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Destructive Processes and Fibrotic Complications in the Liver of Mice with BSG-Induced Granulomatosis Treated with Anti-Tuberculous Drugs

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Three months after infection with *Mycobacterium tuberculosis* (MBT) from BCG vaccine, male BALB/c mice were treated with isonicotinic acid hydrazide, dextrazide (oxidized dextran), and liposome-encapsulated dextrazide intraperitoneally or in inhalations in a dose of 14 mg/kg (calculated for isoniazid) twice a week for 6 months. All these drugs exhibit different antimycobacterial efficiency. In the liver parenchyma, an up to 5-fold decrease in the number of destructed hepatocytes was observed depending on the efficiency of treatment. No destructive processes were observed in granulomas. Type I and III collagens were revealed around the granulomas; their content in the liver parenchyma was negligible. TNFα, IL-6, MMP-1, TIMP1 were expressed only by granuloma macrophages. As the number of damaged hepatocytes and size of inflammatory infiltrates in the liver parenchyma decreased, the content of both types of collagen decreased. No evidence of hepatotoxicity of MBT degradation products in macrophages *in vivo* was obtained; the assumption that fibrotic complications are only the post-destruction process was not confirmed. Fibrotic complications are supposed to be an "excessive" systemic nonspecific adaptive process aimed at the maintenance the so-called structural homeostasis initiated by activated M2-macrophages in granulomas.

Key Words: *tuberculosis granulomatosis; destruction; fibrosis; drugs*

Tuberculosis is the infectious disease caused by intracellular parasite, *M. tuberculosis* (MBT). Tuberculosis manifests in extensive destructive-inflammatory processes, systemic adoptive response (tuberculous granulomatosis), and fibrotic complications, which are presumed to be post-destructive [4]. Fibrotic complications do not disappear in patients even after drug suppression of MBT and have a negative impact on their health throughout the life. This necessitates studying of this problem for prevention and treatment of fibrotic complications.

It is known that MBT have no exotoxins, but their structures, among which are tuberculines and cord-factor, products of MBT degradation in macrophages (MP), exhibit high toxicity. In light of this, the destructive processes can be assumed to increase after drug suppression of MBT in granuloma macrophages. It has been demonstrated [7] that in untreated mice with tuberculous BCG-induced granulomatosis, no destructive processes in MP (the factor contributing to the release of MBT degradation products from MP) were observed in 6 months after infection. Hydropic degeneration was found in up to 45% hepatocytes and necrosis only in 2-3% hepatocytes. Foci with type I collagen also occupied up to 2-3% of the organ parenchyma. However, type I collagen content in granulomas with no destructive processes attained 16-18%. These findings do not confirm exceptionally post-destruction nature of fibrotic processes. On the contrary, destructive and fibrotic processes developed simultaneously in the liver parenchyma, but only fibrotic processes

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without destruction were seen in granulomas. Similar concurrency was observed in the lungs and liver in influenza caused by H5N1 virus [5]. Pathogenic MBT possess higher chemoattractant potential than mycobacteria from BCG vaccine and stronger attract and retain MP in the granuloma. Granulomas induced by pathogenic MBT sometimes grow up to several centimeters in size, while BCG-induced granulomas are usually <75 μ [4]. Long-term persistence of MBT in granuloma MP is associated with granuloma MP transformation into epithelioid cells; of these, type A epithelioid cells possess high hydrolytic potential [8]. They can constitute up to 80% of granuloma cells [4]. This leads to granuloma hypertrophy and degeneration due to the absence of microcirculatory structures. This can initiate spontaneous ischemic destructive processes in the largest granulomas that are the principal cause of destructive processes in organs and fibrotic complications in tuberculous and some other types of granulomatosis [4,6]. However, destructive processes and fibrotic complications in tuberculosis also take place in organ parenchyma with granulomas without necrotic foci. Destructive processes are not the only initiation factor of fibrotic complications. The model of BCG-induced granulomatosis without necrotic process in granulomas best suits for studying these phenomena [4].

