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Immunostimulating Effect of the Synthetic Peptide Octarphin Corresponding to β-Endorphin Fragment 12-19

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Abstract—We have synthesized the peptide TPLVTLFK corresponding to β-endorphin fragment 12-19 (dubbed octarphin) and its analogs (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL). The octarphin peptide was labeled with tritium (specific activity 28 Ci/mol), and its binding to murine peritoneal macrophages was studied. [^{3}H]Octarphin was found to bind to macrophages with high affinity (K_d = 2.3 ± 0.2 nM) and specificity. The specific binding of [^{3}H]octarphin was inhibited by unlabeled β-endorphin and the selective agonist of nonopioid β-endorphin receptor synthetic peptide immunorphin (SLTCLVKGFY) (K_i = 2.7 ± 0.2 and 2.4 ± 0.2 nM, respectively) and was not inhibited by unlabeled naloxone, α-endorphin, γ-endorphin, or [Met^5]enkephalin (K_i > 10 μM). Inhibitory activity of unlabeled octarphin analogs was more than 100 times lower than that of unlabeled octarphin. Octarphin was shown to stimulate activity of murine immunocompetent cells *in vitro* and *in vivo*: at concentration of 1-10 nM it enhanced the adhesion and spreading of peritoneal macrophages as well as their ability to digest bacteria of *Salmonella typhimurium* virulent strain 415 *in vitro*; the peptide administered intraperitoneally at a dose of 20 μg/animal on day 7, 3, and 1 prior to isolation of cells increased activity of peritoneal macrophages as well as spleen T- and B-lymphocytes.

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Key words: β-endorphin, naloxone, peptides, receptors, immune system

It is known that β-endorphin binds (besides the opioid μ- and δ-receptors [1]) to the nonopioid receptor (insensitive to the opioid antagonist naloxone) described for the first time by Hazum et al. [2]. In 1980, Julliard et al. found corticotropin- and β-endorphin-like sequences in the human IgG heavy chain [3]. Then Houck et al. synthesized the tetradecapeptide SLTCLVKGFYPSDI corresponding to the β-endorphin-like sequence of IgG (fragment 364-377 of the heavy chain C_{H3}-domain) and showed that it competed with ^{125}I-labeled β-endorphin for binding to rat brain membranes [4]. Later on, we synthesized and investigated the β-endorphin-like decapeptide SLTCLVKGFY (dubbed immunorphin) corresponding to sequence 364-373 of the human IgG(1-4) heavy chain [5]. Experiments show that immunorphin labeled with ^{125}I or tritium binds with high affinity and specificity to the nonopioid β-endorphin receptor of human T-lymphocytes [6-9], mouse peritoneal macrophages [10, 11], rat brain synaptic membranes [12], and cells of the human T-lymphoblast Jurkat line [13]. The study of biological activity of immunorphin has shown that it enhances the mitogen-induced proliferation of human T-lymphocytes *in vitro* [6-9], activates mouse peritoneal macrophages *in vitro* and *in vivo* [10, 11], stimulates the growth of human T-lymphoblast Jurkat and MT-4 cell lines [13, 14], inhibits the activity of adenylate cyclase of the membranes of rat adrenal cortex and inhibits the secretion of glucocorticoids from adrenal glands into blood [15], and stimulates processes of cell division of early mouse embryos and formation of mature blastocysts *in vitro* [16, 17]. The study of distribution of the nonopioid β-endorphin receptor in rats shows its presence in cells of the immune (macrophages and lymphocytes), nervous (synaptic brain membranes), endocrine (adrenal cortex membranes), and cardiovascular (myocardium membranes) systems [18].

Recently, for the purpose of identifying the shortest β-endorphin fragment that can bind to the nonopioid

Abbreviations: Con A, concanavalin A; CPE, cytopathic effect of bacteria; LPS, lipopolysaccharide; PA, phagocytic activity; PN, phagocytic number.

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receptor with high affinity, we synthesized a panel of β-endorphin fragments and studied the ability of each of them to inhibit specific binding of [^{3}H]immunorphin to mouse peritoneal macrophages. As a result, we established that the synthetic peptide TPLVTLFK corresponding to β-endorphin sequence 12-19 (octarphin) is the shortest fragment of the hormone having practically the same affinity to the nonopioid receptor as immunorphin and β-endorphin [19, 20].

