

IMMUNOLOGY AND MICROBIOLOGY

Effect of Tilorone on the Dynamics of Viral Load and the Levels of Interferons and Interleukin-1 β in the Lung Tissue and Blood Serum of Mice with Experimental Influenza

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We studied the effect of tilorone on the dynamics of IFN α , IFN γ , and IL-1 β levels in the lung tissue and blood serum in relation to viral load in the lungs of BALB/c mice with pneumonia caused by influenza virus A/Aichi/2/68 (H3N2). Tilorone was administered *per os* in doses of 40, 150, and 540 μ g per mouse 6, 30, and 78 h postinfection, which simulated the drug regimen used in the clinic for the treatment of influenza and acute respiratory viral infections in Russia and post-Soviet countries. Tilorone reduced viral load with the maximum amplitude (2-3 lg) after 1-2 administrations. The results of studying the dynamics of the cytokine levels in the infected animals in general support the previous hypothesis that, in repeated dosing, tilorone enhances the IFN response (compensates for its deficiency) at the early stages of acute respiratory viral infections and suppresses (damps) excessive production of IFN and proinflammatory cytokines at the later stages.

Key Words: *tilorone; experimental influenza; interferon- α ; interferon- γ ; interleukin-1 β*

Tilorone is the world's first and perhaps the most studied oral low-molecular-weight "inducer of IFN". It induces the production of all three types of IFN [1] and some other cytokines. Tilorone was developed half a century ago in the United States [7], but its widespread introduction into clinical practice, in particular for the prevention and treatment of influenza and acute respiratory viral infections (ARVI), was implemented in Russia and post-Soviet countries [4,5]. From time to time, the interest to this pharmacological substance revives in connection with emergence of new human

viruses and outbreaks of zoonotic infections affecting humans [6].

Inspiring experimental data were obtained on the antiviral effects of tilorone against Middle East respiratory syndrome coronavirus as well as Ebola, Chikungunya, and Marburg viruses. This drug is currently being considered as a potential treatment option for COVID-19. In cells sensitive to tilorone action, it is co-localized with cytosolic receptors RIG-1 (retinoic acid-inducible gene 1), which presumably reflects a key event in the pharmacological action of this drug [5]. The concept of tilorone as an "IFN inducer" was formulated based mainly on the results of studies of its biological effects in *in vitro* test systems and *in vivo* models with a single administration to intact animals [1,7], but for the treatment of influenza and ARVI, tilorone is administered in a course mode including

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several repeated oral doses. Back in the 1970s, a phenomenon of induced hyporeactivity was described, *i.e.* a decrease in the amplitude and even suppression of cytokine response after repeated administration of tilorone and some other “IFN inducers” [9]. The results of single experimental and clinical studies of the effect of repeated administration of tilorone on levels of IFN and other cytokines in the systemic circulation and affected tissues in ARVI are very contradictory.

Our aim was to study the effect of repeated dosing of tilorone that simulates the regimen of its clinical use for the treatment of influenza and ARVI on the dynamics of levels of IFN α , IFN γ , and IL-1 β in the lungs and blood serum in relation to viral load in the lungs of mice with experimental influenza.

MATERIALS AND METHODS

BALB/c male mice (16–18 g) were obtained from the Andreevka branch of the Scientific Center of Biomedical Technology of the Federal Medical-Biological Agency of Russia. The animals were maintained in the vivarium of the N. F. Gamaleya National Research Center for Epidemiology and Microbiology on a conventional diet. To reproduce non-lethal viral pneumonia, the mice were inoculated intranasally with the A/Aichi/2/68 (H3N2) virus at a preselected dose of 4 lg TCID₅₀. Tilorone (Nizhpharm) was administered orally in 100 μ l of 0.9% NaCl solution in 3 doses: 40, 150, 540 μ g per mouse. The minimum and maximum doses were equivalent to 125 mg for adult humans in terms of body weight and body surface area, respectively, according to the method [8]. Tilorone was administered in 6, 30, and 78 h after infection. In the control group, the infected animals received equivalent volume of 0.9% NaCl *per os* by the same scheme. Each group included 20 animals; 5 mice from each group were sacrificed 24, 48, 72, and 96 h after infection. The blood was first taken from each animal to study the level of circulating cytokines and then the lungs were taken from the exsanguinated animal for cytokine measurement (right lung) and assessment of viral load (left lung).

