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Efficacy of Inhalations of Antituberculous Compositions with Different Length of Experimental Therapy Course in Mice

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The study compared antituberculous efficacy of individual or combined administration of "free" isoniazid and liposomal form of dextrazide (a composition consisted of isoniazid and oxidized dextran) inhaled in standard (15 mg/kg) or low (3 mg/kg) dose. The therapy started 1 month after contamination of outbred ICR male mice with *Mycobacterium tuberculosis* strain H37Rv. Combined inhalation of liposomal form of dextrazide and isoniazid in the low dose was most effective against mycobacterium tuberculosis due to diminished prodestructive pulmonary effect and a low hepatotoxicity. A minor prodestructive effect of this combination was observed starting from 1.5 month after the onset of therapy (12 inhalations, 2 times a week), and it augmented after 24 inhalations administered during 3 months.

Key Words: *liposomal forms of antituberculous compositions; inhalation; efficacy; early therapy stages; mice*

Tuberculosis is a socially challenging infectious disease manifesting itself in diverse clinical forms. The researchers are continuously searching for the drugs, which would effectively suppress *Mycobacteria tuberculosis* (MBT), prevent the toxic, destructive, and fibrotic complications, and possess a broad therapeutic profile in an easy-to-use drug form.

Recently, a composition (dextrazide) had been created by conjugating oxidized dextran (40 kDa, 5% oxidation degree) with isoniazid (INH, isonicotinic acid hydrazide) [3]. In 6-month-long experiments carried out on a model of BCG-induced granulomatosis, dextrazide demonstrated a high potency in suppressing the persistent intracellular MBT due to lysosomal tropicity, antifibrotic effect, and a low hepatotoxicity [5]. However, dextrazide is rapidly captured by hepatocytes and hepatic endothelial sinusoidal cells [5]. In order to enhance the effectiveness of intracellular delivery of dextrazide into macrophages (especially the pulmonary ones with persistent MBT) and diminish its hepatotoxicity, the liposomal form of dextrazide (LFDZ) was produced [6]. Being a corpuscular-size object, LFDZ is not captured by endotheliocytes of hepatic sinusoids and hepatocytes but actively grappled by the macrophages. Within the macrophages, INH is uncaged from the liposomal shell and disengaged from dextran helped by lysosomal hydrolases, thereupon it suppresses MBT persisting in macrophages and partially diffuses into blood and lymph, where it annihilates the circulating MBT [5].

This work was designed to examine the effects of various therapeutic modes of individual or combined inhalation of LFDZ and "free" INH on structural alterations in the lungs and liver of mice contaminated with virulent MBT.

MATERIALS AND METHODS

The experiments were carried out on 2-month-old male ICR mice weighing 20-22 g obtained from Breeding Department of Vector State Research Center of Virology and Biotechnology (Koltsovo, Novosibirsk

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region). The animals were maintained under standard vivarium conditions with water and pelleted food *ad libitum*. The mice were adapted to laboratory environment for 2 weeks. All procedures with animals were carried out in strict adherence to Directive No. 199n of Ministry of Health of the Russian Federation (On Establishing the Rules of Good Laboratory Practice; April 1, 2016) and to European Convention for Protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Strasbourg, 1986).

The mice were contaminated with a 2-week-old virulent MBT culture (strain H37Rv) by a single injection of 0.1 ml isotonic NaCl saline with 0.1 mg culture into the retro-orbital sinus. LFDZ was prepared from phosphatidylcholine (Sigma); the size of liposomes was 200-450 nm [1,6]. LFDZ was administered in a dose of 0.025 ml per 10 g body weight. The infected but untreated mice ones (n=6) were employed as experimental controls.

The mice of experimental groups (6 animals each) received inhalations according to the following regimens: *1*) LFDZ; *2*) LFDZ+INH (the standard dose of 15 mg/kg); *3*) LFDZ+INH (a low dose of 3 mg/kg); *4*) INH (15 mg/kg); and *5*) INH (3 mg/kg). In series I and II, 12 or 24 inhalation sessions were administered, respectively. The mice received inhalations 1 month after infection with MBT. The inhalation sessions were performed at room temperature in a closed chamber equipped with a Musson ultrasonic inhalator. After evaporation of the inhalants, the mice were retrieved from the inhalation chamber.