The goal of the present work was to study the effect of octarphin on the activity of immunocompetent mouse cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

The work was carried out with [Met^5]enkephalin, β-endorphin, naloxone, tuftsin, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (iodogen), cell cultivation media, and calf fetal serum (Sigma, USA); L-glutamine and Hepes (ICN, USA); penicillin and streptomycin (Gibco, USA); agarose, sucrose, BSA, EDTA, EGTA, Tris, phenylmethylsulfonyl fluoride (PMSF), and sodium azide (NaN_3) (Serva, Germany); [methyl-^{3}H]thymidine (specific activity 76 Ci/mmol) and scintillator Unisolv 100 (Amersham, England). N-Methylpyrrolidone, diisopropyl carbodiimide, 1-hydroxybenzotriazole, and thioanizol were purchased from Merck (Germany). All other reagents and solvents were produced domestically and used after appropriate purification.

BALB/c mice were obtained from the nursery of the Branch of the Institute of Bioorganic Chemistry (Russian Academy of Sciences). Immunorphin (SLTCLVKGFY), octarphin (TPLVTLFK), and its analogs (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL) were synthesized in automated synthesizers (models 430A and C250; Applied Biosystems and Vega Coupler (USA), respectively) using the Boc/Bzl strategy of peptide chain tailing and purified by preparative reverse-phase chromatography (Gilson chromatograph; France) in a Waters SymmetryPrep C18 column (19 × 300 mm) (Malva, Greece) as described [10, 21]. The constant protective groups were 2-chlorobenzyl oxycarbonyl and dichlorobenzyl for lysine and tyrosine, respectively. On completion of the assembly of the protected polypeptide chain, the final product was unblocked with simultaneous cleavage of the polymer using anhydrous hydrogen fluoride in the presence of scavengers. Synthesized peptides were characterized by the data of analytical reverse-phase HPLC (Gilson chromatograph, XTerra RP18 column (Malva)), amino acid analysis (hydrolysis with 6 M HCl, 24 h, 110°C; 4151 Alpha Plus amino acid analyzer LKB (Sweden)), and mass-spectral analysis (Finnigan mass spectrometer, USA).

[^{3}H]Octarphin was obtained by the reaction of high-temperature solid-phase catalytic isotope exchange (HTCIE) [22]. Aluminum oxide (50 mg) was added to a solution of 2 mg of octarphin in 0.5 ml of water and evaporated in a rotor evaporator. The aluminum oxide with the applied peptide was mixed with 10 mg of the catalyst (5% Rh/Al_2O_3). The solid mixture was put into a 10-ml ampule. The ampule was evacuated, filled with gaseous tritium up to 250 mm Hg pressure, heated to 170°C, and kept at this temperature for 20 min. Then the ampule was cooled, degassed, purged with hydrogen, and degassed again. The labeled peptide was extracted from the solid reaction mixture using two 3-ml portions of 50% aqueous ethanol; the resulting solution was combined and evaporated. The procedure was repeated twice to remove labile tritium. [^{3}H]Octarphin was purified by HPLC using a 4 × 150 mm Kromasil column (5 μ grains, 20°C, 0.1% trifluoroacetic acid as eluent, methanol gradient 42-70% during 20 min, flow rate 3 ml/min) with monitoring at 254 and 280 nm using a Beckman spectrophotometer. Liquid scintillation counting was used to determine the inclusion of tritium into the peptide.

The reaction of [^{3}H]octarphin binding to mouse peritoneal macrophages was performed in medium 199 containing 25 mM Hepes, 20 mM NaN_3, 0.6 mg/ml PMSF, pH 7.4, by the following scheme: 100 μl of the labeled peptide (10^{-10}-10^{-7} M, three parallel samples for each concentration), 100 μl of the medium (general binding) or 100 μl of 10^{-3} M unlabeled peptide solution in the medium (nonspecific binding), and 800 μl of cell suspension ($1.2 \cdot 10^7$ cells in 1 ml of medium) were introduced into siliconized test tubes. The tubes were incubated at 4°C for 1 h. On completion of the incubation, the reaction mixture was filtered through fiber-glass GF/C filters (Whatman, England) to separate the labeled peptide bound to the cells from unbound (free) labeled peptide. The filters were washed three times with 5 ml of ice-cold saline solution. Radioactivity on the filters was counted using an LS 5801 liquid scintillation counter (Beckman, USA). Specific binding of [^{3}H]octarphin to the cells was detected by the difference between its total and nonspecific binding, while nonspecific binding of [^{3}H]octarphin was detected in the presence of 10^{-4} M unlabeled octarphin (1000-fold excess relative to the highest concentration of labeled octarphin, 10^{-7} M). The parameters of specific binding of the labeled octarphin to macrophages (equilibrium dissociation constant K_d and receptor density n, i.e. the number of peptide specific binding sites per cell) were determined by plotting the dependence of the ratio of molar concentrations of bound (B) and free (F) labeled octarphin on the molar concentration of bound labeled peptide (B). Receptor density (n) was determined using the formula:

$$n = (R_0 A)/N,$$

where R_0 is the molar concentration of receptors, A is Avogadro's number, and N is the cell number in 1 liter [23].

For investigation of the ability of naloxone, unlabeled octarphin analogs, immunorphin, α-, β-, γ-endorphins, and [Met^5]enkephalin to inhibit the specific binding of [^{3}H]octarphin to macrophages, the cells ($1.2 \cdot 10^7$ in 1 ml) were incubated with the labeled octarphin (5 nM) and one of the potential competitors (range of concentrations 10^{-12}-10^{-5} M, three repeats for each concentration) as described above. Inhibition constant was determined using the formula $K_i = [I]_{50}/(1 + [L]/K_d)$ [24], where [L] is the molar concentration of [^{3}H]octarphin; K_d is the equilibrium dissociation constant of the [^{3}H]octarphin–receptor complex; and $[I]_{50}$ is the concentration of unlabeled ligand causing 50% inhibition of specific binding of the labeled octarphin. The $[I]_{50}$ value was determined graphically based on the inhibition curve (diagram of inhibition dependence (in percentage) on molar concentration of inhibitor). The K_d value was determined as described above.

The experiments on phagocytosis were carried out using the virulent strain *Salmonella typhimurium* 415 with typical morphological and functional properties and LD_{50} ~ 100 microbial cells during intraperitoneal infection of white mice. The bacterial culture of Salmonella was grown for 4-6 h at 37°C in Hottinger broth, subcultured on meat-peptone agar, and incubated at 37°C for 18 h.

Macrophages of mouse abdominal cavity were isolated and cultivated according to the recommendations in [25]. The ability of macrophages to adhere to plastic (adhesion) was estimated by cultivation of the cells in a 96-well plate in medium containing 5% calf embryonal serum for 1 h in an atmosphere of 5% CO_2 at 37°C. The monolayer of macrophages was fixed with methanol for 5 min and stained with Giemsa stain. The number of cells adhering to 1 mm^2 of the surface was counted in a Goryaev chamber. The reliability of differences between the control and the experiment was assessed using Student's *t*-criterion.

The effect of octarphin on the spreading of macrophages *in vitro* was studied during incubation of monolayers in the absence (control) and presence of the peptide in the medium without serum for 1 h; then this medium was changed for the medium containing 5% calf embryonal serum and incubation was carried out for 24 h in an atmosphere of 5% CO_2 at 37°C. After that, the monolayer of macrophages was fixed with methanol for 5 min and stained with Giemsa stain. The number of spread macrophages was counted under a microscope using an eyepiece graticule. Macrophages were considered as spread if their longitudinal dimensions were 3-fold greater than their lateral dimensions. Up to 300 cells were counted in each well. The reliability of differences between the control and the experiment was assessed using Student's *t*-criterion.

The influence of octarphin on the ability of mouse peritoneal macrophages for adhesion and spreading *in vivo* was studied by the following scheme: the peptide was injected intraperitoneally at a dose of 20 μg/animal 7, 3, and 1 day before isolation of the cells; in parallel, the control animals received normal saline solution. The experiment was performed in three repeats.