The levels of IFN α , IFN γ , and IL-1 β in biological samples were determined by ELISA (Bender MedSystems GmbH) according to the manufacturer's instructions. Optical density was measured on an iMark microplate photometer (Bio-Rad). Cytokine levels in the lung tissue were calculated per 1 g tissue. To establish the background cytokine levels, the serum and lung samples in 5 intact mice were measured.

Viral load was measured by PCR analysis on a DTlight amplifier (DNA-Technology). Total nucleic acids were isolated from 10% lung suspension using PROBA-NK kits (DNA-Technology). Reverse tran-

scription (RT) was performed using Reverta-L kits (Central Research Institute of Epidemiology of the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing), followed by real-time PCR with hybridization fluorescence detection using specific primers and probes as described earlier [2], and presented as a number of viral genome equivalents (copies) per 1 g lung tissue.

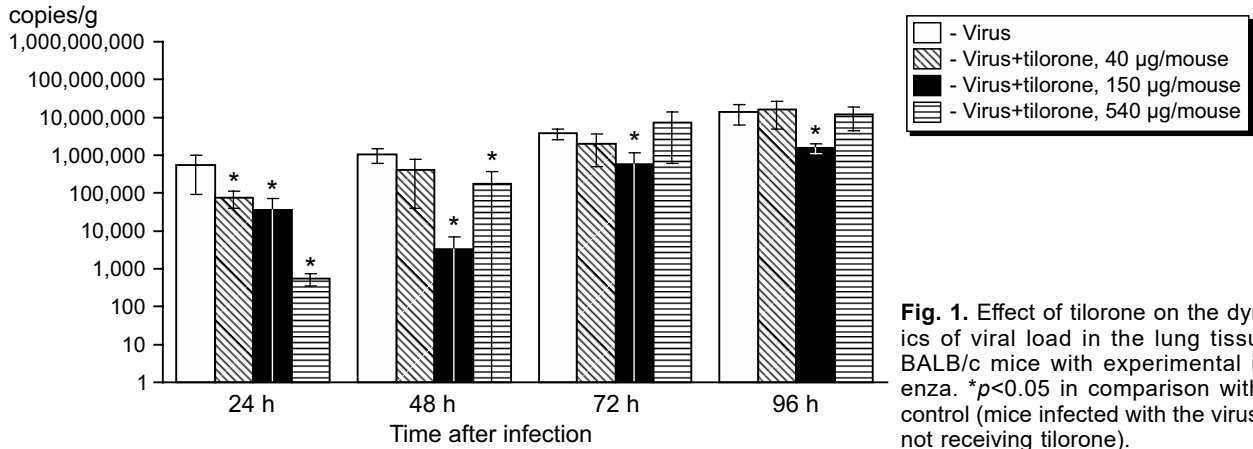
The data obtained were then processed statistically using Statistica 12 Software (StatSoft, Inc.). Significance of differences in quantitative indicators of independent samples was evaluated using the Mann–Whitney *U* test. The differences were significant at $p < 0.05$. On the graphs built using Microsoft Excel 2016, quantitative data were presented as $M \pm SD$.

RESULTS

Under the conditions of experimental influenza in mice not exposed to tilorone, viral load in the lungs steadily increased over 96 h after infection (Fig. 1). In all groups, no deaths during this period and during 10-day follow-up were recorded. The therapeutic course administration of tilorone reduced viral load, especially at the early stages of experimental infection. In 18 h after the first administration of the drug (24 h after infection), tilorone had the most significant dose-dependent antiviral effect, reducing viral load by 3 orders in a dose of 540 μ g per mouse. In 18 h after the second administration, the dose-dependence of the antiviral effects disappeared, and tilorone most strongly (by more than 2 lg) reduced viral load in a dose of 150 μ g per mouse. In this dose the drug suppressed viral replication 72 and 96 h after infection.

