ПРОДУКТЫ КИШЕЧНЫХ БАКТЕРИЙ ВИЧ-ИНФИЦИРОВАННЫХ ПАЦИЕНТОВ ПРЕПЯТСТВУЮТ РЕГЕНЕРАЦИИ CD4$^+$Т-ЛИМФОЦИТОВ

Королевская Л.Б., Сайдакова Е.В., Шмагель Н.Г., Шмагель К.В.

Институт экологии и генетики микроорганизмов Уральского отделения Российской академии наук – филиал ФГБУН «Пермский федеральный исследовательский центр» Уральского отделения Российской академии наук, г. Пермь, Россия

Резюме. У части ВИЧ-инфицированных больных, несмотря на подавление репликации вируса на фоне приема антиретровирусных препаратов, не происходит эффективного прироста числа периферических CD4$^+$Т-лимфоцитов (иммунный неответ на терапию). Одним из значимых факторов в развитии иммунодефицита считается иммунная активация, причиной которой, среди прочих, является поступление в кровоток бактериальных продуктов в результате нарушения целостности кишечного барьера. Кроме того, микрофлора кишечника продуцирует различные растворенные вещества, которые могут накапливаться в крови и проявлять токсические свойства. Целью настоящей работы была оценка влияния микробных продуктов кишечного происхождения – паракрезол сульфата и индоксил сульфата – на число CD4$^+$Т-лимфоцитов ВИЧ-зараженных пациентов, получающих антиретровирусную терапию. Объектом исследования служила периферическая кровь ВИЧ-инфицированных субъектов с различной эффективностью восстановления иммунной системы на фоне проводимой терапии и неинфицированных доноров. Концентрация IL-6 ($p = 0,012$), IP-10 ($p = 0,0004$) и sCD14 ($p = 0,003$) в плазме крови ВИЧ-зараженных иммунных неответчиков была повышена по сравнению с соответствующими показателями лиц с эффективным восстановлением численности CD4$^+$Т-клеток (иммунные ответчики). Хотя обе группы ВИЧ-позитивных субъектов не различались по уровню липополисахарида и I-FABP в плазме крови, содержание паракрезол сульфата ($p = 0,001$) и индоксил сульфата ($p = 0,042$) у иммунных неответчиков было увеличено. В экспериментах *in vitro* было установлено негативное дозозависимое влияние паракрезол сульфата и индоксил сульфата на жизнеспособность и митотическую активность CD4$^+$Т-лимфоцитов. Таким образом, у ВИЧ-инфицированных пациентов с нарушенной регенерацией CD4$^+$Т-лимфоцитов на фоне проводимой антиретровирусной терапии отмечается более высокий уровень системного воспаления, чем у субъектов, отвечающих на лечение приростом численности CD4$^+$Т-клеток. Выраженность повреждения кишечного барьера и нагрузка бактериальными компонентами, выходящими в кровоток, у ВИЧ-зараженных лиц с различной эффективностью восстановления иммунитета в ответ на лечение примерно одинаковая. При этом плазма крови иммунных неответчиков значительно обогащена микробными продуктами ки-

Адрес для переписки:

Королевская Лариса Борисовна
Институт экологии и генетики микроорганизмов
Уральского отделения Российской академии наук
614081, Россия, г. Пермь, ул. Голева, 13.
Тел.: 8 (342) 280-83-34.
Факс: 8 (342) 280-92-11.
E-mail: bioqueen@mail.ru

Address for correspondence:

Larisa B. Korolevskaya
Institute of Ecology and Genetic of Microorganisms,
Ural Branch, Russian Academy of Sciences
13 Golev St
Perm
614081 Russian Federation
Phone: +7 (342) 280-83-34.
Fax: +7 (342) 280-92-11.
E-mail: bioqueen@mail.ru

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шечного происхождения: паракрезол сульфатом и индоксил сульфатом. Выявленное в присутствие данных токсинов существенное снижение пролиферативной способности CD4+Т-клеток, стимулированных в условиях *in vitro*, и индукция их гибели могут быть одной из причин неэффективного восстановления численности CD4+Т-лимфоцитов у ВИЧ-инфицированных лиц, получающих антиретровирусную терапию.

