Mechanisms of Hepatoprotective Effect of Preparation Containing Superlow Doses of Antibodies to Granulocytic Colony-Stimulating Factor

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The hepatoprotective effects of superlow dose preparation of antibodies to granulocytic CSF were studied on a model of CCl_4-induced hepatitis. The preparation exhibited high antiinflammatory and antisclerotic activities determined by stimulation, mobilization, and determined homing of mesenchymal stem cells into damaged liver with subsequent differentiation of these cells into mature hepatocytes.

Key Words: *hepatitis; granulocytic colony-stimulating factor; superlow doses of antibodies; stem cells*

Diseases of the liver and bile ducts are very prevalent all over the world and rank among the first causes of morbidity and mortality in Russia. Health hazards and social significance of liver diseases of different origin dictate constant development of new effective pathogenetically-based methods of drug therapy and prevention of these pathologies. Recent data on the properties and regularities of vital activity of multipotent precursor cells opened new vistas in the development of a novel approach, cell therapy, in the treatment of many diseases [5,8, 11]. The possibility of mobilization of endogenous stem cells with their subsequent homing in damaged tissues by means of pharmacological agents is not doubted; one of these agents, as many scientists think, is granulocytic CSF (G-CSF) [9,10]. On the other hand, it is known that superlow doses of antibodies to some regulators of physiological functions can act similarly as bioactive substances *in vivo* [6].

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We studied hepatoprotective activity of the preparation of superlow doses of antibodies to G-CSF (anti-rhG-CSF) in C12+C30+C200 dilution (equivalent concentration of 10^{-24} fractions of total mass) on the model of CCl_4-induced hepatitis.

MATERIALS AND METHODS

Experiments were carried out on random bred rats (n=50; 250-300 g) and 2-month-old CBA/CaLac mice (n=115; 18-20 g) of both sexes. Conventional certified 1st category linear mice were obtained from Breeding Center of Department for Experimental Biomedical Simulation, Institute of Pharmacology.

In rats, hepatitis was induced by intragastric administration of 50% CCl_4 solution in olive oil in a dose of 2 ml/kg twice a week for 3 weeks (6 doses). In mice the injury to the liver was induced by intragastric administration of 20% CCl_4 in olive oil (0.2 ml/mouse) according to the same protocol. Superlow-dose anti-rhG-CSF preparation (Materia Medica Holding) was administered to rats (1 ml)

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and mice (single daily dose of 0.2 ml) orally starting from the first injection of CCl_4 for 40 days. Control animals received distilled water in equivalent volumes according to the same protocol.

Rat mortality and body weight gain were evaluated throughout the experiment, liver weight coefficient, *i.e.* ratio of the organ weight (mg) to body weight (g), were determined on days 21 and 40. Biochemical studies included measurements of serum ALT, AST, and alkaline phosphatase (AP) on days 7, 14, 21, 28, and 40; liver morphology was studied on days 21 and 40 [4]. Activities of serum enzymes were evaluated by routine methods on a semiautomated biochemical analyzer (Cormay) and using standard kits (Cormay and Vital Diagnostic). Blood for the analysis was collected through a catheter implanted into the femoral artery, with subsequent ligation of vessels. The number of cells in infiltrates was determined on histological sections of the liver stained with hematoxylin and eosin using G. G. Avtandilov ocular grid [1] containing 25 test points. The numbers of infiltrate cells in the test points of the grid were counted in 20 visual fields. Relative area of infiltration was estimated as the ratio of grid points covering the infiltrate cells to all points of the grid in 20 visual fields (500 points). Connective tissue area was evaluated by computer processing of graphic data. To this end, the area occupied by picrofuchsin-stained structures was measured on a standard area of liver section (succession of microphotographs of 10 visual fields, made by Digital micro microvideocam with image transfer to Elecard computer) and the percent ratio to selected standard area was calculated.

Patameters of peripheral blood (total leukocyte count, differential count), structural and functional organization of the bone marrow, binding capacity of bone marrow microenvironment cells towards mesenchymal precursors, content of fibroblast CFU (CFU-F) [3], counts of mesenchymal stem cells in the bone marrow and peripheral blood [8], and count of hepatic precursor cells in the liver were evaluated in mice on days 3, 7, 10, and 14 after the last CCl_4 treatment.

The counts of CFU-F in the bone marrow and peripheral blood, including, apart from stromal precursor cells, mesenchymal stem cells [11], were evaluated by cloning in semisolid methylcellulose medium consisting of 50% DMEM (Sigma), 20% FCS (HyClone), 280 mg/liter L-glutamine (Sigma), 50 mg/liter gentamicin (Serva), and 30% methylcellulose (Sigma). The cells were cultured for 7 days in a CO_2 incubator (Jouan) at 37°C, 5% CO_2, and 100% humidity. After incubation the colonies (cell aggregations of at least 50 cells) were counted under an inverted microscope.

