Role of Stem Cells in Adaptation to Hypoxia and Mechanisms of Neuroprotective Effect of Granulocytic Colony-Stimulating Factor

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The role of regional cerebral neural precursor cells in restoration of the CNS activity during the development of hypoxic encephalopathy and the participation of the bone marrow mesenchymal stem cells in the formation of blood system reactions in hypoxia are shown. The mechanisms of neuroprotective effect of granulocytic colony-stimulating factor in severe oxygen insufficiency are studied. The specific psychopharmacological effect of granulocytic colony-stimulating factor is due to mobilization and determined homing of the bone marrow mesenchymal stem cells in damaged CNS zones with subsequent differentiation of these cells into specialized elements and due to activation of erythropoiesis.

Key Words: *hypoxia; encephalopathy; mesenchymal stem cells; neural precursor cells; granulocytic colony-stimulating factor*

Hypoxia as a typical pathological process is associated with virtually all known diseases. The specific metabolism of nerve tissue cells determines greater sensitivity of the brain to oxygen deficit and the maximum damage of primarily CNS structures in hypoxia. Decompensation of the adaptation mechanisms leads to progressive neurological and visceral disorders — development of encephalopathies [1].

Intensive development of biomedical science in the sphere of cell technologies led to discovery of resident cells (stem cells — SC) in an adult organism. These cells are characterized by a unique capacity to self-maintenance and, if necessary, to differentiation into virtually all specialized elements. Stem cells are a population of self-renewing cells intended for tissue regeneration in response to physiological loss of elements or their death caused by a damaging factor [6]. On the other hand, the role of regional neural precursor cells of the brain and possible participation of bone marrow mesenchymal stem cells (MSC) in compensation for the disorders in CNS activity (due to their migration into damaged zone) under conditions of oxygen deficiency and during adaptation to hypoxia in general remains unclear. In addition, the possibility of improving the adaptation reserve in oxygen deficiency by means of pharmacological activation of the "deep reserve" mechanisms (endogenous bone marrow MSC) remains not studied.

We studied the mechanisms of adaptation to hypoxia with participation SC and the effect of granulocytic CSF (G-CSF) on these mechanisms.

MATERIALS AND METHODS

The study was carried out on 357 CBA/CaLac mice (18-20 g). Certified conventional 1st category animals were obtained from Breeding Center of De-

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partment for Biomedical Simulation, Institute of Pharmacology. Hypoxic hypoxia and two variants of hemic hypoxia were simulated. Hypoxic hypoxia was induced by single and double (with 10-min interval) exposure of animals in a 500-ml sealed chamber. The mice were removed from the chamber within 10-15 sec after the end of generalized convulsions and/or respiration arrest (determined visually). Hemic hypoxia was induced by intraperitoneal injection of phenylhydrazine hydrochloride (30 and 150 mg/kg) and by bleeding (puncture of the retroorbital sinus and blood letting through heparin-washed graduated Pasteur pipette): 30% circulating blood volume (single portion) or 70% circulating blood volume (3 portions over 2-3 h). The volume of bleeding was estimated from the approximate volume of circulating blood volume in rodents ($^1/_{13}$ of body weight). Single hypoxic hypoxia in a sealed chamber, injection of hemolytic poison in a dose of 30 mg/kg, and the loss of 30% circulating blood volume did not lead to significant changes in the psychoneurological status. Severe oxygen deficiency, injection of 150 mg/kg phenylhydrazine hydrochloride, and loss of 70% circulating blood volume were associated with the formation of encephalopathy, which was seen from the development of amnesia during reproduction of conditioned passive avoidance reflex and disturbed orientation and exploratory behavior of animals in the open field [2]. Recombinant human G-CSF (rhG-CSF) neupogen (Hoffman la Roche), dissolved in 0.2 ml RPMI-1640, was injected directly after induction of severe hypoxia of different genesis subcutaneously in a single daily dose of 125 μg/kg for 5 days. Peripheral blood and bone marrow hemopoiesis values were evaluated by standard hematological methods [5] in animals with encephalopathy and in mice injected with rhG-CSF on days 1, 3, 5, and 7. On days 1, 3, and 7 the counts of MSC in the bone marrow and peripheral blood were determined by the method of limiting dilutions [10], numbers of erythroid and granulomonocytic colony-forming units (CFU-E and CFU-GM) in the bone marrow [5] and content of neural precursor cells in the cerebral paraventricular area [4] were determined in all experimental groups. The direct effect of rhG-CSF on neural precursors was evaluated by the level of neurosphere formation in brain cell cultures, derived from intact mice and mice with posthemorrhagic encephalopathy, after adding 5 g/liter neupogen. Cell cultures without cytokine, derived from respective animals, served as the control. The results were processed by methods of variation statistics using Student's *t* test and nonparametric Wilcoxon—Mann—Whitney *U* test. The incidence of MSC in the bone marrow and peripheral blood was evaluated using generalized linear model for Poisson distribution. The correspondence of the limiting dilutions data to Poisson unidimensional 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 MSC and x_i number of cells put into a well [8]. Statistica 6.0 software was used.

