REGULATION OF MEDULLARY HEMATOPOIESIS BY OPIOID PEPTIDES DURING STRESS

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The study of the mechanisms of formation of the adaptive response of hematopoietic tissue using a model of immobilization stress has demonstrated the important role of hormones of the pituitary-adrenal system and also of intercellular interactions (T lymphocytes, macrophages, etc.) in the control of proliferation and differentiation of the ancestral blood cells, responsible for development of compensatory and adaptive changes in medullary hematopoiesis [1, 2, 4, 7]. Under these circumstances the central neuroendocrine mechanisms of stress are the factor triggering adaptive responses in the blood system [6, 11]. In turn, these mechanisms are under the control of the endogenous opioid peptide system [10, 12, 14]. Enkephalins have been shown to be capable of exerting a modulating influence on hematopoiesis under conditions of immobilization stress [3]. The investigation described below was conducted to study the possible ways whereby opioids may act on the response of hematopoietic tissue to stress and, in particular, to examine their connections with other known regulatory mechanisms.

EXPERIMENTAL METHOD

Experiments wee carried out on 294 male (CBA × C57BL/6)F_1 mice and 312 noninbred male mice weighing 18-20 g, immobilized for 10 h and 6 h respectively. The experimental animals received a single intraperitoneal injection of Leu-enkephalin or its synthetic analog dalargin 6 h after the beginning of exposure in a dose of 100 μg/kg (the preparations were obtained in the Laboratory of Peptide Synthesis, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR). Animals of one group also received the enkephalinase inhibitor D-β-phenyl-α-alanine for 7 days before exposure to stress in a daily dose of 300 mg/kg [13]. Mice of the control group received injections of physiological saline under the same conditions. At various times after immobilization the total number of myelokaryocytes (per femur) was determined. The myelogram was counted on bone marrow films. Functional activity of hematopoietic precursor cells was assessed by cloning the bone marrow of the experimental animals in a plasma clot in diffusion microchambers, implanted intraperitoneally into recipient mice, which received cyclophosphamide in a dose of 200 mg/kg 24 h previously [2]. The number of colony-forming units was calculated per 10^3 myelokaryocytes. The qualitative composition of the colonies thus formed was determined in total preparations stained with azure II-eosin. In a separate series of experiments, to study the possibility that dalargin may have a direct action on medullary nucleated cells, the preparation was added directly to the culture medium during cloning of myelokaryocytes taken from stressed animals on the 5th day after the beginning of exposure, in diffusion microchambers.

A solution of dalargin in concentration of 10^{-6}, 10^{-7}, and 10^{-8} M accounted for 1/10 of the total volume of medium. The specificity of the effect of dalargin on proliferation and differentiation of hematopoietic precursors was studied in vitro by the use of naloxone ("Narcan," USA), an antagonist of opiate receptors. For this purpose bone marrow cells taken from mice on the 5th day of immobilization were diluted to a concentration of 10^{-9} M with semisolid nutrient medium containing 10% embryonic calf serum, 5% anemic mouse serum, and 2% of conditioning medium of spleen cells, incubated with pokeweed mitogen, 73-83% of synthetic α-MEM medium, and 10% dalargin solution in a concentration of 10^{-6} M; naloxone in a concentration of 10^{-4} M, or the two preparations. The cell suspension was incubated at 37°C for 1 h. In the case of combined addition of naloxone and dalargin, the latter was added to the medium after preincubation for 30 min with naloxone. The cells were cultured in 24-well panels ("Costar," USA),