Ключевые слова: ВИЧ-инфекция, бактериальные токсины, кишечник, CD4+Т-лимфоциты, антиретровирусная терапия, регенерация иммунитета

IN HIV-INFECTED PATIENTS, INTESTINAL BACTERIA-DERIVED PRODUCTS INTERFERE WITH CD4+T CELL REGENERATION

Korolevskaya L.B., Saidakova E.V., Shmagel N.G., Shmagel K.V.

Institute of Ecology and Genetic of Microorganisms, Ural Branch, Russian Academy of Sciences, Branch of Perm Federal Research Center, Ural Branch, Russian Academy of Sciences, Perm, Russian Federation

Abstract. Despite successful suppression of viral replication by antiretroviral drugs there is no significant increase in the number of peripheral CD4+T lymphocytes in some HIV-infected patients (immune non-response to therapy). One of the crucial factors for immunodeficiency aggravation is immune activation developing in response to the bacterial products entry into the bloodstream through the damaged intestinal barrier. Additionally, the intestinal microflora produces various solutes that accumulate in the blood and exhibit toxic properties. This work aimed to evaluate the effect of intestinal microbial products (para-cresol sulfate and indoxyl sulfate) on the number of CD4+T lymphocytes in HIV-infected patients receiving antiretroviral therapy. The object of the study was the peripheral blood of HIV-infected subjects with different immune system restoration efficiency during the therapy. Uninfected donors were enrolled as healthy controls. Plasma concentrations of IL-6 ($p = 0.012$), IP-10 ($p = 0.0004$), and sCD14 ($p = 0.003$) in HIV-infected immune non-responders were increased compared with those in individuals with effective restoration of CD4+T cells (immune responders). Although both groups of HIV-positive subjects did not differ in plasma lipopolysaccharide and I-FABP levels, para-cresol sulfate ($p = 0.001$) and indoxyl sulfate ($p = 0.042$) concentrations were increased in immune non-responders. *In vitro* experiments showed a negative dose-dependent effect of para-cresol sulfate and indoxyl sulfate on the viability and mitotic activity of CD4+T lymphocytes. Thus, in HIV-infected patients with impaired regeneration of CD4+T lymphocytes during antiretroviral therapy, a higher level of systemic inflammation is noted than in subjects responding to treatment with an increase in the number of CD4+T cells. The severity of the intestinal barrier damage and the load of bacterial components released into the bloodstream are approximately the same in HIV-infected individuals with different efficiency of immune recovery in response to treatment. Simultaneously, the blood plasma of immune non-responders is significantly enriched with microbial products of intestinal origin: para-cresol sulfate and indoxyl sulfate. The significant decrease in the proliferative capacity of CD4+T cells stimulated *in vitro* and the induction of their death in the presence of these toxins may be a reason for the ineffective restoration of the number of CD4+T lymphocytes in HIV-infected individuals receiving antiretroviral therapy.

Keywords: HIV infection, bacterial toxins, intestine, CD4+T lymphocytes, antiretroviral therapy, immune regeneration

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Introduction

In HIV-infected patients, the use of antiretroviral therapy (ART) leads to suppression of viral replication and a subsequent increase in the peripheral CD4+T lymphocyte count (standard response) [2]. However, up to 30% of patients referred to as "immune non-responders" (INRs), develop a discordant response to treatment, in which, despite an effective viral load suppression, the number of blood CD4+T cells remains low [10]. INRs compared with subjects giving a standard response to ART have a significantly increased risk of morbidity and mortality from both AIDS-associated and non-AIDS-associated diseases [9].

Immune activation is considered one of the most significant factors in the development of immunodeficiency during HIV-infection. The generally accepted concept of immune activation is based on the idea that intestinal barrier integrity is disrupted and the microbial products enter the bloodstream, maintaining high chronic systemic inflammation level [3, 4]. HIV-infected individuals develop intestinal dysbiosis, which manifests in a reduction in bacterial community diversity [6, 11]. Additionally, the intestinal flora produces gut

bacteria-derived solutes, including the so-called "uremic toxins", the accumulation of which in patients with chronic kidney disease is associated with clinical progression and cardiovascular complication development [8, 15]. The most studied uremic toxins are para-cresol sulfate (PCS) and indoxyl sulfate (IS). Their precursors, para-cresol and indole, are formed as a result of amino acid fermentation by intestinal bacteria. After sulfation, these products enter the bloodstream in the form of PCS and IS. Although PCS and IS have pro-inflammatory effect on a number of cell populations [1, 5], data on their effects on T lymphocytes are limited.