The objective of the study was to investigate the relation between destructive process and the extent and localization of fibrotic complications in the liver of mice with BCG-induced granulomatosis treated with different anti-tuberculous drugs.

MATERIALS AND METHODS

The study was performed on 50 male BALB/c mice weighing 19-21 g (nursery of the Federal Research Centre Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences). The mice were kept under standard conditions with free access to water and food. The experiment was carried out according to Order No. 199n of Ministry of Health of Russian Federation (On the Principles of Good Laboratory Practices; February 1, 2016). We used the model of BCG-induced granulomatosis and drugs for its treatment (in the lungs) that exhibit different efficacy in suppression of MBT in MP and promote the formation of different amounts of MBT degradation product in MP [3].

The mice were infected by retroorbital injection of BCG vaccine (0.5 mg MBT microbial bodies in 0.2 ml of 0.85% NaCl per animal) and divided into groups. Group 1 mice received no treatment, group 2 mice were given isonicotinic acid hydrazide (INH) intraperitoneally; group 3 animals were treated with dextrazide intraperitoneally, group 4 received liposome-encapsulated dextrazide (LEDZ) intraperitoneally and group 5 received LEDZ via inhalation. In each group, the dose corresponded to 14 mg/kg INH. The mice were treated with the above drugs twice a week for 6 months; the treatment was started in 3 months after infection. LEDZ inhalations were performed in a Musson chamber with ultrasonic nebulizer at room temperature. The mice were taken out of the camera immediately after the evaporation of LEDZ.

Dextrazide is a composition of oxidized dextran with molecular weight of 40 kDa (5% oxidation) and INH. INH has very low molecular weight and, consequently, is rapidly excreted from cells, while increasing the dose leads to strengthening of its hepatotoxic effect. Conjugation of INH with oxidized dextran allows increasing its molecular weight prolongs excretion period up to 7 days [4], and its encapsulation in phosphatidylcholine liposomes (Sigma) with a size of 200-450 nm increased its dose in MP and decreased in hepatocytes [3,4].

The animals were sacrificed by cervical dislocation under ether anesthesia, the liver was isolated and fixed in 10% isotonic formalin. Standard histological processing was performed using an STP-120 tissue processor (ThermoScientific) and an EC-350 paraffin embedding center (ThermoScientific). Histological sections (4-5 μ) were sliced on a Microm microtome (ThermoScientific). To take into account all types of fibrous structures (collagens) the sections were stained with hematoxylin and eosin, after van Gieson, and by silver impregnation. To evaluate potential detection of the same fibrous structures of extracellular matrix, immunohistochemical staining for types I and III collagens was used. The data were summarized to get a holistic view of fibrotic complications extent and the potential of each method of visualization of type I and III collagens. In addition, indirect peroxidase method with monoclonal antibodies was used for detection of cells expressing TNFα, IL-6, MMP-1, and TIMP-1 (1:100; Abcam). Histological samples were examined under an AxioImager A1 microscope quipped with an AxioCam MRc camera and analyzed using AxioVision 4.7 software (Carl Zeiss). Volume densities (Vv) of collagen and reticulin fibers, destruction foci, cellular infiltrations, and granuloma MP were determined using a closed test system consisting of 100 regularly positioned testing points covering the area of 3.64×10^5 μ^2. Volume density of cells expressing TNFα, IL-6, MMP-1, and TIMP-1 and volume density of type I and III collagen fibers were determined using ImageJ software.

Statistica 10.0 (StatSoft, Inc.) was used for statistical processing of morphometry data, significance of differences between the means was assessed using

the Student's *t* test. The differences were considered significant at $p<0.05$.