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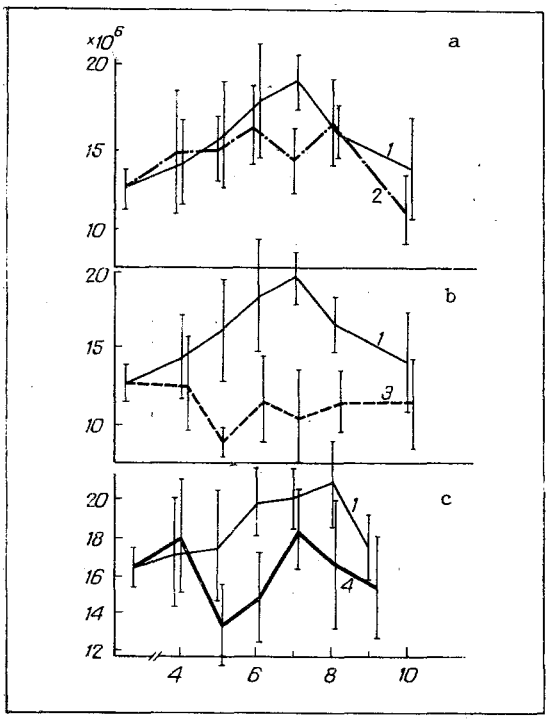

Fig. 1. Time course of total number of myelokaryocytes in noninbred mice exposed to immobilization for 6 h and receiving injections of physiological saline (1), Leu-enkephalin (2), dalargin (3), and D-phenylalanine (4) before (c) and after (a, b) immobilization. Abscissa, time of investigation (in days); ordinate, number of cells ($\cdot 10^6$/femur). Confidence intervals at p = 0.05.

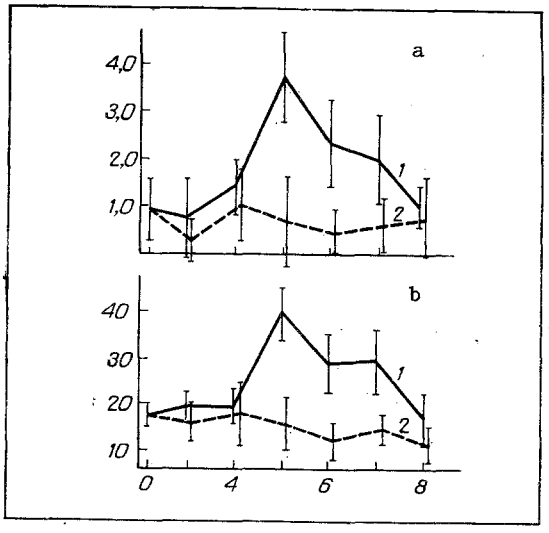

Fig. 2. Time course of number of CFU_{dc} (a) and $C1FU_{dc}$ (b) in (CBA × C57BL/6)F_1 hybrid mice immobilized for 10 days and receiving physiological saline (1) and dalargin (2) 6 h after beginning of immobilization. Abscissa, times of investigation (in days); ordinate, number of CFU_{dc} and $C1FU_{dc}$ (per 10^3 medullary nucleated cells). Confidence intervals at p = 0.05.

incubated for 7 days in a GPI-I incubator at 37°C, 100% humidity, and in an atmosphere with 5% CO_2, after which the number of number of colonies in the wells was counted and expressed per 10^6 medullary nucleated cells. The plasma

TABLE 1. Time Course of Expression of Markers of T Lymphocyte Subpopulations in Bone Marrow of (CBA × C57BL/6)F_1 Mice Immobilized for 10 h and Receiving Injection of Physiological Saline or Dalargin after 6 h (X)

Time of investigation, days	Index of cytotoxicity of antibodies, %			
	Anti-Lyt-1 + C		Anti-Lyt-2 + C	
	physiological saline	dalargin	physiological saline	dalargin
Before immobili	0,3	0,3	0,7	0,7
3-d	3,7	0	5,1	0
p_u	<0,01	>0,05	<0,05	>0,05
4-th	4,2	1,0	3,3	0,25
p_u	<0,01	>0,05	>0,05	>0,05
5-th	7,8	1,3	10,2	0,5
p_u	<0,05	>0,05	<0,05	>0,05
6-th	1,8	3,0	5,0	0
p_u	<0,05	<0,01	>0,05	>0,05