The counts of mesenchymal cells in the bone marrow (at all terms of the study) and peripheral blood (on day 3 after the last CCl_4 treatment) were evaluated by the method of limiting dilutions [8] in our modification. The cells (peripheral blood myelokaryocytes and mononuclears) in suspensions of different concentration (maximum 750,000/ml, minimum 2500/ml) were placed in a medium of the following composition: 90% DMEM (Sigma), 10% FCS (HyClone), 280 mg/liter L-glutamine (Sigma), 50 mg/liter gentamicin (Serva), 8000 U/liter heparin (Biochemie), and 15 ng/ml fibroblast growth factor (FGF-basic; Sigma). Twelve samples of each cell dilution were put into 96-well plastic plates (Costar) with bottoms coated with 1% gelatin and incubated for 4 weeks in a CO_2 incubator (Jouan) at 37°C, 5% CO_2, and 100% humidity; the medium was changed twice a week. After incubation fibroblast-like cells were counted in each well. If their number was at least 10, the well was considered as "positive", if less than 10, the well was considered "negative".

The content of regional stem cells in the liver was evaluated by hepatic tissue cloning in culture medium: 90% DMEM (Sigma), 10% noninactivated FCS (HyClone), 6 g/liter glucose, 280 mg/liter L-glutamine (Sigma), 50 mg/liter gentamicin (Serva), 8000 U/liter heparin (Biochemie), 25 mg/liter porcine multicomponent insulin (Novo Nordisk), and 10 ng/ml stem cell factor (SCF; Sigma). The material was incubated for 10 days in a CO_2 incubator (Jouan) at 37°C, 5% CO_2, and 100% humidity. After incubation the colonies (round or irregularly-shaped formation of at least 30 cells) were counted under an inverted microscope.

The results were processed by methods of variation statistics using Student's *t* test and nonparametric Wilcoxon—Mann—Whitney *U* test. The incidence of mesenchymal stem cells in the bone marrow and peripheral blood was evaluated using generalized linear model for Poisson distribution. Correspondence of the limiting dilutions data to unidimensional Poisson model was evaluated by linear log-log regression. The theoretical fraction of negative wells (μ_i) was distributed as $\mu_i=\exp(-fx_i)$, where f is the incidence of mesenchymal stem cells and x_i is the number of cells put into well [7,8]. Statistica 6.0 software was used.

RESULTS

Rat mortality in groups receiving distilled water and anti-G-CSF started simultaneously (after the 3rd CCl_4 treatment) and by the end of the experiment

was 21.2 and 15.1% in the control and experimental groups, respectively. The liver in dead animals was enlarged, yellow, of kaolin-like consistency with focal hemorrhages and macroscopic signs of acute degeneration. The percentage of body weight gain in rats treated with anti-G-CSF on day 14 of the experiment was significantly higher than in the control. No differences in the weight index of the liver in these two groups were detected.

Biochemical tests of the serum showed increased ALT activity in control and experimental groups on days 7, 14, and 21 and AST activity on days 7 and 14 of the experiment (after the start of CCl_4 treatment). However, serum concentrations of these enzymes were significantly lower in rats treated with anti-G-CSF than in animals injected with the solvent at all terms. Simulation of chronic liver injury increased serum levels of AP in all groups on days 7, 14, and 21 after the start of CCl_4 treatment. On the other hand, serum concentrations of AP were lower than in the control group after treatment with superlow doses of antibodies to G-CSF at all terms of the experiment.

Examination of histological preparations of rat liver stained with hematoxylin and eosin showed pronounced disorders in the lobular structure of the liver in control group on day 21. Fields of granulation tissue replacing dead hepatocytes with the formation of new vessels and hepatic ducts were seen in the preparations. Numerous Counselman bodies were seen; pronounced large-droplet fatty degeneration was observed in retained hepatocytes. Some cells fused to form fatty cysts. On the other hand, significant regeneration hypertrophy of hepatocytes and abundant hepatocyte mitoses were seen. Infiltration was diffuse, its relative area being 25.48± 3.67%. Relative area of the connective tissue was 7.53±1.61%.