This work **aimed** to evaluate the effect of para-cresol sulfate and indoxyl sulfate on $CD4^+$T lymphocytes in HIV-infected patients receiving ART.

Materials and methods

The study was approved by the ethics committee of the Perm Regional Center for Protection against AIDS and Infectious Diseases (No. IRB00008964). Each subject signed informed consent. The study enrolled HIV-infected patients with a suppressed viral load (< 50 copies/ml of blood) who were adherent to ART for more than two years. The immune system regeneration was assessed by the number of blood $CD4^+$T lymphocytes and was compared to the established threshold value of 350/μL [10]. Patients were divided into groups: 1) immune non-responders (INRs; n = 16) with $CD4^+$T cell count < 350/μL; and 2) immune responders (IRs; n = 21) with $CD4^+$T cell count > 350/μL. The third group enrolled relatively healthy controls without HIV-infection (HC; n = 20). All the examined subjects had no clinical signs of kidney dysfunction.

Blood was sampled into vials containing ethylenediaminetetraacetic acid (Weihai Hongyu Medical Devices Co Ltd, China). The peripheral blood $CD4^+$T lymphocyte number was assessed with a CytoFLEX S flow cytometer (Beckman Coulter, USA) using a commercial Immunocytometry Systems (BDIS) Simultest™ kit (Becton Dickinson, USA). The blood plasma concentrations of interleukin-6 (IL-6), interferon-gamma induced protein 10 (IP-10), soluble CD14 (sCD14), and fatty acid-binding intestinal protein (I-FABP) were determined using R&D Systems ELISA kits (USA). The content of bacterial lipopolysaccharide (LPS) in blood plasma was assessed with the LAL assay QCL-1000 chromogenic kit (Lonza, USA). The studies were conducted according to the manufacturers' instructions. Analysis of PCS and IS in plasma was performed using high-performance liquid chromatography. The effect of PCS and IS on the T lymphocytes proliferative capacity and their resistance to apoptosis was assessed *in vitro* using mononuclear leukocytes obtained from healthy volunteers. The isolated cells were stained with CellTrace Violet (Invitrogen, USA) according to the manufacturer's instructions.

T lymphocyte proliferation was induced by stimulation of the CD3/CD28 complex using a polymer nanomatrix conjugated with CD3 and CD28 agonists (T Cell TransAct; Miltenyi Biotec, USA). Unstimulated cells served as control. Samples were incubated for 72 h in the presence of PCS (ApexBio, USA) or IS (Sigma-Aldrich, USA) at final concentrations of 1 μM, 10 μM, 25 μM, 50 μM or 0.1 μM, 1 μM, 10 μM, respectively. Control samples with stimulated and unstimulated cells did not contain these reagents. After incubation, the cells were harvested and stained with anti-CD3-PE and anti-CD4-V450 antibodies (Becton Dickinson, USA). The proliferation of T lymphocytes was assessed with flow cytometry according to the dilution of the tracking dye in daughter cell generations. The level of $CD4^+$T cell apoptosis was measured by flow cytometry using a commercial FITC AnnexinV Apoptosis Detection Kit I (BD, USA).

Statistical analysis of the obtained data was carried out using nonparametric methods. The median and interquartile ranges (25-75th percentiles) were calculated. The significance of differences between groups was determined using the Mann–Whitney U test or the Kruskal–Wallis test.

Results and discussion

All three groups had no differences in age or gender composition. The INR and IR groups did not differ in HIV-infection or ART duration. In accordance with the selection criteria, the absolute number of $CD4^+$T lymphocytes in INRs was significantly lower than that in the comparison groups ($p < 0.001$). The number of $CD4^+$T cells in IRs was still reduced compared with the corresponding rate in HC ($p < 0.001$).

The study of the inflammation, bacterial translocation, and intestinal epithelium damage indices in the blood plasma of HIV-infected subjects revealed the following (Figure 1).

