Effect of Antiserotonin Drug on the Development of Lung Fibrosis and Blood System Reactions after Intratracheal Administration of Bleomycin

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The effects of antiserotonin preparation on the development of the connective tissue in the lungs, reaction of the blood system, and the content of hemopoietic stem cells, committed hemopoietic and stromal precursors in BM, spleen, and peripheral blood were studied on C57Bl/6 mice with experimental toxic lung fibrosis caused by intratracheal administration of bleomycin. It was demonstrated that the antiserotonin drug inhibits the growth of the connective tissue in the lungs and attenuates the course inflammatory process primarily due to inhibition of the granulocytic lineage, which was related to suppression of hemopoietic stem cells. Reduced content of the stromal precursor cells in BM and spleen was noted.

Key Words: *antiserotonin preparation; lung fibrosis; hemopoiesis; stem cells; committed precursors*

The lungs actively participate in the regulation of blood level of bioactive substances and maintain the aggregate state of the blood by synthesizing thromboplastin, heparin, and plasminogen activator [3]. Enzymes inactivating norepinephrine, acetylcholine, prostaglandins, bradykinin, and angiotensin I were found in the lung tissue. Serotonin (5-HT) is most intensively bound and metabolized by the lungs (up to 95%).

5-HT participates in physiological processes, *e.g.* regulation of cell migration and proliferation, cytokine production, and vasoregulation, which is determined by abundance of 5-HT receptors (5-HT_1–5-HT_7) [10]. Disorders in 5-HT-activating mechanism stimulate fibroblast proliferation and promote the development of the connective tissue, which is believed to be a cause of retroperitoneal fibrosis and liver fibrosis [8,11]. Increased concentration of 5-HT and enhanced expression of its receptors (5-HT_{2A}, 5-HT_{2B}) in the lungs were observed during the development of lung fibrosis in mice after administration of bleomycin [7,10].

Mitogenic effects of 5-HT *in vitro* on hemopoietic precursors are now actively discussed [6,13,15]. Experimental studies showed that 5-HT increases the content of granulocytic-erythroid-macrophage-megakaryocyte (CFU-GEMM), granulocytic-macrophage (CFU-GM), erythroid (CFU-E), megakaryocyte (CFU-MG) CFU in umbilical cord blood [15]. There are interesting reports that 5-HT added to the culture medium stimulates the growth of fibroblast colonies (CFU-F) from the pulmonary artery of hypoxic rats and from adherent BM cells of cyclophosphamide-treated mice [6,13]. In this context we hypothesized that 5-HT plays an important role in the pathogenesis of lung fibrosis and involves hemopoietic and mesenchymal cells into the pathological process.

Here we studied the effect of antiserotonin drug on the development of lung fibrosis and on SC and

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committed hemopoietic and stromal precursors under conditions of intratracheal administration of bleomycin.

MATERIALS AND METHODS

The experiments were carried out on 2-2.5-month-old C57Bl/6 mice weighing 20 g (n=210, conventional mouse strain obtained from the nursery of Institute of Pharmacology, Tomsk Research Center, Siberian Division of Russian Academy of Medical Sciences).

Lung fibrosis was modeled by single intratracheal administration of 80 μg bleomycin (Bleomycetin, Lensfarm) in 30 μl physiological saline. Controls received an equivalent volume of physiological saline under the same conditions (control). Antiserotonin drug cyproheptadine (Peritol, EGIS pharmaceutical plant) was administered intraperitoneally in a daily dose of 2 mg/kg/day in 200 μl physiological saline until sacrifice. Intact animals served as the background (intact control).

On days 3, 7, 14, 21, and 25 after the start of bleomycin administration, the total leukocyte count and absolute count of their forms in the peripheral blood were evaluated using standard hematological methods. After that, the mice were sacrificed by CO_2 overdose and morphological picture of the lungs, BM cellularity, and the content of morphologically discernible cells of the granulocytic, lymphoid, and erythroid lineages were analyzed [1]. For histological studies, lung samples were fixed in 10% formalin. After standard histological processing, the specimens were embedded in paraffin blocks and histological sections (5 μ) were sliced. For visualization of the connective tissue, differential van Gieson staining was used (picrofuchsin, a component of the dye, stains collagen fibers into magenta color) [5]. The area of collagen fibers was measured in pixels using computer graphical analysis, the area of lung tissue was also measured in pixels, and then the percent of collagen fibers from the total area of lung tissue was calculated.

