypoxia-inducible factor HIF-1α. In LR animals, the content of HIF-1α in different organs is higher [1-3]. However, the established differences are revealed after testing the individual resistance to hypoxia. The existing methods for assessing the initial resistance to hypoxia involve only direct exposure to hypoxic conditions in a decompression chamber, *i.e. in vivo*. For these purposes, a sublethal hypoxic exposure is used, which can lead to pathological and inflammatory changes in the brain and internal organs; therefore, it is recommended to include animals in the experiment no earlier than 1 month after hypoxic exposure in a decompression chamber [4]. At present, there is no alternative method for identifying HR and LR animals. This necessitates the search for biomarkers that make it possible to determine the individual organism resistance to hypoxia without exposure to sublethal oxygen deficiency.

To determine biomarkers of the resistance to acute mountain sickness and other altitude-related diseases, physiological and blood parameters are stu-

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died in people before and after altitude climbing or equivalent decompression chamber exposure and clinical manifestations are monitored. It was demonstrated that after 4- and 9-h exposure to a simulated "altitude" of 4875 m in a decompression chamber, subjects tolerant to acute mountain sickness had a higher level of IL-1Ra, an IL-1 receptor agonist [5]. After 4-h hypoxic exposure, the level of MIP-1 (macrophage inflammatory protein-1) was higher in people susceptible to acute mountain sickness in comparison to tolerant. Biomarkers such as IL-6, IL-8, IL-10, vascular endothelial growth factor, TNFα, MCP-1, and MMP-9 in plasma and serum did not differ between subjects tolerant and susceptible to acute mountain sickness, both before and after hypoxia exposure [5]. According to published reports, short-term (up to 2 days) exposure to hypoxic conditions (at an altitude>3800 m above sea level) promotes an increase in the blood levels of IL-1β, IL-6, IL-10, and TNFα [6]. In addition, we have previously demonstrated [2] that in response to the administration of 1.5 mg/kg LPS in LR rats, a more pronounced inflammatory reaction is observed in the liver and lungs, which is accompanied by an increase in the IL-1β content in the blood serum. Under physiological conditions, the levels of pro- and anti-inflammatory cytokines do not differ in HR and LR animals [2], and to stimulate their production *in vivo*, inductors of systemic inflammatory response (SIR) are administered, which is unacceptable for humans according to bioethical rules. Evaluation of cytokine production by blood cells in *ex vivo* system allows to overcome this limitation and is used to study the functional state of the immune system in both healthy individuals and patients with various diseases [7-9].

Previously, the levels of spontaneous and mitogen-stimulated production of IL-1β and IL-10 *ex vivo* was assessed in animals with different resistance to hypoxia during SIR caused by intraperitoneal administration of LPS at a dose of 1.5 mg/kg; the mitogens used for cell stimulation were LPS, phytohemagglutinin (PHA), concanavalin A (ConA) [9]. It was demonstrated that during SIR, the spontaneous and stimulated production of IL-1β and the spontaneous production of IL-10 decreased only in HR rats. High IL-1β/IL-10 index in LR rats of the control group decreased with the development of SIR, while in HR rats, on the contrary, no changes in this indicator were observed. These data attest to a high proinflammatory potential of blood cells in LR rats, which apparently determines the development of a more severe course of SIR. Thus, the levels of spontaneous and stimulated production of proinflammatory and anti-inflammatory cytokines differ in HR and LR animals and largely determine the SIR severity. They may serve as potential biomarkers for determining the resistance to hypoxia without hypoxia simulation in a decompression chamber.

The aim of this work is to identify the characteristics of spontaneous and *ex vivo* stimulated production of cytokines by blood cells of male Wistar rats with subsequent evaluation of the resistance to hypoxia in a decompression chamber.

MATERIALS AND METHODS

The experiment was carried out on male Wistar rats (n=50, age 2-3 months, body weight 220-260 g) obtained from Stolbovaya Animal Breeding Facility, Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia. The study was performed in compliance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and Directive 2010/63/EU of the European Parliament and of the Council (September 22, 2010; On the Protection of Animals Used for Scientific Purposes). The study was approved by the Bioethics Commission of the Avtsyn Research Institute of Human Morphology (Protocol No. 36/12 of March 28, 2022).