RESULTS

Hypoxic exposure of different nature causing no coarse disorders in the psychoneurological status led to increase in the MSC count in the bone marrow on day 1 of the experiment (up to 193.5, 264.5, and 309.7% of the basal level in pressure hypoxic hypoxia, hemolytic anemia, and blood loss, respectively) with subsequent (on days 3 and 7) normalization of the parameter. The count of MSC in the peripheral blood was retained at the initial level (Fig. 1). Changes in the pool of multipotent SC were in all cases paralleled by an appreciable increase in the content of CFU-E (days 3 and 7) and CFU-GM (days 1, 3, and 7) in the bone marrow (Fig. 2), followed, as we showed previously [3], by hyperplasia of the erythroid and, to a lesser extent, granulocytic hemopoietic stems in these variants of hypoxia. On the other hand, the count of neural precursor cells in the bone marrow tended to increase in all models. In hypoxic hypoxia and loss of 30% circulating blood volume these shifts reached statistical significance on day 3 of the experiment (up to 135.3 and 136.9% of basal levels, respectively; Fig. 3).

Similar shifts were observed in oxygen insufficiency causing disorders in CNS activity. The development of encephalopathy had virtually no effect on the shifts in MSC counts in the bone marrow on day 1 after hypoxic exposure, except a decrease in the count of these cells during simulation of posthemorrhagic encephalopathy. In hypoxic hypoxia and injection of phenylhydrazine in a high dose the count of MSC significantly increased in comparison with the basal level and attained the level of the parameter in hypoxia models not associated with the formation of cerebral abnormalities. On the other hand, more severe exposure significantly increased MSC count in the bone marrow on day 3 of the experiment: up to 240.4% of basal level in hypoxic hypoxia and hemolytic anemia and up to 264.5% of initial level in severe hemorrhage. These changes did not lead to the increase in MSC count in the peripheral blood (Fig. 1). The pool of unipotent hemopoietic precursors in the hemo-