Phagocytic activity of macrophages was studied according to our strategy [26]. Monolayers of macrophages on cover glasses obtained by appropriate preparative procedures were cultivated at 37°C in sterile glass test tubes filled with medium 199 containing streptomycin and penicillin (100 μg/ml of medium) and inactivated calf embryonal serum (5%). In 24 h, macrophages were infected with medium 199 containing the serum and the bacterial culture of *S. typhimurium* 415 in appropriate amounts for reaching the final dilution of 10^8 microbial cells per ml of the medium. In 2 h, the contact between macrophages and the microbes was interrupted, and the medium was changed for the cultivation medium with antibiotics. For preventing the capture by macrophages of the microbes released from destroyed phagocytes, the medium was henceforth hourly replaced by fresh medium. Macrophages were fixed on cover glasses in 1, 2, 4, 7, and 12 h (methanol, 3 min). Then the preparations were stained with 0.1% aqueous solution of azure II-eosin dye for 5 min. Three hundred cells were examined on each glass under a microscope at magnification of ×1350. The following parameters were determined: phagocytic activity (PA), i.e. the share (in percentage) of macrophages involved in phagocytosis; cytopathic effect of bacteria (CPE), i.e. the share (in percentage) of phagocytes destroyed by intracellular bacteria; and phagocytic number (PN), i.e. the average number of microbes per macrophage.

For investigation of the effect of octarphin on the activity of spleen T- and B-lymphocytes *in vivo*, the animals received an intraperitoneal injection of the peptide (20 μg/animal) or normal saline solution (control) 7, 3, and 1 day before the isolation of splenocytes. The control and experimental groups contained three animals each. The experiment was repeated twice. The reaction of blast transformation was performed by the following scheme: 1 ml of cell suspension ($2.5 \cdot 10^6$ cells in 1 ml) was added into the wells of the plate for cell cultivation. The experimental and control tests were made in five repeats. The T-cell population of spleen lymphocytes was stimulated with Con A (5 μg/ml); B-cells were activated with *Salmonella typhi* LPS (10 μg/ml). Splenocytes were cultivated for 48 h at 37°C in an atmosphere of 5% CO_2. Five hours before the end of the incubation, [methyl-^{3}H]thymidine was added into each well (0.5 μCi). After the end of incubation, the contents of each well were transferred onto a GF/C fiber-glass filter (Whatman) and washed with 15-fold volume of cooled saline solution and 5-fold volume of 5% TCA using a water-jet pump. Radioactivity on the filters was counted with an LS 5801 liquid scintillation counter (Beckman).

Table 1. Basic characteristics of the peptides (purity >97%)

Peptide	Amino acid analysis data	Molecular weight, Da*
SLTCLVKGFY (immunorphin)	Thr 0.89; Ser 0.92; Gly 1.00; Val 1.00; Leu 1.94; Tyr 1.03; Phe 1.00; Lys 0.91	1129.3 (1130.0)
TPLVTLFK (octarphin)	Thr 1.96; Pro 0.97; Leu 2.02; Val 1.00; Phe 1.00; Lys 0.95	917.9 (918.24)
LPLVTLFK	Thr 0.95; Pro 0.99; Leu 2.98; Val 0.99; Phe 0.97; Lys 0.95	930.4 (930.29)
TLLVTLFK	Thr 1.98; Leu 2.99; Val 0.98; Phe 0.98; Lys 0.97	934.4 (934.28)
TPLVLLFK	Thr 0.99; Pro 0.98; Leu 3.04; Val 1.00; Phe 1.02; Lys 0.97	930.5 (930.29)
TPLVTLLK	Thr 1.97; Pro 0.96; Leu 3.00; Val 1.01; Lys 0.98	884.6 (884.22)
TPLVTLFL	Thr 1.98; Pro 0.98; Leu 2.96; Val 0.99; Phe 1.03	902.9 (903.22)

* Calculated value is given in parenthesis.

RESULTS

The basic characteristics of synthesized peptides (purity, amino acid analysis data, and molecular weights) are given in Table 1.

[^{3}H]Octarphin with the specific activity of 28 Ci/mol was obtained by the HTCIE reaction after purification. The retention time of [^{3}H]octarphin and unlabeled octarphin on the Kromasil C18 column (conditions of chromatography are described in "Materials and Methods") was 15 min in both cases; absorption ratios at 254 and 280 nm for the labeled and unlabeled peptides were also the same, demonstrating preservation of the chemical structure of octarphin during substitution of tritium for hydrogen.