The blood (1 ml) was taken from the caudal vein under Zoletil anesthesia (2 mg/kg; Virbac Sante Animale) into tubes with heparin (Slavyanskaya Apteka) at a final concentration of 15 IU/ml. To 100 μl of heparinized blood, 900 μl of DMEM medium with L-glutamine, 1 mg/ml glucose, and 100 μg/ml gentamicin (PanEco) was added. To assess the spontaneous production of cytokines, blood cells were incubated for 24 h at 37°C in a CO_2 incubator and the production of IL-6, IL-10, and TNFα was assayed by ELISA using highly sensitive kits from Invitrogen (IL-10) and Cloud-Clone Corp. (IL-6, TNFα) on an Anthos 2010 analyzer. To assess the stimulated production of IL-1β and IL-10 cytokines, a complex mitogen consisting of *E. coli* LPS, PHA ,and ConA (Sigma) was added to blood cells at final concentrations 2, 4, and 4 μg, respectively [8,9]. PHA is a lectin isolated from red bean (*Phaseolus vulgaris*), has potent cell agglutinating and mitogenic activity, binds to T-cell membranes, and stimulates their metabolic activity and proliferation [10]. LPS activates Toll like receptor 4 also expressed on the surface of granulocytes, monocytes, and B lymphocytes [11]. ConA is a lectin from the seeds of the bean plant (*Canavalia ensiformis*), a non-catalytic protein that reversibly binds specific carbohydrates (monomers and oligomers of mannose and glucose) with high specificity and moderate affinity. It acts as a mitogen that preferentially activates T lymphocytes and induced production of cytokines by these cells [12,13]. Thus, the activator complex is a mitogen that stimulates the

production of cytokines by all blood leukocytes. The concentrations of the complex correspond to those in the Cytokine-Stimul-Best (Vector-Best) test system, which is widely used in laboratory practice to study the production of cytokines by blood cells in humans for evaluation of the functional state of the immune system under physiological conditions and in various diseases [7-9]. Stimulated blood cells were incubated for 24 h at 37°C in a CO_2 incubator and the production of IL-1β and IL-10 was measured by ELISA using highly sensitive kits from Cloud-Clone Corp. and Invitrogen, respectively. To record the intensity of the color reaction, the Anthos 2010 microplate ELISA analyzer was used.

Two weeks after blood collection, the resistance to hypoxia was assessed. To this end, the rats in a ventilated decompression chamber were elevated to a critical "altitude" of 11,500 m [1-3]; the temperature was maintained at 20-22°C, air humidity 50-65%. The resistance to hypoxia was evaluated by the "gasping time" at "altitude", *i.e.* the time from the moment of ascent to taking a lateral position. Rats with "gasping time" at "altitude" >240 sec and <80 sec were classified as HR (n=7) and LR (n=11), respectively; rats with normal resistance to hypoxia (n=32) were excluded.

Statistical analysis was performed in GraphPad Prism 8 (GraphPad Software). The data are expressed as the median (Me) and interquartile range (25%; 75%). Since the data were not normally distributed, the significance of differences between the parameters was determined using the nonparametric Mann–Whitney U test. The differences were considered statistically significant at $p<0.05$.

RESULTS

To identify biomarkers of individual resistance to hypoxia, spontaneous and stimulated production of cytokines by blood cells was studied before the experiment in the decompression chamber. Spontaneous production of proinflammatory cytokines IL-6 and TNFα, as well as anti-inflammatory IL-10, was higher in LR animals (Table 1). The increased levels of these cytokines in LR rats may be due to their "proinflammatory phenotype". In particular, we have previously demonstrated that in LR Wistar rats, non-activated (M0) macrophages are characterized by increased expression of *Tnfa* and *Il1b* in comparison with HR animals [14].

The level of cytokines in the serum or plasma reflects the immune system functional state, while in situations associated with deficiency or imbalance of regulatory factors, it is necessary to assess the ability of blood cells to secrete cytokines. Spontaneous cytokine production reflects the existing *in vivo* level of activation of blood cell, while mitogen-induced production allows assessing the potential of cytokine secretion in response to an additional stimulus, which is important for assessing the functional state of the immune system [7]. The use of whole blood in this case has a number of advantages, because it does not require cell isolation and preparation for cultivation, which simplifies the analysis and eliminates the possibility of unwanted cell activation or death during sample preparation. In addition, blood cell culturing occurs in a natural microenvironment under conditions of preserved balance of all humoral factors operating *in vivo* [15]. Although, in the opinion of most researchers, these methods should occupy a central place in modern immunodiagnostics, at present, due to significant differences in the results of different laboratories, it is very difficult, and sometimes impossible, to correctly assess the disturbance of the cytokine profile in a particular clinical case. Currently, *ex vivo* assessment of the cytokine secretion ability of blood cells is widely used [7].