The formation of CFU-GEMM, CFU-G, and CFU-E was studied in culture of non-adhered nuclear fraction from BM, spleen, and peripherals blood. The intensity of CFU-F growth was studied in culture of adherent BM, spleen, and peripheral blood cells [1,2]. In all tissues, the content of polypotent hemopoietic precursors that formed colonies consisting of non-differentiated hemopoietic cells (CFU-N) and MSC was determined by the method of limiting dilutions.

The data were processed by standard methods of variation statistics. Significance of differences was evaluated using parametric Student's t test and non-parametric Mann–Whitney U test. For data expressed in fractions, Fisher exact test was used. The incidence of CFU-N and MSC was evaluated using generalized linear model for Poisson distribution.

RESULTS

According to modern concepts, alteration of the lung tissue after bleomycin administration proceeds in several stages. The first two stages of alteration are characterized by apoptosis of epithelial cells, massive infiltration of the lung tissue with lymphocytes, neutrophils, and plasma cells, and intensive release of various mediators [14]. Hyperproduction of collagen fibers disordering the lung tissue architectonics is observed during phase 3 of the pathological process.

In our experiments, similar changes in morphological picture of the lungs in C57Bl/6 mice after intratracheal administration of bleomycin were observed. Bleomycin administration led to interstitial infiltration of the alveoli and alveolar ducts with inflammatory cells (lymphocytes, neutrophils, plasma cells); venous plethora and hemorrhages were seen (on days 3-7; Fig. 1). Connective tissue growth was observed on day 14 of the study; on day 25, pronounced pneumofibrosis was seen (Table 1).

Simultaneously with activation of the inflammatory processes in the lungs, increased peripheral blood lymphocyte count (on days 3 and 14) and increased content of neutrophil granulocytes (on days 3, 7, 14, 21, and 25) and erythrokaryocytes (on day 14) in BM were detected (Fig. 2). Changes in the content of morphologically discernible blood cells in response to extreme influences of different nature are determined by activity of different classes of hemopoietic precursors [2,5]. Cell culture experiments showed that bleomycin treatment increased the content of polypotent (days 3 and 14), CFU-GEMM (day 3), CFU-E (day 3), and CFU-G (days 3, 7, 14, and 21) in BM (Fig. 2).

Thus, intratracheal administration of bleomycin stimulates hemopoiesis (primarily, granulocyte lineage), which attests to the involvement of the blood system into the development of lung tissue fibrosis.

The hypothesis on the mesenchymal-epithelial transition, according to which MSC can acquire characteristics of alveolar cells under the influence of various mediators and replenish their population in the lung tissue, is now confirmed in many experimental studies [12]. The decrease in the content of MSC in BM and spleen against the background of bleomycin treatment on day 3 (in comparison with intact control) was demonstrated by the method of limiting dilutions, which is probably related to cell migration to the site of injury and initiation of reparative processes.

Course treatment with cyproheptadine against the background of bleomycin administration reduced infiltration of alveoli with inflammatory cells (lympho-