Binding of [^{3}H]octarphin and its analogs to mouse peritoneal macrophages. The experiments showed that under the selected conditions (see "Materials and Methods") [^{3}H]octarphin specifically bound to mouse peritoneal macrophages. Specific binding of [^{3}H]octarphin was determined by the difference between its total and nonspecific binding. Nonspecific binding of [^{3}H]octarphin was estimated in the presence of 10^{-4} M unlabeled octarphin; it was 7.8 ± 0.6% of total binding of the labeled peptide. Figure 1a shows the dependence of

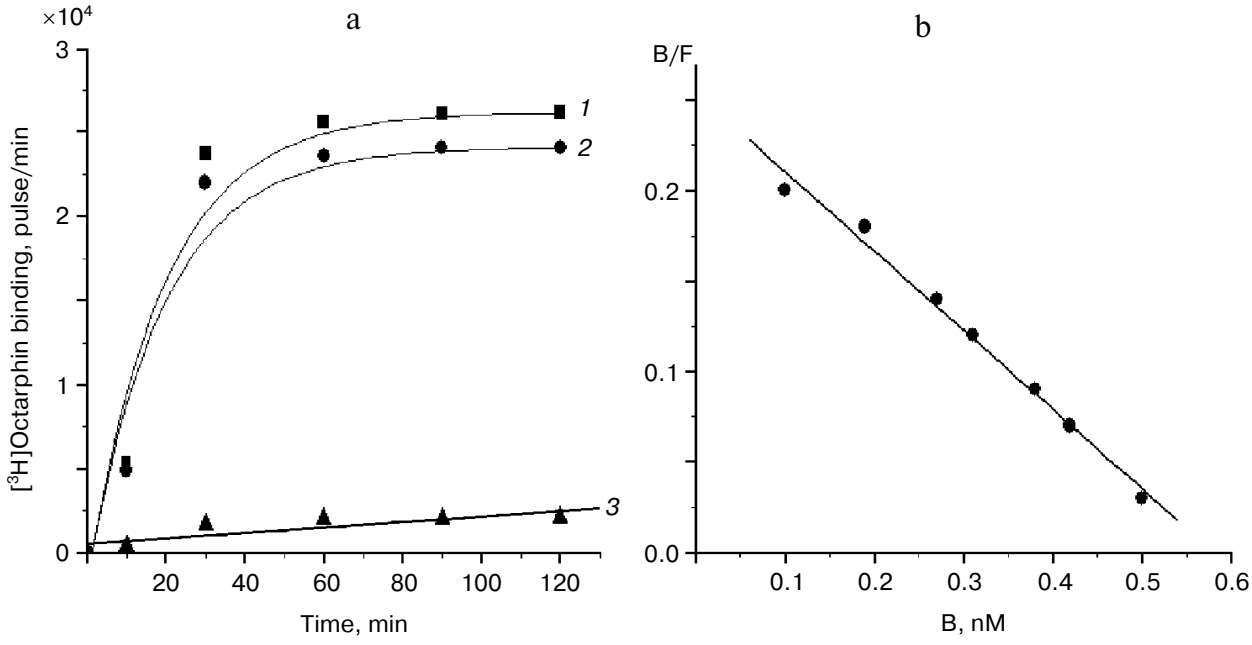

Fig. 1. a) Dependence of total (*1*), specific (*2*), and nonspecific (*3*) binding of [^{3}H]octarphin to mouse peritoneal macrophages on incubation time. b) Analysis in Scatchard coordinates of specific binding of [^{3}H]octarphin to mouse peritoneal macrophages. B and F are molar concentrations of the bound and free labeled peptide, respectively.

specific binding of [^{3}H]octarphin to mouse macrophages at 4°C on incubation time. It can be seen that the dynamic equilibrium in the [^{3}H]octarphin–receptor system is established in 1 h and maintained for at least 2 h; therefore, the reaction of binding of [^{3}H]octarphin to the macrophages was performed during 1 h.

The analysis of specific binding of [^{3}H]octarphin to the macrophages in Scatchard coordinates (Fig. 1b) shows the presence of a single class of binding sites (receptors) of this peptide on the macrophage surface: the graph is a straight line, $K_d = 2.3 \pm 0.2$ nM, being evidence of high affinity of octarphin to the receptor. Receptor density, i.e. the number of labeled peptide specific binding sites per macrophage, was 43,600 ± 3900.