After stimulation of blood cells with LPS, ConA, and PHA, the production of IL-1β by blood cells increased only in LR rats (Fig. 1). In addition, the IL-1β/IL-10 ratio upon stimulation increased only in LR animals. We have previously demonstrated that 1 month after evaluation of the resistance to hypoxia, the production of IL-1β and IL-10 upon stimulation with LPS, ConA, and PHA increased in both HR and LR rats, and the IL-1β/IL-10 ratio in LR animals decreased [9]. Moreover, the IL-1β/IL-10 ratio without stimulation was higher in LR rats. This suggests that measurement of hypoxia resistance in a decompression chamber has an immunomodulatory effect persisting even 1 month after exposure, which confirms the data on the effect of oxygen deficiency on immune cells [16].

Thus, it was demonstrated that the spontaneous production *ex vivo* by blood cells of the proinflammatory cytokines IL-6 and TNFα, as well as the anti-inflammatory IL-10, was higher in LR Wistar rats before evaluation of the hypoxia resistance in a decompression chamber. In addition, after stimulation of blood

TABLE 1. Level of Spontaneous Cytokine Production (pg/ml) by Peripheral Blood Cells of Wistar Rats before Determining Resistance to Oxygen Deficiency in a Decompression Chamber (Me (25%; 75%))

Cytokine	HR rats (n=7)	LR rats (n=11)
IL-6	7.6 (7.3; 8.9)	15.3 (10.6; 20.9) p=0.04
TNFα	3.1 (2.5; 7.0)	17.1 (14.6; 20.3) p=0.0006
IL-10	12.5 (12.5; 49.9)	62.4 (37.4; 74.9) p=0.03

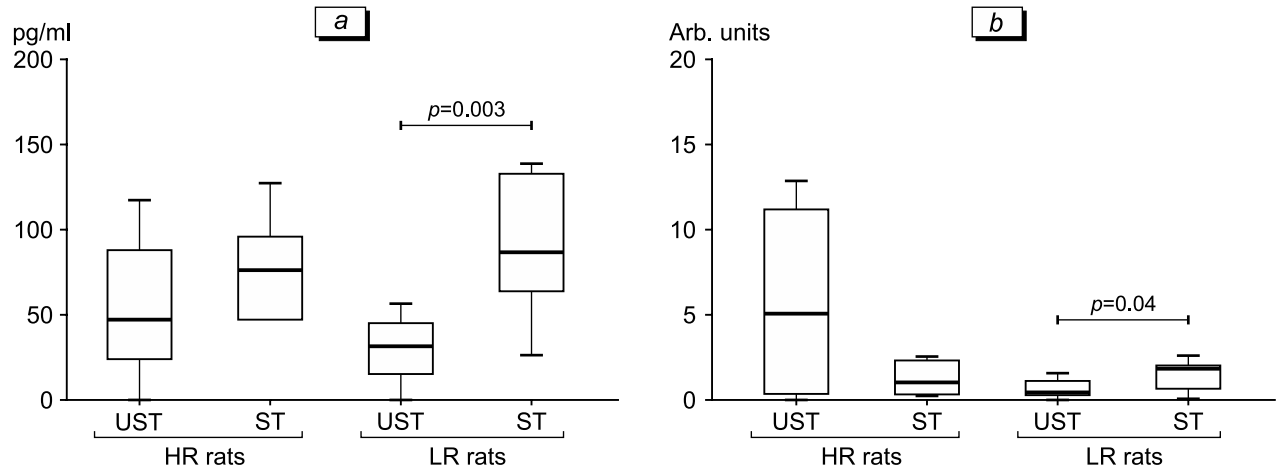

Fig 1. Unstimulated (UST) and stimulated (ST) IL-1β production (*a*) and the IL-1β/IL-10 ratio (*b*) in Wistar rats before evaluation of the resistance to oxygen deficiency in a decompression chamber.

cells with a complex of mitogens (LPS, ConA, and PHA), the production of IL-1β and the IL-1β/IL-10 ratio increased only in LR rats. This allows considering these indicators as potential biomarkers of hypoxia resistance, which in the future can be used to determine individual resistance to oxygen deficiency without sublethal hypoxic exposure in a decompression chamber.

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Conflict of interest. The authors have no conflicts of interest to declare.

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