The lung and liver specimens were obtained after sacrificing the mice under ether narcosis by cervical dislocation. The specimens were fixed in 10% isotonic formalin water solution and embedded in paraffin. The histological sections (4-5 μ) were cut with a Microm HM3555 microtome and stained with hematoxylin, eosin, and picrofuchsin according to Van Gieson. They were examined under AxioImager A light microscope equipped with an AxioCam MRc5 digital camera operated under AxioVision 4.8 software (Carl Zeiss). The morphometry was performed in a closed test system of 100 regularly positioned points occupying the area of 3.6×10^5 μ^2, which determined the population ("numerical", Nai) density of tuberculous granulomas (*i.e.*, their number in the test area) and granuloma diameter (μ). A decrease of these parameters attested to diminished chemoattractant concentration gradient generated by living MBT [5]. In addition, the volume densities (Vv) of destruction foci (necroses and vacuolar dystrophy), inflammatory infiltrates, and connective tissue fibers were calculated (in % of the test area).

The data were analyzed statistically using Student's t test at p<0.05. The results are summarized as *m±SEM*.

RESULTS

In 1 month after contamination of the mice, the typical tuberculous granulomas [5] were formed in the lung and liver from monocytes, macrophages, and a small population of epithelioid cells. In addition, there were

TABLE 1. Pulmonary Morphometry of Mice Infected with MBT H37Rv and Treated with Antituberculous Inhalants (*m±SEM*, *n*=6)

Group	Numerical density of granulomas (Nai)	Granuloma diameter, μ	Vv of infiltrates, %	Vv of destruction foci, %	Vv of fibrous connective tissue, %
Series I (12 inhalations, 1.5 months)					
Untreated	6.10±0.21	38.82±2.54	30.44±4.26	27.74±2.36	1.32±0.18
LFDZ	4.95±0.28*	32.32±1.18*	20.44±1.82*	22.35±1.23*	1.24±0.12
LFDZ+INH, 3 mg/kg	3.16±0.19*	23.14±1.36*	15.26±1.24*	19.72±1.23	1.26±0.15
LFDZ+INH, 15 mg/kg	3.12±0.22*	27.61±1.15*	15.42±1.38*	19.33±1.21*	1.25±0.21*
INH, 3 mg/kg	2.46±0.18*	27.36±2.56*	19.95±1.37*	17.79±1.31	1.32±0.26
INH, 15 mg/kg	1.82±0.16*	22.89±1.76*	23.48±1.24*	23.58±2.12	1.51±0.26
Series II (24 inhalations, 3 months)					
Untreated	7.32±0.22	32.18±3.14	29.44±2.26	27.74±1.36	3.28±0.32
LFDZ	3.15±0.12*	23.11±1.11*	24.48±1.42*	22.15±2.23*	2.14±0.32*
LFDZ+INH, 3 mg/kg	2.16±0.28*	19.71±1.23*	13.45±1.34*	19.27±1.17*	2.24±0.31*
LFDZ+INH, 15 mg/kg	1.59±0.33*	18.31±1.45*	7.98±0.08*	17.76±1.42*	2.85±0.31*
INH, 3 mg/kg	1.69±0.16*	20.18±1.35*	13.95±1.53*	25.37±3.25	3.51±0.32
INH, 15 mg/kg	1.27±0.19*	18.75±1.17*	13.14±1.24*	29.19±2.16	3.78±0.27*

Note. Here and in Table 2: *p<0.05 in comparison with untreated mice.

destruction foci such as vacuolar dystrophy as well as necrosis of alveolar epitheliocytes and centrilobular hepatocytes; also, we observed inflammatory infiltrates around these foci and near the blood vessels and bronchi.

The 1.5-month course of LFDZ inhalations (12 inhalation sessions) diminished the number of pulmonary granulomas by 19% and decreased their size by 17%. At this, Vv of destructive foci and inflammatory infiltrates decreased by 20 and 33%, respectively. In contrast, the Vv of connective tissue fibers did not change (Table 1). In liver, Vv of destruction foci of hepatocytes diminished by almost 2-fold. In contrast, Vv of inflammatory infiltrates increased by 1.6 times (Table 2). At this, Vv of connective tissue fibers decreased by 1.7 times (Table 2).