RESULTS

Inflammatory infiltrates in the liver parenchyma were located around the destruction foci and were mainly presented by lymphocyte-monocyte lineage cells with single neutrophils and had mainly perivascular and periportal localization. Liver sinusoids were moderately plethoric, the Disse spaces were moderately dilated. Granulomas were mainly located in the perivascular and periportal liver parenchyma. The granulomas primarily contained MP with single epithelioid cells, no micronecrosis zones were seen. Some granulomas were surrounded by collagen fibers. Single fibroblasts were located on the periphery of the granulomas. Necrotized hepatocytes and hepatocytes in the state of fatty and hydropic degeneration formed groups were diffusely scattered in the liver parenchyma. They had maximum size in untreated mice (Table 1), which is typical for this model [2,4,7]. In animals treated with INH, the number of destruction foci was by $^1/_3$ lower than in untreated animals (Table 1). This can be attribute to the effect of INH [4]. INH stops MBT proliferation by blocking DNA synthesis and promotes MBT destruction by lysosome enzymes. Consequently, toxic MBT degradation products appear in granuloma MP; in addition, toxic INH metabolites also affected hepatocytes. According to the concept of toxicity of MBT degradation products, the destructive processes are assumed to be more intensive in mice treated with INH than in untreated mice, but this was not the case (Table 1). In mice receiving dextrazide and LEDZ (intraperitoneally and in inhalations), these processes had to be even more intensive, because these drugs and administration routes were more effective against MBT then INH. This was seen from lower volume densities of granuloma MP (Table 1) retained by the chemoattractant potential of live MBT [4]. However, in both groups of animals treated with LEDZ, the destruction processes were least pronounced (Table 1). Thus, the concept of high toxicity of products of MBT degradation in MP *in vivo* seems insufficiently substantiated.

Destructive non-drug complications in the liver parenchyma in tuberculosis remain an unsolved issue (Table 1), because destructive processes in the liver parenchyma were most extensive in untreated mice. It remains unclear which and how toxins enter the hepatocytes from MBT. Tuberculins and cord-factor are MBT structures and molecules released during degradation, but what is the mechanism? Endocytosis of live or dead MBT ultimates in their hydrolysis, which does not leave structures and molecules preserved. It cannot be excluded that some metabolic products produced by live MBT possess toxicity.

The size of inflammatory lymphocyte infiltration after treatment increased by 4-5 times and approached the size destruction foci in these groups (Table 1). Most likely their sizes were determined by the sizes of destruction foci. However, proinflammatory cytokines TNFα and IL-6 were expressed only by granuloma MP, though there were no destructive processes in granulomas. The number of granuloma MP expressing TNFα and IL-6 decreased along with the decrease in volume densities of destruction foci and infiltration (Table 1). Cell composition of granu-

TABLE 1. Results of Morphological Studies of the Liver in BCG-Infected Mice after 6-Month Treatment (*M±m*)

Volume density of studied components, %	Group 1 (untreated)	Group 2 (INH intraperitoneally)	Group 3 (dextrazide intraperitoneally)	Group 4 (LEDZ intraperitoneally)	Group 5 (LEDZ inhalations)
Granuloma macrophages	3.70±0.23	2.6±0.2	2.3±0.4	1.5±0.2*	1.3±0.1
Infiltration	2.90±0.17	1.90±0.14	1.5±0.2+	0.70±0.15*+	0.50±0.09*+
Destruction foci	20.7±1.6	13.8±0.9	7.4±0.8*+	4.10±0,12*+	3.9±0.3*+
Cells expressing TNFα	16.1±1.1	12.4±1.3	3.4±0.6*+	1.8±0.11*+	2.01±0.20*+
Cells expressing IL-6	12.3±0.8	8.20±0.35	3.7±0.4*+	1.10±0.13*+	1.30±0.15*+
Collagen fibers	3.8±0.5	5.2±0.6	2.9±0.3*	2.08±0.20*	2.1±0.6*
Reticulin fibers	8.3±0.9	6.2±0.7	2.7±0.2*	2.10±0.18*	2.01±0.15*
Type I collagen	4.70±0.24	5.90±0.33	4.00±0.48	3.20±0.63	2.80±0.65
Type III collagen	3.90±0.64	2.20±0.43	1.70±0.41	1.20±0.19	1.10±0.15
Cells expressing MMP-1	5.20±0.34	15.6±0.9	4.30±0.37*	2.7±0.3*+	2.50±0.29*+
Cells expressing TIMP-1	6.10±0.44	8.6±0.6	6.2±0.3*	6.40±0.24*	5.8±0.4*