In infected animals not treated with tilorone, IFN α levels in the lung tissue steadily increased over 96 h (Fig. 2, *a*) and directly correlated with viral load. In 18 h after the first administration of tilorone, a tendency to an increase in the level of this cytokine was found; it was most pronounced ($p = 0.056$) when the drug was used in a dose of 150 μ g per mouse. Then, the increasing tendency was replaced by a decreasing one, and 72 h after infection a significant decrease in IFN α levels was observed in all groups of animals exposed to tilorone. The maximum amplitude of the decrease in the level of this cytokine in the lung tissue was recorded 96 h after infection, *i.e.*, 18 h after the third injection.

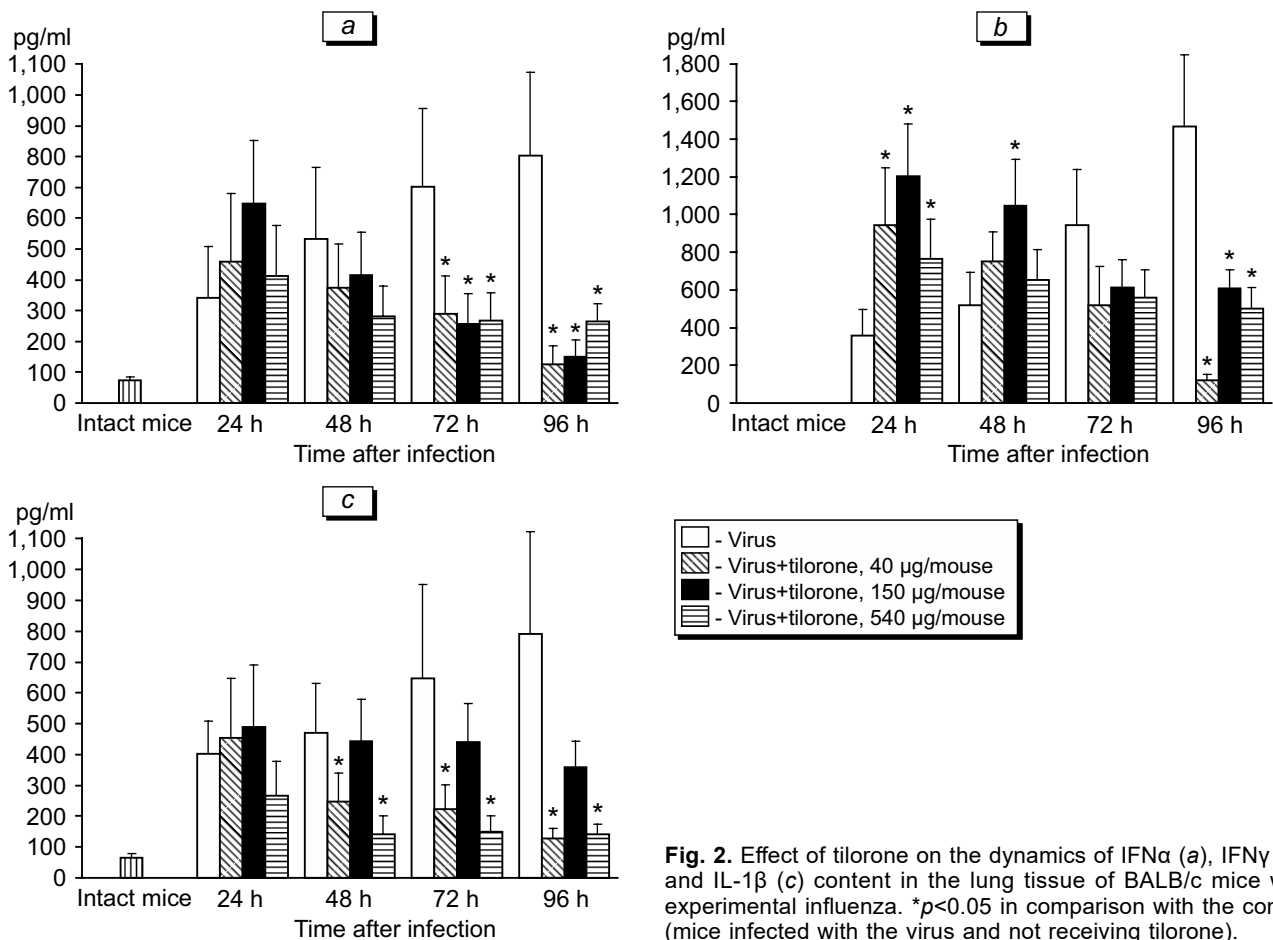
Changes in IFN γ levels in the lung tissue of animals with experimental influenza that received or did not receive tilorone were similar to those for IFN α (Fig. 2, *b*). In 24 h after infection, tilorone in all doses increased the IFN γ level by 2–3 times in comparison to that in infected animals not treated with tilorone. The drug exhibited the highest stimulating activity in a dose of 150 μ g per mouse. In 18 h after the second