Evaluation of possible effect of the drug on liver morphology showed that treatment with anti-G-CSF during simulation of CCl_4 hepatitis did not impair the lobular structure of the liver. Counselman bodies were seen in few cases, and fatty dege-

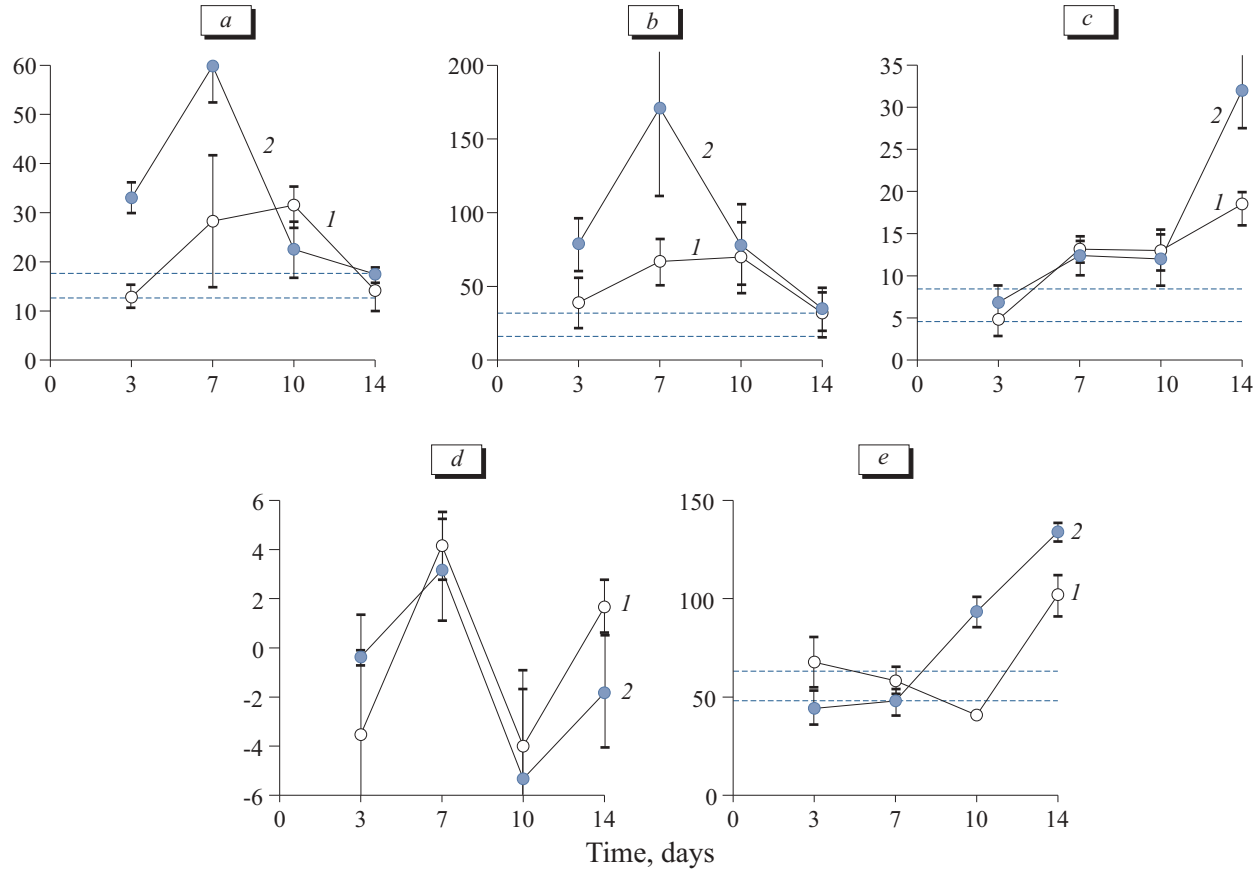

Fig. 1. Time course of CFU-F (*a*), mesenchymal stem cells (*b*) in the bone marrow, CFU-F in the peripheral blood (*c*), capacity to bind CFU-F by bone marrow stromal cells (*d*), and CFU count in the liver (*e*) in CBA/CaLac mice with CCl_4 hepatitis injected with distilled water (*1*) and anti-G-CSF (*2*). Ordinate: *a*) per 250,000 myelokaryocytes; *b*) per 10^6 myelokaryocytes; *c*) per 250,000 myelokaryocytes; *d*) arb. units; *e*) per 10^5 nuclears. Confidence intervals at $p<0.05$. Space between intermittent lines: confidence interval region the parameter in intact mice at $p=0.05$.

neration of hepatocytes was mainly small-droplet. On the other hand, infiltration of the portal tracts was less significant, but still remarkable. As a rule, infiltration did not penetrate into the lobules. On the whole, the relative area of infiltration was significantly lower than in the control group (12.08± 1.06%). In addition, the superlow-dose preparation of anti-G-CSF reduced the connective tissue area almost 3-fold (1.94±0.36%).

On day 40 of the experiment morphological signs of hepatitis in the liver were less pronounced in both groups compared to day 21. Fatty degeneration of hepatocytes persisted, but became small-droplet in the control group as well. The relative area of liver parenchyma infiltration (and hence, the inflammatory process) decreased in both groups. However, it was significantly lower in rats treated with anti-G-CSF (11.66±0.70%) compared to animals receiving the solvent (15.4±1.2%). The connective tissue occupied significantly lesser area (1.44±0.17%) in animals treated with superlow doses of antibodies to G-CSF compared to the control (2.75± 0.42%).