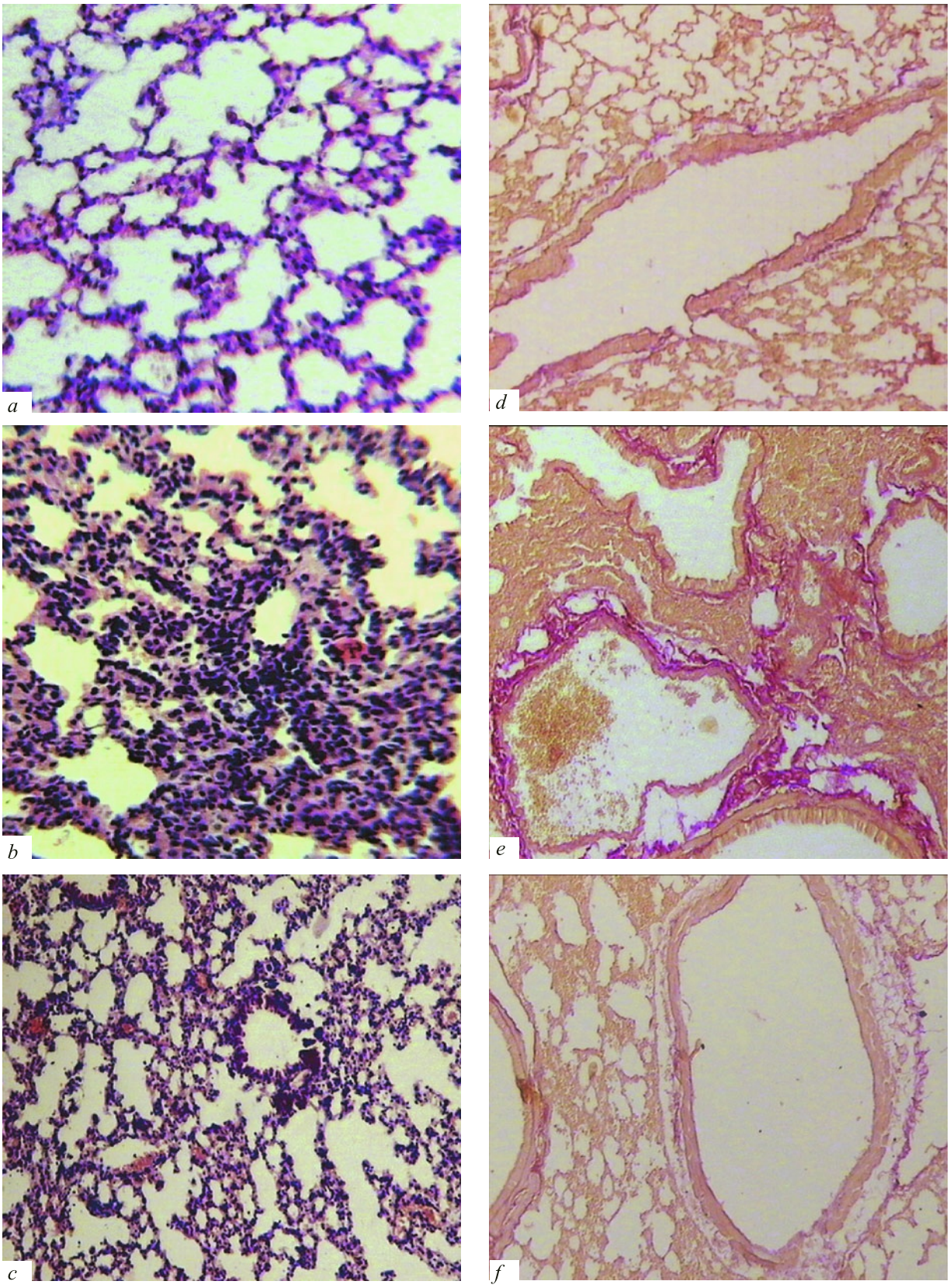

Fig. 1. Morphological picture of the lungs in C57Bl/6 mice after intratracheal treatment with bleomycin and administration of antiserotonin drug. *a, d*) lung of a control mouse; *b, e*) lung of a bleomycin-treated mouse; *c, f*) lung of a mouse receiving antiserotonin drug against the background of lung fibrosis modeling. *a-c*) hematoxylin and eosin staining, ×300. *d-f*) van Gieson staining with picrofuchsin, ×150.