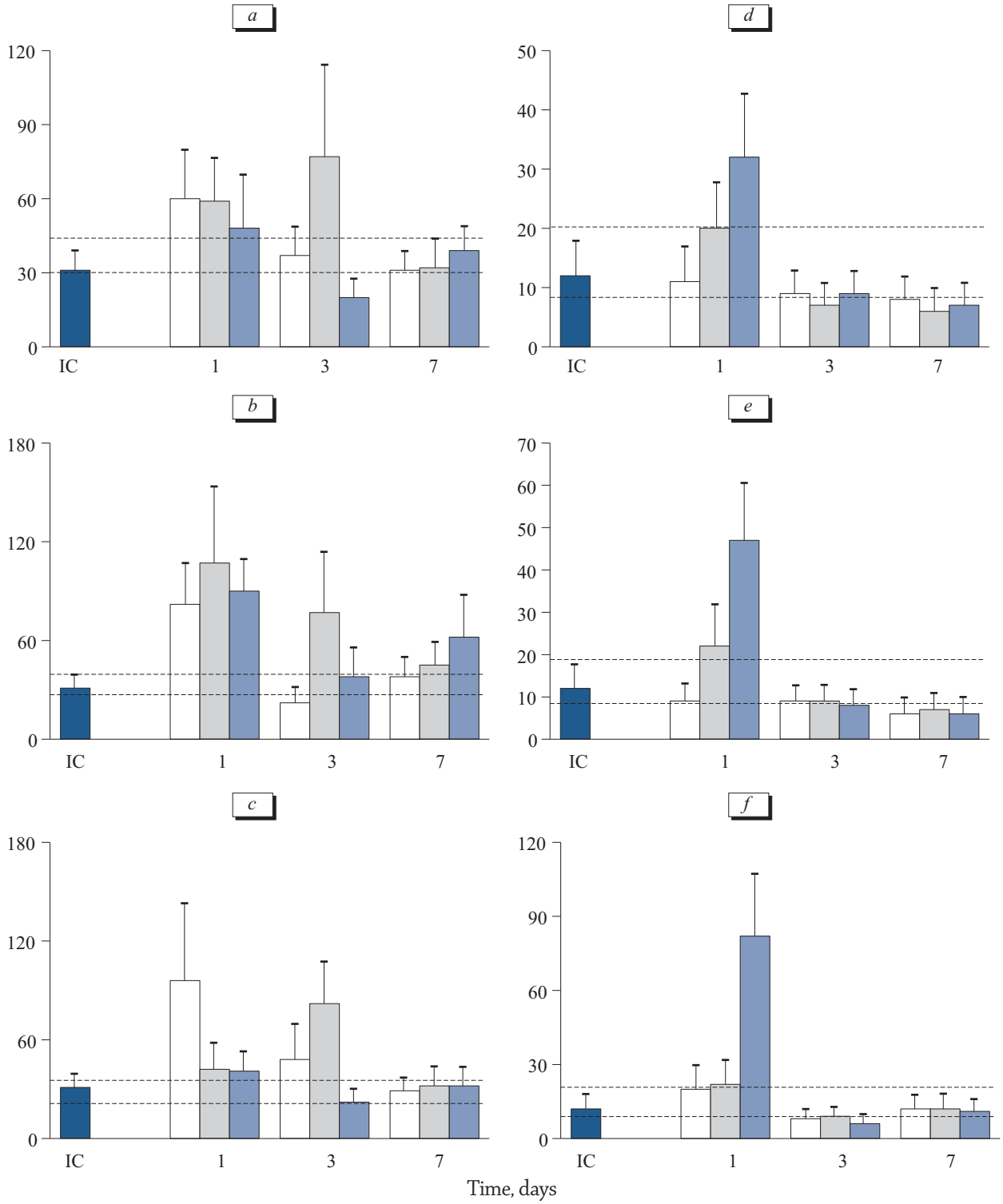

Fig. 1. Time course of the count of mesenchymal stem cells in the bone marrow (*a-c*) and peripheral blood (*d-f*) of CBA/CaLac mice in hypoxic hypoxia (*a*, *d*), hemolytic anemia (*b*, *e*), and blood loss (*c*, *f*) causing no disorders in CNS activity (open bars), associated with encephalopathy development (gray bars), and in animals injected with G-CSF during development of brain pathology (blue bars). Ordinate: the value per 10^6 peripheral blood myelokaryocytes (*a-c*) and per 10^6 mononuclears (*d-f*). Here and in Figs. 2, 3: space between intermitted lines: confidence interval for the value in intact controls (IC) at $p<0.05$.

poietic tissue decreased in all groups (Fig. 2), because of their damage induced by hyperactivation of the adrenergic systems [3]. The counts of neural precursor cells in the brain (paraventricular area) in animals exposed to severe hypoxia increased in all cases, reaching statistical significance on day 3 in