Hence, biochemical and morphological studies of the liver in rats detected significant hepatoprotective activity of the preparation of superlow doses of antibodies to G-CSF. Moreover, this preparation exhibited pronounced antiinflammatory and antisclerotic effects.

The next stage in the study of possible hepatoprotective effects of the preparation was experiments on mice, aimed at investigation of its effect on the pool of bone marrow, circulating, and regional stem cells.

Changes in the composition of peripheral blood cells usually reflect functional activity of stem cells, the earliest shifts being observed in the quantitative and qualitative composition of leukocytes as the most mobile blood cells [3]. Experiments showed that treatment with CCl_4 paralleled by distilled water administration led to a significant reduction in the total leukocyte count at the expense of a decrease in the counts of stab and segmented neutrophils in the peripheral blood. On the other hand, treatment with anti-G-CSF after the end of hepatitis induction on days 10 and 14 led to a still greater drop in the total count of leukocytes. However, these changes were largely due to the decrease in the count of mononuclear cells (monocytes on day 7 and lymphocytes virtually during all periods of the study), but not in the count of peripheral blood polymorphonuclear cells. The dynamics of the counts of various leukocyte forms in the control and experimental groups seemed to be determined by the toxic effect of CCl_4 on the hemopoietic tissue and activation (most pronounced during administration of the preparation) of the redistribution reactions in the blood system, concerning primarily migration of mononuclears (fraction of cells including stem cells).

Culture studies of the mechanisms of compensation of the "deep" reserve showed increased count of CFU-F in the bone marrow tissue of controls on days 7 and 10; CFU-F fraction consisted of stromal element precursors and true stem cells, which was confirmed by increased count of mesenchymal stem cells in hemopoietic tissue during the same periods of the study (to 279.1 and 291.7% of the basal level on days 7 and 10, respectively). Changes in the stem cell pool seemed to be nonspecific and caused by activation of the stress-realizing systems during the formation of liver disease induced by the toxic agent. Moreover, the counts of fibroblast precursor cells in the peripheral blood on days 7, 10, and 14 of the experiment and that of mesenchymal stem cells on day 3 increased, indicating mobilization of stem cells of different degree of maturity in this condition of the liver representing an extreme exposure. Release of these elements into the blood was observed against the background of increased count of macrophage-positive (days 7 and 10) and macrophage-negative (day 7) hemopoietic islets, despite increased capacity of cell components of the bone marrow microenvironment to bind stem cells, which suggests other mechanisms of mesenchymal precursor mobilization under conditions of CCl_4 intoxication. On the other hand, the study of the time course of the content of regional precursor cells in the liver detected a decrease in CFU count in the liver, reaching a statistically significant level on day 10 of the experiment and replaced by a significant increase in their count only by the end of the experiment (day 14), when the toxic effect of CCl_4 were presumably eliminated (Fig. 1). These results indicate activation of the mechanisms of compensation of the "deep reserve" (bone marrow stem cells) in chronic CCl_4 intoxication, these reserves being, however, insufficient and/or incompetent for hepatic tissue reparation, leveling the damaging effect of CCl_4.

The study of possible stimulation of these compensatory adaptive mechanisms on the model of liver disease with antibodies to G-CSF in superlow doses showed that it led to accumulation of committed and true (mesenchymal) stem cells in the bone marrow, in comparison with the control, on days 3 and 7 of observation with subsequent more pronounced, in comparison with the control (1.3 times), release of mesenchymal stem cells into the peripheral blood (up to 203.2% of the basal level, day 3) and of CFU-F (day 14) containing mesen-

chymal stem cells. Similarly as in animals receiving no preparation, these shifts did not depend on binding capacity of the bone marrow stromal elements and its structural and functional organization. However, these shifts in the stem cell pool of the hemopoietic tissue and peripheral blood indicating mobilization and migration of mesenchymal stem cells were paralleled by their homing in the hepatic tissue, manifesting in a pronounced increase in the count of precursor cells in the liver on days 10 and 14 of the experiment (up to 228.9 and 131.4%, respectively, in comparison with animals receiving the solvent; Fig. 1).

Hence, the preparation of superlow anti-G-CSF doses led to pronounced changes in the mesenchymal stem cell function, aimed at stimulation of reparation of damaged liver.

Therefore, the preparation of superlow doses of antibodies to G-CSF exhibited a pronounced hepatoprotective effect, realized due to stimulation, mobilization, migration, and determined homing of mesenchymal stem cells into the liver and their subsequent differentiation into mature hepatocytes.

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