Note. $p<0.05$ in comparison with *group 2, +group 1.

lomas was presented by epithelioid cells and MP (during morphometry, epithelioid cells were classified as MP). Fibroblasts and fibrous connective tissue were located around the granulomas (Figs. 1 and 2). The collagen content in the groups treated with dextrazide and LEDZ (both routes) little differed (Table 1). In the group of untreated mice and mice treated with INH, the total collagen content was similar and much higher than in other groups (Table 1). It should be noted that granulomas did not contain destructive cells, but fibroblasts and fibrous connective tissue of both types were observed at their periphery. Granulomas and their MP, in addition to the function "shielding" the body from MBT, can be considered as temporary organs acting as cleaners. Collagens of both types in granulomas were observed in the liver parenchyma in all 5 groups (Table 1). The total content of collagens of both types around the granulomas decreased as the size of destruction foci and the number of MP in granulomas decreased (Table 1). This attested to similar dynamics of the content of both types of collagens in terms of their correlation with the sizes of destruction foci and infiltrations in the liver parenchyma in mice of all groups.

The correlation between the decrease in the number of MPs expressing MMP1 in granuloma with the decrease in collagen content in mice of groups 3-5 is noteworthy (Table 1). The expression of MMP1 by MP significantly decreased during treatment of BCG-induced granulomatosis with INH and its compositions with oxidized dextran. Similar dynamics of MMP1 expression level was found in MP expressing TIMP-1 in mice of the compared groups (Table 1). This can be viewed as a process of homeostasis maintenance in the

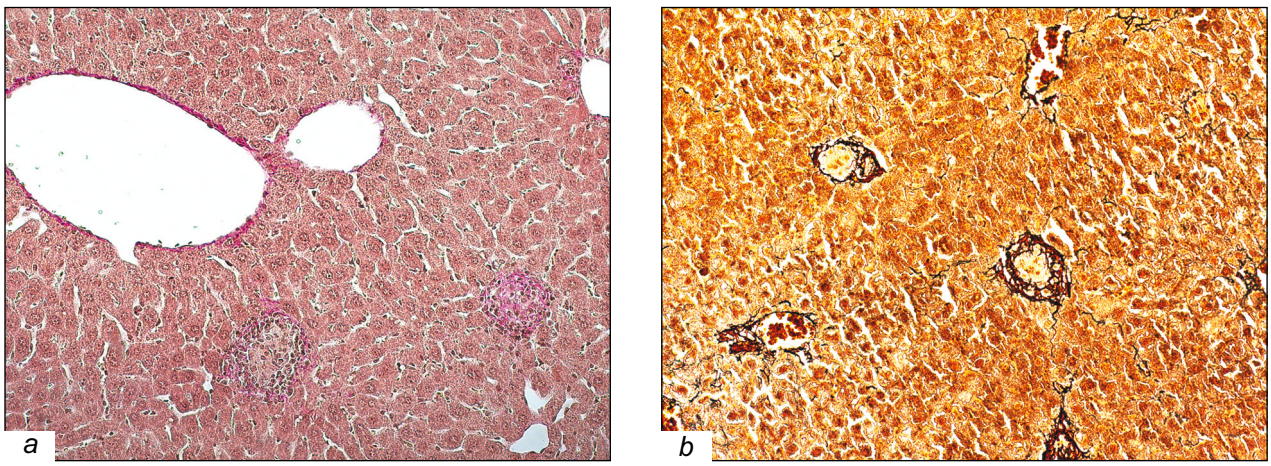

Fig. 1. Liver of group 1 mouse (no treatment). van Gieson staining (*a*), silver impregnation (*b*), ×200. *a*) Collagen fibers around and in the center of the granuloma, *b*) reticular fibers along the periphery of the granuloma.