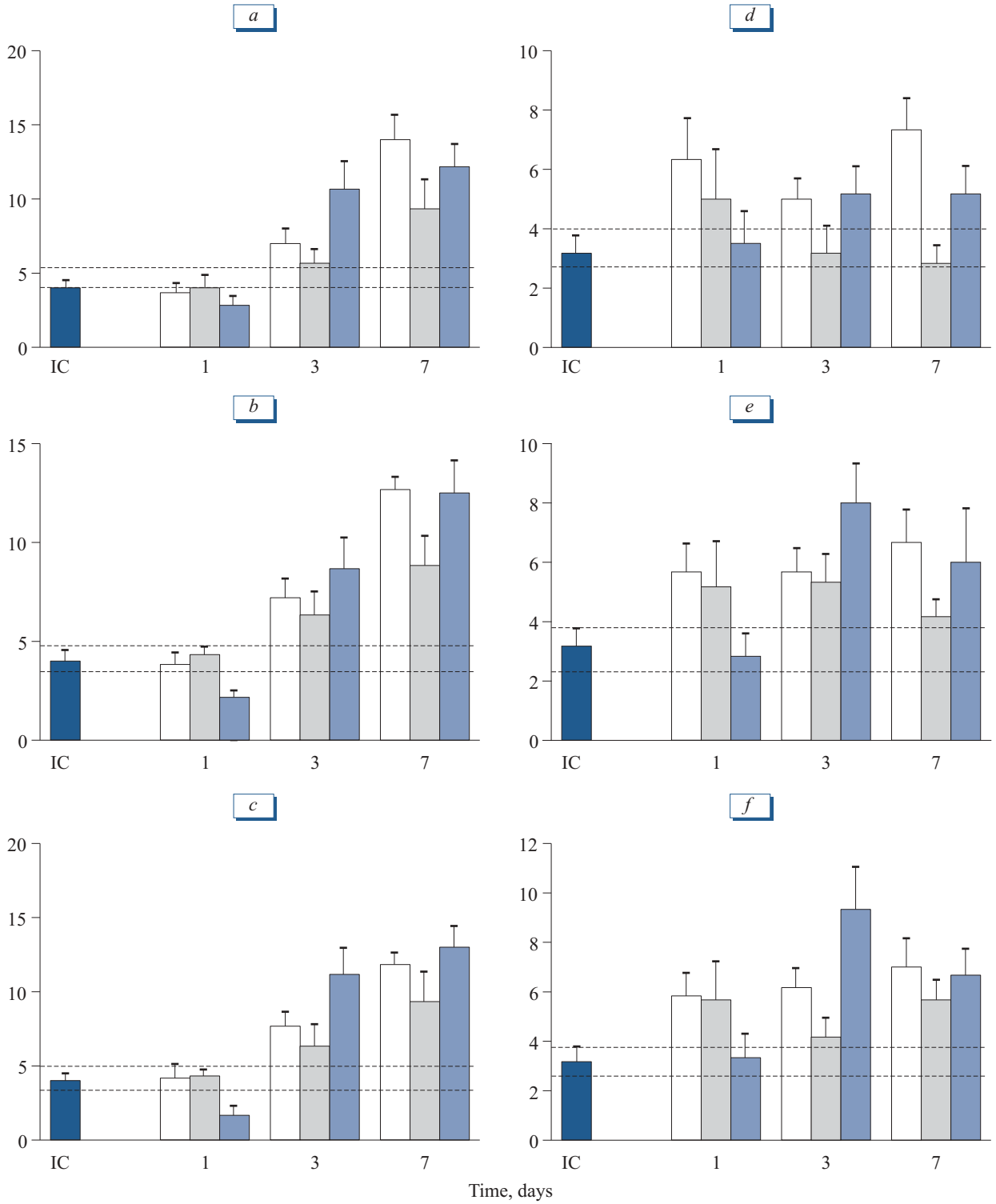

Fig. 2. Time course of the count of CFU-E (*a*-*c*) and CFU-GM (*d*-*f*) in the bone marrow of CBA/CaLac mice in hypoxic hypoxia (*a*, *d*), hemolytic anemia (*b*, *e*), and blood loss (*c*, *f*) causing no disorders in CNS activity (open bars), associated with encephalopathy development (gray bars), and in animals injected with G-CSF during development of brain pathology (blue bars). Here and in Fig. 3: values per 10^5 karyocytes.

pressure chamber hypoxia, on day 7 after injection of a high dose of hemolytic poison, and on days 3 and 7 after blood loss. On the other hand, the content of SC in the nervous tissue decreased on day 1 of the experiment in animals with hypoxia induced by phenylhydrazine hydrochloride, which

was presumably due damage to these cells and the used poison [7] (Fig. 3).

The results indicate the involvement of the true bone marrow MSC into the reactions of the blood system during oxygen deficiency of different nature, especially in severe forms. On the other hand, even pronounced increase in functional activity of bone marrow MSC in severe hypoxia fails to compensate for the decrease in the count of committed hemopoietic precursors in the hemopoietic tissue, causing "erythropoietic distress" in cases with encephalopathy development and consisting in a decrease of hyperplasia of the hemopoietic erythroid stem and production of pathological forms of erythrocytes [3]. Hence, increased content of SC in the bone marrow is associated with stimulation of the regional neural precursor cells and does not depend on the status of MSC pool in the bone marrow.