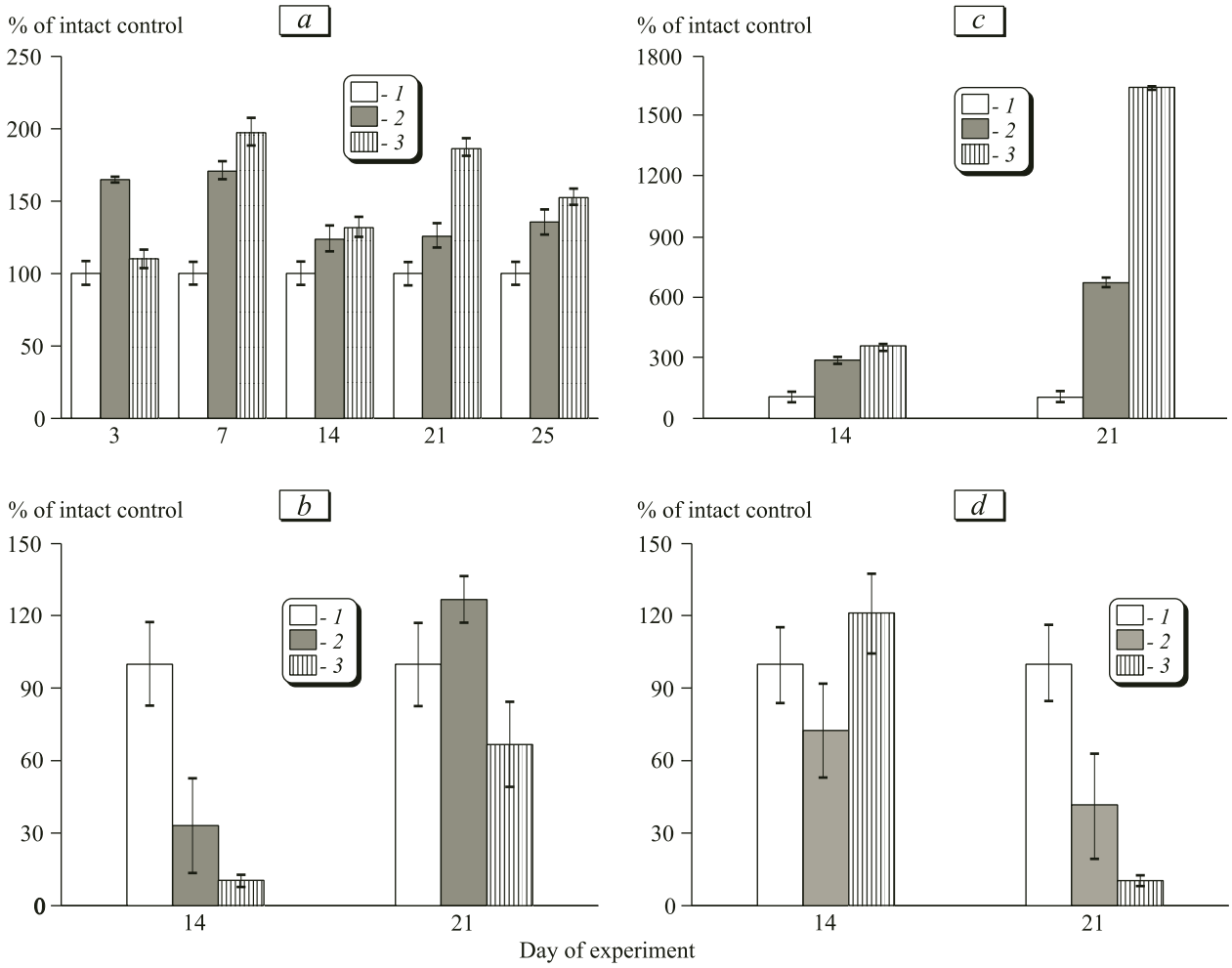

Fig. 2. Dynamics of the content of mature neutrophil granulocytes (*a*), CFU-GEMM (*b*), CFU-G (*c*), and CFU-F (*d*) in culture of BM cells from C57Bl/6 mice against the background of intratracheal bleomycin treatment and administration of antiserotonin drug. Here and in Fig. 3: ordinate: cell count; *1*) intact control; *2*) mice receiving bleomycin; *3*) administration of antiserotonin drug against the background of intratracheal bleomycin treatment.

cytes, neutrophils, and plasma cells); the content of mature neutrophil granulocytes in BM decreased under these conditions (day 3; Fig 1). At latter terms, the antiserotonin drug inhibited the growth of the connective tissue in the lungs. Thus, the content of collagen fibers was significantly lower than in the group of bleomycin control on day 14, 21, and 25 of the experiment and was comparable to that in the group of intact control on days 14 and 21 (Fig. 1, Table 1).

According to current views, antifibrotic effects of antiserotonin preparations (ketaserin, terguride, and selective blockers) are determined by blockade of 5-$HT_{2A,2B}$-receptors in the lungs and reduced collagen production by fibroblasts [8,11]. The preparations reduce the level of mRNA for transforming growth factor-β, plasminogen activator inhibitor-1, and connective tissue growth factor, *i.e.* the bioactive substances provoking the development of fibrosis. In light of this we did not exclude that the inhibitory effects of cyproheptadine on the development of fibrosis is related to its antiserotonin activity.