TABLE 2. Number of CFU_{dc} and $ClFU_{dc}$ (per 10^3 myelokaryocytes) in (CBA × C57BL/6)F_1 Mice on 5th Day after Immobilization for 10 h and during Culture with Dalargin ($\bar{X} \pm m$)

Experimental conditions	CFU_{dc}	$ClFU_{dc}$
Control (stress)	3,76±0,42	39,4±3,6
Dalargin, M		
10^{-7}	0,63±0,33	11,2±2,8
p	<0,001	<0,001
10^{-8}	0,46±0,33	10,8±1,8
p	<0,001	<0,001
10^{-9}	0	5,9±1,2
p	<0,001	<0,001

TABLE 3. Number of CFU_c (per 10^6 myelokaryocytes) in (CBA × C57BL/6)F_1 Mice on 6th Day after Immobilization for 10 h, and with Addition of Dalargin, Naloxone, or Both Ligands in Vitro ($\bar{X} \pm m$)

Experimental conditions	CFU_c
Control (stress)	12,0±2,4
Dalargin, 10^{-7} M	1,6±0,8
p	<0,01
Naloxone, 10^{-5} M	5,6±2,4
p	>0,05
Naloxone + dalargin	6,0±0,8
p	<0,05

11-OHCS level in immobilized mice was determined fluorometrically [9] on a "Hitachi" spectrofluorometer. Migration of T lymphocytes into bone marrow was studied with the aid of monoclonal antibodies to specific Lyt-1 and Lyt-2 antigens of mouse T lymphocytes. Supernatants from H-11-86.2.1 (to Lyt-1.1, mouse, μ.K.) and H-35-17.2 (to Lyt-2, rat, γ2B, K), were added to a cell suspension containing 10^9 myelokaryocytes to 1 liter of medium RPMI-1640. Nontoxic guinea pig serum was used as the source of complement. The cytotoxic test was set up with the use of trypan blue [8]. The results were subjected to statistical analysis by Student's and the Whitney—Wilcoxon—Mann tests.

EXPERIMENTAL RESULTS

In the different series of experiments immobilization led to the formation of a uniform reaction of stimulation of medullary hematopoiesis on the 5th-8th day of investigation, with an increase in the total number of

myelokaryocytes up to 141-156% of the initial level (Fig. 1). The increase in the cell count in the bone marrow was preceded by an increase in its colony- and cluster-forming activity up to 408 and 226% of the initial value respectively on the 5th day of the investigation (Fig. 2). Analysis of the qualitative composition of colonies detected in total preparations showed that immobilization stimulated the formation not only of granulocytic, macrophagal, and combined granulocytic-macrophagal colonies, but also of erythroid colonies (up to 28%), which was not observed when bone marrow from unimmobilized mice was cultured in a plasma clot. The increase in the number of hematopoietic precursor cells committed to erythro- and granulo-monocytopoiesis in the bone marrow of the stressed animals was accompanied by stimulation of different branches of hematopoiesis, revealed by analysis of the myelograms. For instance, the number of immature forms of neutrophilic granulocytes was increased on the 5th-7th day of the experiment to 137-184% of the initial value in different series of experiments. The total number of erythrokaryocytes of different degrees of maturity increased in the bone marrow on the 4th-7th day of the experiment to 245% of the initial values.