Hence, hypoxia of different etiology leads to activation of the "deep reserve" mechanisms — SC, which, however, were insufficient and incompetent in severe hypoxia eventuating in encephalopathy and dysadaptation of the hemopoietic tissue.

The next stage of the study was investigation of G-CSF effect on the pool of regional neural and mesenchymal bone marrow MSC in hypoxia in order to clear out possible neuroprotective mechanisms of rhG-CSF effect preventing the development of encephalopathy [4].

Injection of rhG-CSF to animals with severe oxidative insufficiency of different genesis virtually did not modify the content of MSC in the bone marrow on day 1 after exposure. However, in contrast to "pure" encephalopathy models, rhG-CSF appreciably increased MSC release into the peripheral blood on day 1 (to 266.7, 391.7, and 683.3% of basal level) in hypoxic hypoxia, hemolytic anemia, and blood loss, respectively, and did not modify SC count in the blood at later terms. On the other hand, on day 3 the count of MSC in the bone marrow was significantly lower than in animals with encephalopathy, the shifts were significant in animals with hypoxic hypoxia and blood loss, while in hemolytic anemia on day 7 the count of bone marrow long-repopulating cells increased again in comparison with the basal level. The shifts in the

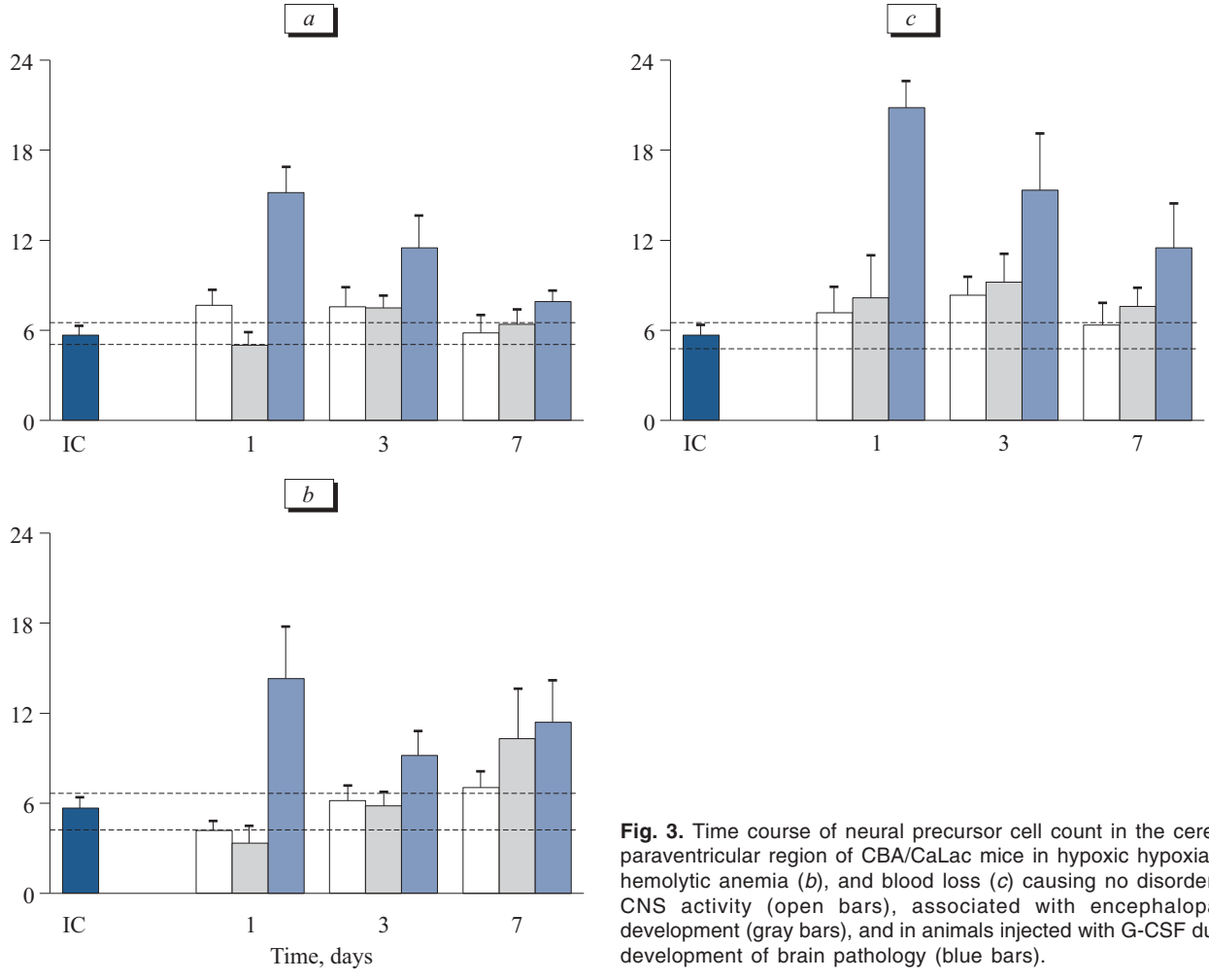

Fig. 3. Time course of neural precursor cell count in the cerebral paraventricular region of CBA/CaLac mice in hypoxic hypoxia (*a*), hemolytic anemia (*b*), and blood loss (*c*) causing no disorders in CNS activity (open bars), associated with encephalopathy development (gray bars), and in animals injected with G-CSF during development of brain pathology (blue bars).

bone marrow MSC pool, caused by rhG-CSF and consisting in pronounced mobilization of MSC into peripheral blood led to their determined homing in the CNS (Fig. 1). The penetration of bone marrow SC through the blood-brain barrier is now proven [11]. The content of neural precursors increased significantly in the brain on days 1, 3, and 7 of the experiment in hypoxic hypoxia and blood loss and on days 1 and 3 in oxidative insufficiency induced by phenylhydrazine hydrochloride, which, in turn, was associated with disappearance of signs of psychoneurological disorders (recovery of conditioned reflex and orientation and exploratory behavior; Fig. 3). On the other hand, rhG-CSF added *in vitro* to cultured cerebral cells derived from intact mice and animals with posthemorrhagic encephalopathy had no direct effect on neural precursors.