administration of tilorone in this dose (48 h after infection), the $IFN\gamma$ levels doubled, other doses of the drug induced insignificant increase in the levels of the cytokine. In 72 h after infection, there was a tendency to a decrease in the $IFN\gamma$ level in all groups of mice treated with tilorone. In 96 h, course administration of tilorone in all doses led to a significant drop in the levels of this cytokine.

In addition to the effect on $IFN\alpha$ and $IFN\gamma$ levels, the influence of tilorone on the dynamics of levels of

the key proinflammatory mediator $IL-1\beta$ in the lung tissue was studied using an influenza model. The level of this cytokine in animals that did not receive tilorone increased steadily throughout the experiment (Fig. 2, c) in direct correlation with viral load. In 24 h after infection, tilorone in doses of 40 and 150 $\mu\text{g}/\text{ml}$ did not change the levels of $IL-1\beta$; in the highest dose, the drug caused a tendency towards a decrease in the levels of this cytokine. At later terms, we observed either a significant drop in $IL-1\beta$ levels under the in-



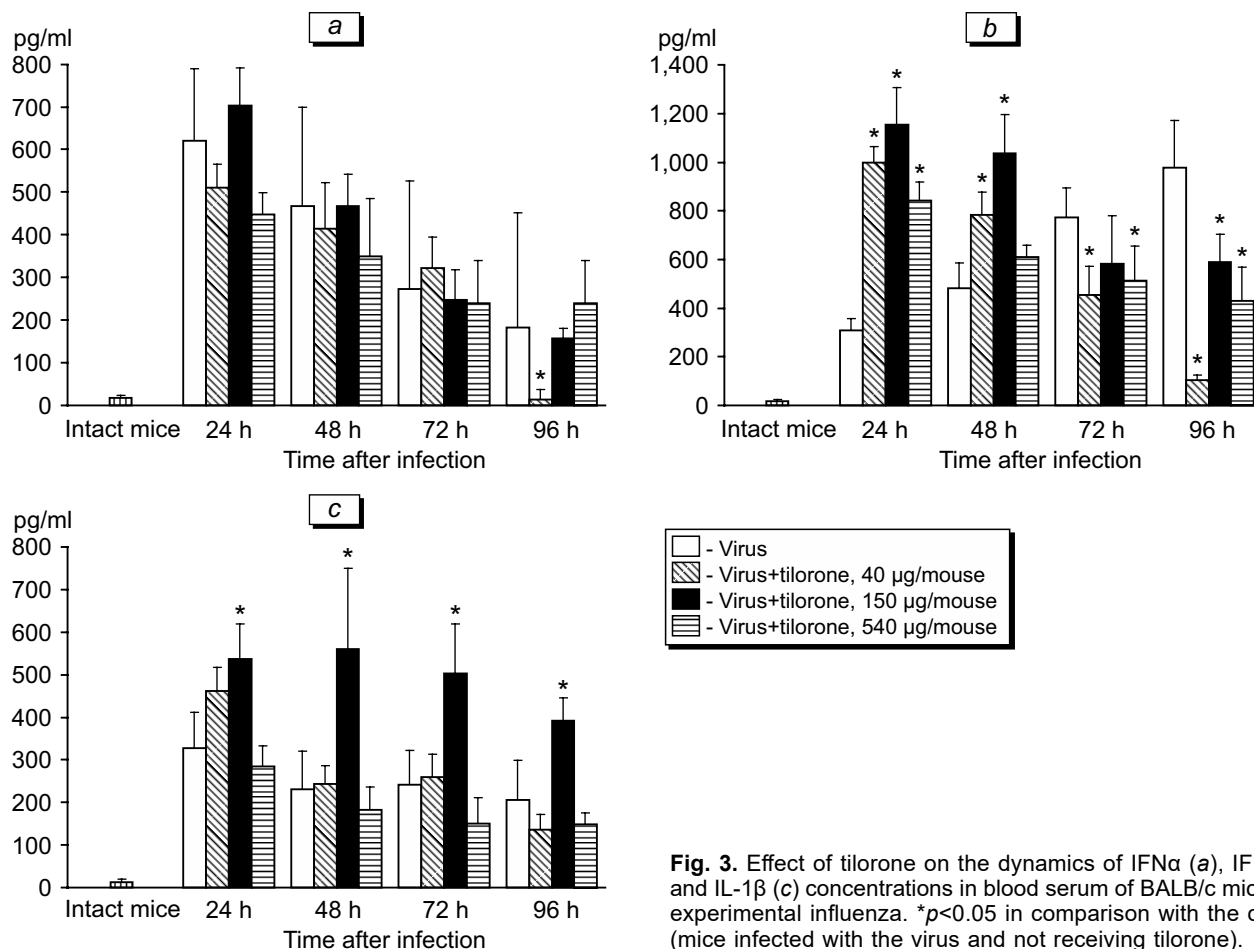


Fig. 3. Effect of tilorone on the dynamics of IFN α (a), IFN γ (b) and IL-1 β (c) concentrations in blood serum of BALB/c mice with experimental influenza. * $p < 0.05$ in comparison with the control (mice infected with the virus and not receiving tilorone).

fluence of tilorone (40 and 540 μ g/ml), or a tendency towards a decrease in the level of this proinflammatory mediator in the lung tissue (150 μ g/ml).

In all groups of infected animals, the maximum rise in serum concentration of IFN α in 24 h postinfection followed by gradual decrease in its level was observed (Fig. 3, a); no significant differences between the groups were revealed, except for a decrease in the concentration of IFN α at 96 h after infection in animals treated with tilorone in a dose of 150 μ g per mouse in comparison with the control group.

The dynamics of serum levels of IFN γ in infected animals was similar to that in the lung tissue (Fig. 3, b). In the control group, the concentration of this cytokine remained elevated throughout the experiment. Under the influence of tilorone, the level of IFN γ surpassed the control 24 and 48 h after infection and was below the control at later terms (72 and 96 h).