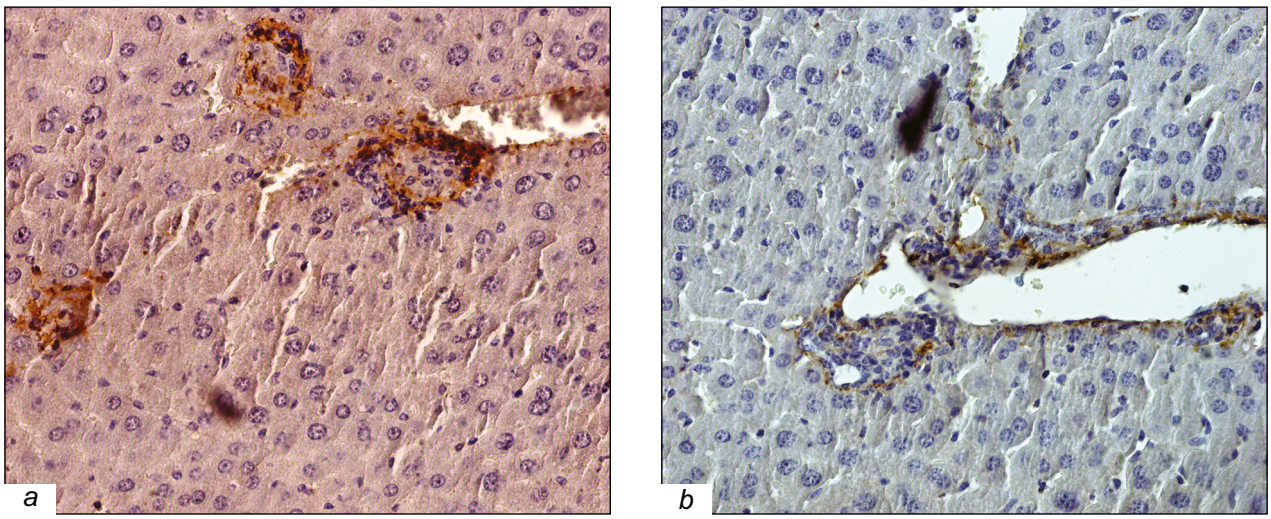

Fig. 2. Liver of group 1 mouse (without treatment). Collagen fibers around the granuloma. Immunohistochemical staining for types I (*a*) and III (*b*) collagens, ×200.

system of numerous intracellular matrix molecules [6]. The structures of live or killed by drugs MBT produce a hepatotoxic effect, but the mechanisms of this effect remain to be explored. However, it is evident that destructive processes have no strict typological relationship with fibrotic processes in granulomas in the used model. Fibrotic complications in liver in BCG-induced granulomatosis, especially in granulomas, are not determined by destructive processes in the liver parenchyma and granulomas. They are not a post-destructive reparative process either, because do not compensate lost structures and their functions. Granulomas consist of activated MP, the "major regulators" of inflammatory processes of various genesis. The ration of M1 and M2 phenotypes in MP population can be shifted in case of structural homeostasis disturbances. For instance, in similar experimental simulation, the number of M2-MP 3-fold surpassed that of M1-MP, which could be determined by the necessity of increasing the cleaning function under conditions of the risk of destructive process [1]. MP initiate the synthesis of intracellular matrix molecule protecting the inner structures of the body from contacts with external factors [2,6]. With a high value of intracellular matrix system for body, this non-specific adaptive process is realized by several types of cells with fibroplastic functions and is regulated by intricate mechanisms. Evidently, if regulation fails, this non-specific adoptive process can become "excessive" and transform into fibrotic complications. As the congestive phenomena in the liver were moderately expressed (moderate dilation of the Disse spaces) in all the studied groups, there is no sufficient reason to believe that this could be the main factor initiating the process of collagen synthesis in granulomas.

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