In all cases injection of the preparation was associated with a decrease in the count of hemopoietic precursor cells in the bone marrow on day 1, evidently caused by their mobilization and migration into the spleen [9], and further appreciable increase in the count of hemopoietic CFU on days 3 and 7 of the experiment. On day 3 the content of CFU-E in hemopoietic tissue in all models and of CFU-GM in severe hemolytic anemia and massive blood loss far surpassed the corresponding levels in animals exposed to hypoxia causing no CNS abnormalities (Fig. 2). The increase in the number of hemopoietic precursors paralleled by a decrease in the bone marrow MSC count and the absence of their release into the blood suggest that rhG-CSF stimulated MSC differentiation into hemopoietic precursors during this period (day 3). These changes in the pool of committed hemopoietic precursor cells increased erythrokaryocyte count in the bone marrow on day 7 in hypoxic hypoxia and hemolytic anemia and on days 5 and 7 in blood loss and induced accumulation of immature (on days 5, 1 and on days 5, 1, and 3 of the experiment) and less so of mature neutrophilic granulocytes (on days 5, 7, and 1) in hypoxic hypoxia, hemolytic anemia, and blood loss, respectively. On the other hand, the count of erythrocytes in the peripheral blood increased significantly on day 7 in phenylhydrazine hydrochloride-induced anemia and loss of 70% circulating blood volume. Neutrophil count increased only on day 1 of the experiment, which attested to their mobilization after the first injection of the preparation and to presumably ineffective granulocytopoiesis under the effect of this preparation in hypoxia at later terms.

The results indicate pronounced neuroprotective effect of G-CSF in hypoxia of different origin. Apart from the decrease in the glutamate-dependent excitotoxicity [12], G-CSF stimulates, mobilizes and ensures determined homing of endogenous bone marrow MSC into damaged CNS zones with subsequent differentiation of these cells into specialized elements and activation of extramedullary [9] and bone marrow erythropoiesis, associated with increased production of erythrocytes ensuring the optimal gaseous homeostasis in the body, essential for rapid recovery of CNS activity.

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