The serum concentration of IL-1 β in animals of the control group increased 24 h postinfection, then somewhat decreased, but remained at an increased plateau in comparison with intact animals until the end of the experiment. Tilorone in the lowest and highest doses did not significantly change the levels of

this cytokine during the experiment, and in a dose of 150 μ g/mouse increased the concentration of IL-1 β .

The antiviral activity of tilorone was confirmed under conditions of experimental influenza *in vivo*, which is consistent with the data of other studies in models of viral infections and clinical trials of the drug in patients with ARVI/influenza [4,5]. A sufficiently high amplitude of a decrease in viral load (2-3 lg) after the first or second administration of the drug was revealed, which coincided with the time of increase in the level of the cytokines in the lung tissue and blood serum.

The pleiotropy of tilorone is worthy of note: it modulated systemic (blood serum) and local (lung tissue) concentrations of cytokines produced by lymphocytes (IFN γ), plasmacytoid dendritic cells, which are mainly of lymphoid origin (IFN α), and myeloid lineage cells (IL-1 β).

The traditional understanding of the mechanisms of the clinical efficacy of tilorone for ARVI only as a classical "IFN inducer" has required a revisiting for a long time. Earlier, we hypothesized that the effectiveness of tilorone at different stages of ARVI is associated with multidirectional changes in the production

of IFN and some other cytokines. With a therapeutic course administration, the drug can compensate for the deficiency of the cytokine response, including IFN, at the early stages of ARVI, *i.e.* during the period of the most active virus replication, but at later stages of the disease, tilorone can suppress (damp) the excess production of IFN and proinflammatory cytokines, when their high level can become an endogenous damaging factor (including provoking a secondary bacterial infection) in the respiratory and other systems of the infected organism [3,4].

Despite the specific features of the influenza model we have reproduced, in which during 96 h of infection there was a steady increase in viral load in the lungs, which on days 3-4 of the experiment was only partially restrained by the course administration of tilorone in a dose of 150 µg/ml, the data obtained largely support the previous hypothesis.

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