Compared with IRs, INRs had significantly increased content of IL-6 ($p = 0.012$) and IP-10 ($p = 0.0004$), which indicates a higher level of systemic inflammation. Although the sCD14 concentration in INRs exceeded that in IRs ($p = 0.003$), the LPS level did not differ between the two groups. It is known that the CD14 molecule on the membrane of monocytes/macrophages is a co-receptor for LPS, and its presence in the bloodstream in a soluble form (sCD14) serves as an indicator of bacterial translocation [4]. Based on the fact that CD14 is shed from the cell surface during cell stimulation [14], the presence of sCD14 in blood plasma can also be regarded as a result of monocyte activation. According to some [4], activation, and inflammation in HIV-infection may be due to the release of bacterial products into the bloodstream

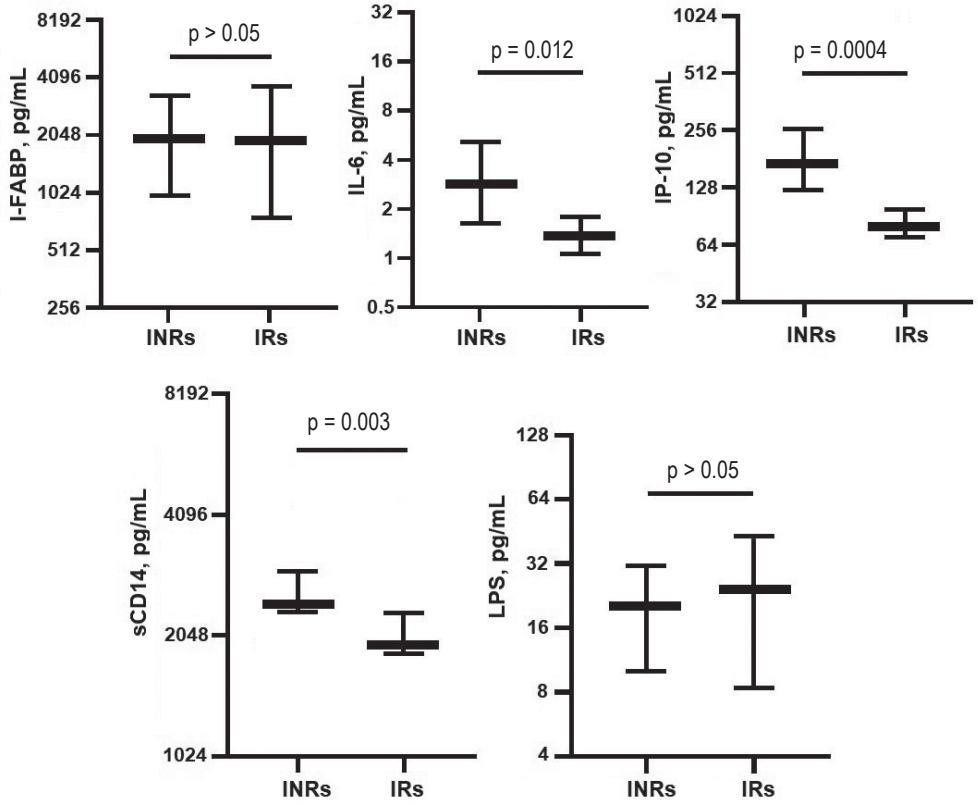

Figure 1. Systemic inflammation, bacterial translocation, and intestinal epithelium damage indices in the blood plasma of HIV-infected patients

Note. INR, immune non-responders; IR, immune responders. Medians (horizontal lines) and interquartile ranges (vertical bars) are shown. Statistical calculations were made using the Mann–Whitney method.

following increased intestinal permeability. When assessing the intestinal epithelium destruction index (I-FABP) we did not reveal any differences between the INR and IR groups. Thus, one can assume that intestinal permeability is not a major reason for the increased systemic activation in HIV-infected individuals with impaired $CD4^+$T lymphocyte regeneration. The same was confirmed by the level of plasma LPS. In addition to bacterial components, various waste products of the intestinal flora can enter the bloodstream [7, 12]. Their entry into the circulation does not require an increase in intestinal permeability since it occurs in a healthy body during the fermentation of food compounds.