Administration of Leu-enkephalin or dalargin to the experimental animals 6 h after the beginning of immobilization, and also elevation of the endogenous plasma Leu-enkephalin level of the mice under the influence of D-phenylalanine (by 6.3 and 27 times respectively 6 and 12 h after the beginning of immobilization compared with that in the groups of control animals, receiving physiological saline) changed the pattern of medullary hematopoiesis. In particular, Leu-enkephalin reduced the total number of bone marrow cells compared with that in the control group of mice on the 7th day of the experiment to 72%; D-phenylalanine reduced it to 75 and 74% on the 5th and 6th days respectively, and dalargin the same on the 5th-8th days of the experiment (Fig. 1). In the last case, moreover, moderate hypoplasia of medullary hematopoiesis actually developed on the 5th day of the investigation (to 72% of the initial level) on account of a preponderant decrease in the number of cells belonging to the erythroid branch of hematopoiesis. Thus injection of dalargin, a stable synthetic Leu-enkephalin analog, actually led to more marked abolition of the phenomenon of medullary hyperplasia than the action of exogenous and increased concentrations of endogenous Leu-enkephalin. The effect of dalargin was due to its effect on proliferation and differentiation of hematopoietic precursor cells. For instance, in animals receiving dalargin no increase in colony- and cluster-forming activity of the bone marrow was observed (Fig. 2). In total preparations under these circumstances mainly colonies of macrophagal type were found, and no erythroid colonies.

An important role in the regulation of functional activity of hematopoietic precursors is played by intercellular cooperation, involving the participation of T lymphocytes with the Lyt-1^+, 2^+L3T4 phenotype and migrating into the bone marrow during stress under the influence of glucocorticoids [4, 7]. In the present experiments immobilization led to an increase in the number of T-lymphocytes with the Lyt-1^+, 2^+ phenotype in the bone marrow on the 3rd day after the beginning of the investigation, which preceded stimulation of medullary hematopoiesis (Table 1). Meanwhile, in animals receiving dalargin, migration of regulatory T lymphocytes into the bone marrow was reduced. Expression of Lyt-1 and Lyt-2 antigens on lymphocytes of the bone marrow under these circumstances varied as a rule within the limits of the initial values, and was significantly depressed compared with that in the control group of mice, receiving physiological saline, on the 3rd and 4th days of the investigation (Table 1). This effect of dalargin was evidently due to weakening of activity of the pituitary-adrenal system during stress under the influence of dalargin. For instance, an increase in the plasma 11-OHCS concentration of mice subjected to stress was observed 6 h after the beginning of exposure (up to 171% of the initial value) and it lasted until the 3rd day of investigation. Meanwhile in mice receiving this opioid peptide 6 h after the beginning of immobilization no increase was found in the glucocorticoid concentration at later stages of observation. The concentration of the hormone in animals of the experimental group was significantly reduced compared with that in mice receiving physiological saline under analogous conditions until the 3rd day of observation, i.e., in the period preceding accumulation of regulatory T lymphocytes in the bone marrow. The facts described above are evidence in support of an indirect (through glucocorticoids and T lymphocytes) effect of opioids on hematopoiesis in stress. The writers previously demonstrated the important role of glucocorticoids in the mechanisms of adaptive regulation of hematopoiesis in stress. Glucocorticoids have a stimulating effect on proliferation and differentiation of committed precursor cells of myelopoiesis indirectly, through the system of T cells [5]. Meanwhole the direct effect of enkephalins on nucleated bone marrow cells cannot be ruled out. For instance, addition of dalargin to the culture medium during cloning of bone marrow taken from immobilized animals in diffusion microchambers on the 5th day after the beginning of exposure led to a dose-dependent decrease in the cloning efficiency of CFU_{dc} and $ClFU_{dc}$ or even to complete suppression of colony growth in medium containing 10^{-9} dalargin (Table 2). A similar action of dalargin also was observed on its addition to the culture medium in vitro (Table 3). Moreover, in the last case, preliminary incubation of myelokaryocytes with naloxone reduced the suppressive action of dalargin on colony formation, evidence that the effect of the opioid peptide was realized with the participation of specific opiate receptors.

Enkephalins may thus have a modulating effect on medullary hematopoiesis in stress by affecting activity of other regulatory systems and, in particular, the glucocorticoid and T-lymphocyte systems. Besides, the effect of opioids

can be realized in principle directly through receptors on hematopoietic precursors or on cells forming the hematopoiesis-inducing microenvironment (T lymphocytes, macrophages, stromal mechanocytes), on account of a change in production of short-distance humoral factors regulating proliferation and differentiation of hematopoietic precursor cells.

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