Longer treatment with LFDZ (24 inhalation sessions during 3 months) decreased the number of pulmonary granulomas and their size by 2.3 times and by 28%, respectively (Table 1). At this, Vv of destruction foci, infiltrates, and connective tissue fibers decreased by 20, 17, and 35%, respectively. In liver, Vv of destruction foci decreased almost 3-fold, while Vv of inflammatory infiltrates and connective tissue fibers did not differ from corresponding parameters of untreated mice (Table 2).

The 1.5-month inhalation course (12 sessions) employing LFDZ with INH in the standard dose of 15 mg/kg decreased the number and size of pulmonary granulomas by 2 times and 29%, respectively (Table 1). At this, Vv of destruction foci and inflammatory infiltrates decreased by 30 and 49%, respectively, while Vv of connective tissue fibers changes insignificantly. In liver, Vv of parenchymal destruction foci diminished by 23%, while Vv of inflammatory infiltrates increased by 22% in parallel with a 2-fold decrease in Vv of connective tissue fibers (Table 2).

Longer treatment (24 sessions over 3 months) with LFDZ and INH in the standard dose of 15 mg/kg decreased the number and size of granulomas in the lungs by 4.6 times and 43%, respectively (Table 1). Vv of destruction foci, inflammatory infiltrates, and connective tissue fibers decreased by 36%, 3.7 times and 13%, respectively, in comparison with untreated mice (Table 1). In the liver, Vv of destruction foci diminished by 47%, while Vv of inflammatory infiltrates was similar to those in untreated mice; at this, Vv of connective tissue fibers was smaller by 32% in comparison with untreated mice (Table 2).

The short-term inhalation course (12 sessions) with LFDZ and low dose of INH decreased the number of granulomas by almost 2-fold and reduced their size by 40%. Vv of destruction foci and inflammatory infiltrates decreased by 29 and 50%, respectively, while Vv of connective tissue fibers did not change significantly. In the liver, Vv of parenchymal destruction foci and collagen fibers were nearly 2-fold smaller than the respective parameters of untreated mice, but Vv of inflammatory infiltrates differed insignificantly in untreated and experimental groups (Table 2).

Long-term inhalation course (24 sessions over 3 months) with LFDZ and low dose of INH decreased the number of pulmonary granulomas by 3.4 times and reduced their size by 39% (Table 1). Vv of destruction foci, inflammatory infiltrates, and connective tissue fibers decreased by 31, 54, and 32%, respectively (Table

TABLE 2. Hepatic Morphometry of Mice Infected with MBT H37Rv and Treated with Antituberculous Inhalants (*m*±*SEM*, *n*=6)

Group	Vv of destruction foci, %	Vv of infiltrates, %	Vv of fibrous connective tissue, %
Series I (12 inhalations, 1.5 months)			
Untreated	23.40±1.63	2.70±0.30	2.00±0.26
LFDZ	12.10±0.98*	4.30±0.53*	1.20±0.23*
LFDZ+INH, 3 mg/kg	12.40±1.12*	2.20±0.23	1.00±0.21*
LFDZ+INH, 15 mg/kg	18.10±1.45*	3.30±0.29*	1.00±0.19*
INH, 3 mg/kg	19.40±1.00*	2.70±0.30	2.00±0.27
INH, 15 mg/kg	21.90±1.47	3.00±0.41	3.60±0.40*
Series II (24 inhalations, 3 months)			
Untreated	30.90±0.92	1.50±0.20	3.40±0.39
LFDZ	10.60±0.78*	1.60±0.17	3.30±0.29
LFDZ+INH, 3 mg/kg	12.90±0.91*	1.80±0.15	3.50±0.32
LFDZ+INH, 15 mg/kg	16.5±1.12*	1.50±0.13	2.30±0.28*
INH, 3 mg/kg	17.6±1.17*	1.10±0.11*	2.50±0.30*
INH, 15 mg/kg	28.3±0.94*	1.70±0.12*	3.80±0.31

1). In liver, Vv of destruction foci decreased by 58%, while Vv of connective tissue fibers and inflammatory infiltrates did not significantly differ from the respective parameters of untreated mice (Table 2).