Analysis of the gut-derived bacterial products in the blood plasma revealed the following. Median PCS concentrations in INRs, IRs, and HCs were 65.4 μM, 31.7 μM, and 28.5 μM, respectively. Statistically significant differences were found only between the INR and IR groups ($p = 0.001$). Compared with PCS, the content of IS in the blood plasma of all examined subjects was significantly lower. The median concentrations of this microbial toxin in INRs, IRs, and HCs were 8.1 μM, 6.1 μM, and 4.2 μM, respectively. These indices in INRs were significantly higher than the corresponding values in IRs ($p = 0.042$) and HCs ($p = 0.015$). It is known that uremic toxin accumulation in blood occurs due to the kidney function impairment [5, 15]. As there were no signs of kidney dysfunction in any subject, the obtained data indicate the enrichment of the blood plasma of INRs with uremic toxins: compared with IRs in INRs the content of PCS was almost 2 times higher, and the level of IS was 1.3 times higher. Since both substances are products of the intestinal microflora metabolism, then, apparently, the bacterial composition in IRs and INRs differ and is characterized by the predominance of PCS and IS producers in the latter. These toxins may additionally contribute to increased inflammation in INRs and affect $CD4^+$T cells.

The impact of PCS and IS on the viability and proliferative capacity of $CD4^+$T lymphocytes was assessed *in vitro*. The concentrations of PCS and IS (see Materials and Methods) were applied on the basis of literature data on their physiological values in serum/plasma [7, 13]. An assessment of $CD4^+$T lymphocytes binding AnnexinV revealed the following (Figure 2).

In control samples incubated without the PCS or IS, the frequency of cells prone to apoptosis was relatively low. But, in cell samples containing PCS, the level of $CD4^+$T lymphocytes that bound AnnexinV increased significantly (Figure 2A). The effect was

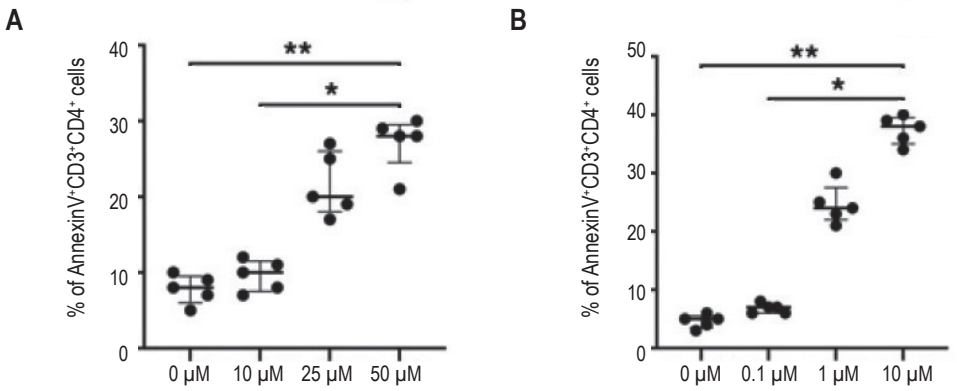

Figure 2. Effect of para-cresol sulfate (A) and indoxyl sulfate (B) on the CD4⁺T lymphocyte viability

Note. The abscissa shows the concentrations of the uremic toxins in the sample. *, $p < 0.05$; **, $p < 0.01$. Statistical calculations were performed using the Kruskal–Wallis method.

dose-dependent. At a PCS concentration of 50 μM, the frequency of cells prone to apoptosis exceeded 25%. Similar results were revealed when assessing the effect of IS on the CD4⁺T lymphocyte viability (Figure 2B).

Furthermore, PCS and IS had a negative impact on the stimulated CD4⁺T cells proliferation (Figure 3).

The number of stimulated CD4⁺T lymphocytes that underwent more than one cycle of division while being incubated in the presence of toxins for 72 h was significantly reduced. The effect of PCS and IS was dose-dependent. At the maximum concentrations used (50 μM of PCS and 10 μM of IS), almost all stimulated cells were unable to proceed to the second round of mitosis. Therefore, both PCS and IS cause CD4⁺T lymphocyte death and disrupt their mitotic activity. The effect of these gut-derived microbial products on CD4⁺T cells is strongly dose-dependent.

Conclusion

Thus, in contrast to HIV-infected subjects with a standard response to treatment, patients with a discordant response to ART are characterized by higher levels of systemic inflammation. Simultaneously, in subjects with a standard and discordant response to ART, the severity of intestinal barrier destruction and the amount of bacterial components entering the bloodstream are approximately the same. However, the content of gut-derived microbial products, such as para-cresol sulfate and indoxyl sulfate, is increased in the blood plasma of HIV-infected immune non-responders. Apparently, in patients with a discordant response to ART, this may be the result of the unique intestinal microflora composition, enriched in the taxa-producers of para-cresol sulfate and indoxyl sulfate. We have shown for the first time the negative impact of these gut-derived microbial products on CD4⁺T lymphocytes, namely, a significant decrease in the proliferative capacity of cells stimulated *in vitro* andthe induction of their death. This may be a reason for the ineffective restoration of the CD4⁺T lymphocyte counts in HIV-infected patients receiving ART.