On day 21 of the experiment, the count of stab neutrophils in the peripheral blood of animals receiving cyproheptadine decreased to $9\pm4\times10^7$/liter (*vs.* $4.5\pm1.4\times10^8$/liter in the group of bleomycin control and $2.5\pm0.8\times10^9$/liter in intact control) and the content of mature neutrophil granulocytes in BM increased. Hemopoietic BM precursors demonstrated an opposite response to the preparation. The number of CFU-GEMM decreased, but the count of CFU-G surpassed the control value (without the drug; Fig. 2). The observed changes in hemopoiesis indexes are probably related to the anti-inflammatory effects of the antiserotonin drug, the blocker of H_1-histamine receptors [4].

5-HT stimulates proliferation of fibroblast cells of different origin under normal and pathological conditions (hypoxia, cytostatic myelosuppression) [6,13,15]. In light of this, reduced formation of CFU-F from

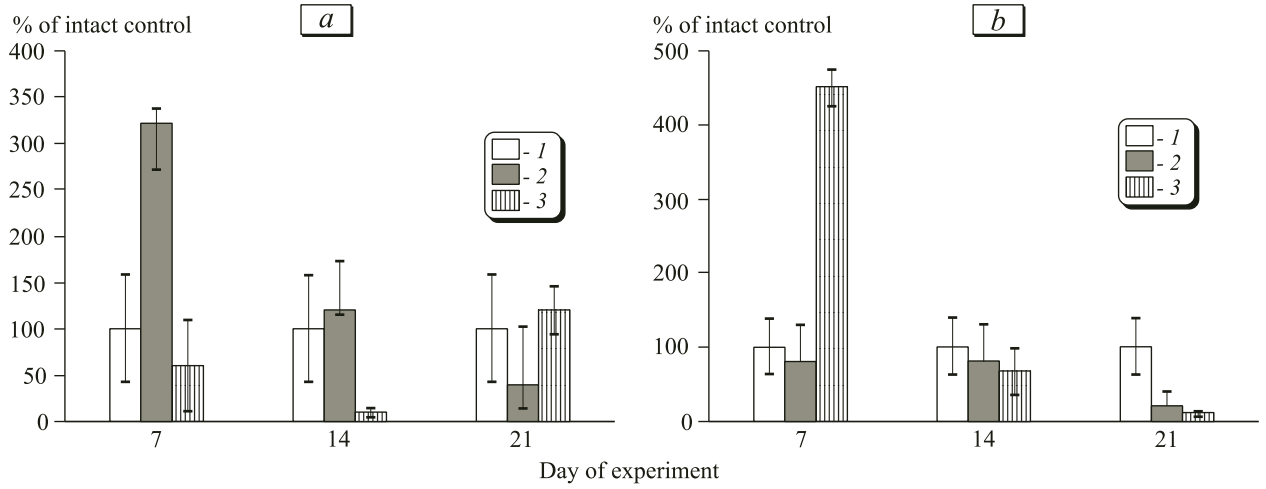

Fig. 3. Dynamics of the content of CFU-GEMM (*a*) and CFU-F (*b*) in culture of splenic cells from C57Bl/6 mice against the background of intratracheal bleomycin treatment and administration of antiserotoninc drug.

adherent BM cells (on day 21) and spleen (on day 14) under conditions of cyproheptadine suppression of the serotoninergic system is an expected phenomenon (Fig. 3). Taking into account the well-known dependence of collagen synthesis by fibroblasts on HT-5 [7], we can conclude that the revealed phenomenon is determined by systemic inhibitory effect of the drug on stromal cells.

Thus, cyproheptadine treatment against the background of bleomycin administration suppressed the inflammatory response and inhibits connective tissue growth in the lungs by reducing activity of committed stromal precursor cells.

TABLE 1. Percent of Collagen Fibers of the Total Area of Lung Tissue in C57Bl/6 Mice under Conditions of Intratracheal Bleomycin Treatment and Cyproheptadine Administration against the Background of Lung Fibrosis Modeling (*M*±*m*)

Day of experiment	Bleomycin	Cyproheptadine
Intact control	1.86±0.25	1.86±0.25
Day 7	1.98±0.58	1.27±0.62
Day 14	3.09±0.23*	2.28±0.31^{+}
Day 21	2.94±1.04*	1.99±0.82^{+}
Day 25	5.33±1.14*	3.22±0.45^{*+}

Note. $p<0.05$ in comparison with: *intact control, $^{+}$mice receiving bleomycin alone (bleomycin conrol).

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