Short-term inhalation course (12 sessions over 1.5 months) with standard dose of INH (15 mg/kg) decreased the number of pulmonary granulomas by 3.4 times and reduced their size by 41% (Table 1). Vv of inflammatory infiltrates decreased by 23%. However, Vv of destruction foci and connective tissue fibers did not significantly differ from the respective parameters of untreated mice (Table 1). In liver, Vv of destruction foci and inflammatory infiltrates did not significantly differ from the respective data of untreated mice, while the inhalation course increased Vv of connective tissue fibers by 1.8 times (Table 2).

Long-term inhalation course (24 sessions over 3 months) with standard dose of INH decreased the number of pulmonary granulomas by 5.8 times and reduced their size by 1.7 times, but did not significantly change Vv of destruction foci (Table 1). However, it produced a small but significant increase in Vv of connective tissue fibers and reduced Vv of inflammatory infiltrated by 2.2 times (Table 1). In liver, Vv of destruction foci significantly decreased by 9%, while Vv of inflammatory infiltrates significantly increased by 13% (Table 2). At this, Vv of connective tissue fibers did not change significantly.

Short-term inhalation course (12 sessions over 1.5 months) with low dose of INH decreased the number of pulmonary granulomas by 2.5 times and reduced their size by 30% (Table 1). At this, Vv of destruction foci and inflammatory infiltrates decreased by 36 and 34%, respectively. However, Vv of connective tissue fibers did not differ from the respective parameter of untreated mice (Table 1). In hepatic parenchyma, Vv of destruction foci decreased by 17%, although Vv of inflammatory infiltrates and connective tissue fibers were the same as in untreated mice (Table 2).

The long-term inhalation course (24 sessions during 3 months) with low dose of INH decreased the number of pulmonary granulomas by 4.3 times and reduced their size by 37% (Table 1). In parallel, Vv of inflammatory infiltrates dropped by more than 2-fold, while Vv of destruction foci and connective tissue fibers did not significantly differ from corresponding parameters of untreated mice. In liver, Vv of parenchymal destruction foci, inflammatory infiltrates, and collagen fibers significantly decreased by 43, 27, and 26%, respectively, in comparison with the corresponding parameters of untreated mice (Table 2).

Thus, INH inhaled for 3 months even at low dose demonstrated a high antimycobacterial potency attested by a pronounced decrease in the number and size of pulmonary granulomas known to be determined by the value of chemoattractant concentration gradient formed by living MBT [5]. Notably, a small dose of this antibiotic is characterized by a low hepatotoxicity despite a high prodestructive activity in the lungs (Tables 1, 2). Therefore, the inhalations of LFDZ with a low-dose INH could be successfully used in antituberculosis therapy due to antimycobacterial activity (Table 1) and diminished hepatotoxicity (Table 2) of these remedies with due account for the fact that duration of routine treatment of various forms of tuberculosis surpasses by far the length of experimental therapy in the present study. The routine use of dextrazide is explained by the potency of oxidized dextran (characterized by a high biocompatibility [1]) to elevate the incidence of MBT-loaded endosome fusion with macrophagal lysosomes [5,6].

Interestingly, the 3-month-long inhalation course of LFDZ with a low-dose INH decreased Vv of connective tissue fibers in the lungs by 32% (Table 1). Probably, this phenomenon would be more pronounced in experiments with longer inhalation course. Similar phenomenon we observed previously [4,5] with dextrazide employed *1*) outside the liposomes on the models of BCG-induced granulomatosis in more lengthy experiments and *2*) inside the liposomes in relation to its effect on expression of MMP-1 and MMP-9 [2].

The present study established a low hepatotoxicity, high therapeutic effectiveness, and easiness to use of LFDZ combined with a low dose of INH. The therapeutic effect of this composition was effectively manifested even at the early stages of the therapy attesting to a rather rapid "discharge" of INH from the liposomes and unbinding from oxidized dextran in macrophages. These processes are known to be accompanied by activation of the plastic processes in macrophages: synthesis of CSF-GM, DNA in MBT, and macrophagal lysosomal hydrolases [5] needed for realization of microbicide potential of INH, as well as synthesis of lysosomal proteases in macrophages targeted against MBT, including MBT with probable drug resistance. Because of low molecular weight, some INH molecules released from the conjugate with dextran after its hydrolysis can diffuse from the macrophages and together with administered "free" INH suppress MBT circulating in blood and lymph with the potential threat to persist in macrophages.

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