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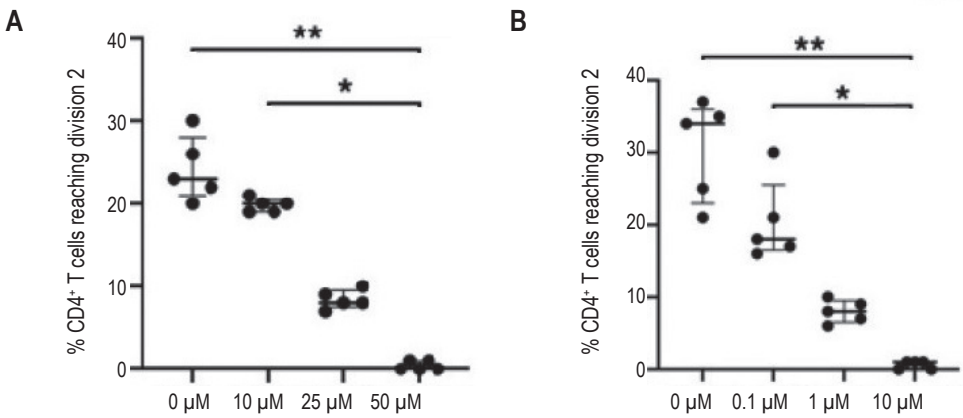

Figure 3. Effect of para-cresol sulfate (A) and indoxyl sulfate (B) on the CD4⁺T lymphocyte proliferative capacity

Note. As for Figure 2.

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Авторы:

Королевская Л.Б. *— к.м.н., научный сотрудник лаборатории экологической иммунологии, Институт экологии и генетики микроорганизмов» Уральского отделения Российской академии наук — филиал ФГБУН «Пермский федеральный исследовательский центр» Уральского отделения Российской академии наук, г. Пермь, Россия*

Сайдакова Е.В. *— д.б.н., заведующая лабораторией молекулярной иммунологии, Институт экологии и генетики микроорганизмов» Уральского отделения Российской академии наук — филиал ФГБУН «Пермский федеральный исследовательский центр» Уральского отделения Российской академии наук, г. Пермь, Россия*

Шмагель Н.Г. *— д.м.н., старший научный сотрудник лаборатории экологической иммунологии, Институт экологии и генетики микроорганизмов» Уральского отделения Российской академии наук — филиал ФГБУН «Пермский федеральный исследовательский центр» Уральского отделения Российской академии наук, г. Пермь, Россия*

Шмагель К.В. *— д.м.н., заведующий лабораторией экологической иммунологии, Институт экологии и генетики микроорганизмов» Уральского отделения Российской академии наук — филиал ФГБУН «Пермский федеральный исследовательский центр» Уральского отделения Российской академии наук, г. Пермь, Россия*

Authors:

Korolevskaya L.B., *PhD (Medicine), Research Associate, Laboratory of Ecological Immunology, Institute of Ecology and Genetic of Microorganisms, Ural Branch, Russian Academy of Sciences, Branch of Perm Federal Research Center, Ural Branch, Russian Academy of Sciences, Perm, Russian Federation*

Saidakova E.V., *PhD, MD (Biology), Head, Laboratory of Molecular Immunology, Institute of Ecology and Genetic of Microorganisms, Ural Branch, Russian Academy of Sciences, Branch of Perm Federal Research Center, Ural Branch, Russian Academy of Sciences, Perm, Russian Federation*

Shmagel N.G., *PhD, MD (Medicine), Senior Research Associate, Laboratory of Ecological Immunology, Institute of Ecology and Genetic of Microorganisms, Ural Branch, Russian Academy of Sciences, Branch of Perm Federal Research Center, Ural Branch, Russian Academy of Sciences, Perm, Russian Federation*

Shmagel K.V., *PhD, MD (Medicine), Head, Laboratory of Ecological Immunology, Institute of Ecology and Genetic of Microorganisms, Ural Branch, Russian Academy of Sciences, Branch of Perm Federal Research Center, Ural Branch, Russian Academy of Sciences, Perm, Russian Federation*

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