Inhibition of specific binding of [^{3}H]octarphin to mouse peritoneal macrophages by naloxone and unlabeled peptides. Experimental results presented in Table 2 show that the specific binding of [^{3}H]octarphin to mouse peritoneal macrophages is inhibited by immunorphin and β-endorphin ($K_i = 2.4 \pm 0.2$ and 2.7 ± 0.2 nM, respectively). Naloxone, α- and γ-endorphins, and [Met^5]enkephalin did not inhibit the binding ($K_i > 10$ μM). The inhibitory activity of unlabeled analogs LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, and TPLVTLFL was, respectively, 102, 124, 105, 146, and 175 times lower that the activity of unlabeled octarphin (Table 2).

Effect of octarphin on activity of mouse peritoneal macrophages *in vitro*. The experiments have shown that octarphin increases the adhesion and spreading of mouse peritoneal macrophages: at the peptide concentrations of 1 and 10 nM, the ability of macrophages to adhere to plastic increases compared to the control by 25.8 and 33.0% ($p \le 0.01$), respectively, and the number of spread cells is by 37.2 and 73.0% greater than in the control ($p \le 0.01$).

Octarphin stimulated the bactericidal activity of peritoneal macrophages during phagocytosis of the bacterium *S. typhimurium* 415. Tuftsin and immunorphin were taken as a positive control. Table 3 shows the basic parameters (PA, CPE, and PN) characterizing phagocytosis of this bacterium by macrophages in the control and in the presence of one of the peptides under study. The control experiments showed that macrophages actively consumed the bacteria of this strain: in 2 h, phagocytosis involved >2/3 of the total number of macrophages (PA 75.6 ± 1.7%); at the same time, each phagocyte contained 10 microorganisms on average (PN 10.3 ± 1.3). However, there was no digestion of the consumed microbes. Moreover, they continued to actively reproduce within phagocytes, as was demonstrated by the increase in PN from 10.3 ± 1.3 to 15.7 ± 1.7 between hour 2 and 7 of phagocytosis. Infection of the monolayer lasted for 2 h; then the infection medium was replaced by cultivation medium; therefore, beginning with that moment, PN could increase only due to reproduction of previously consumed microbes. Mass mortality of phago-

Table 2. Inhibition of specific binding of [^{3}H]octarphin to mouse peritoneal macrophages by naloxone and unlabeled peptides

Ligand	$[I]_{50}$, nM	K_i, nM
Naloxone	> 10 000	> 10 000
β-Endorphin	8.6 ± 0.5	2.7 ± 0.2
Immunorphin	7.7 ± 0.6	2.4 ± 0.2
α-Endorphin	> 10 000	> 10 000
γ-Endorphin	> 10 000	> 10 000
[Met^5]Enkephalin	> 10 000	> 10 000
Tuftsin	> 10 000	>10 000
Octarphin	10.9 ± 0.8	3.4 ± 0.3
LPLVTLFK	1113.9 ± 78.2	348.1 ± 22.6
TLLVTLFK	1356.0 ± 106.7	423.8 ± 29.7
TPLVLLFK	1147.2 ± 80.3	358.5 ± 18.1
TPLVTLLK	1596.4 ± 111.8	498.9 ± 34.9
TPLVTLFL	1899.6 ± 133.0	593.6 ± 41.6

Table 3. Effects of octarphin, immunorphin, and tuftsin on phagocytosis of bacteria of the virulent strain *S. typhimurium* 415 by mouse peritoneal macrophages *in vitro**

Peptide (concentration, nM)	PA, %	CPE, %	PN
Control	66.2 ± 3.2	14.0 ± 1.8	3.8 ± 0.91
	75.6 ± 1.7	12.2 ± 2.3	0.3 ± 1.3
	64.7 ± 1.9	32.7 ± 2.4	10.7 ± 1.3
	28.2 ± 2.5	69.6 ± 4.1	15.7 ± 1.7
	0.1 ± 0.2	99.7 ± 4.4	0.2 ± 0.4
Octarphin (1)	85.9 ± 1.9**	1.3 ± 0.9**	8.2 ± 1.6**
	93.9 ± 2.4**	1.8 ± 1.5**	9.6 ± 1.3
	65.7 ± 2.8**	7.4 ± 1.3**	5.2 ± 1.4**
	24.3 ± 2.7	9.0 ± 1.3**	0.9 ± 0.6**
	2.8 ± 0.9**	2.3 ± 1.1**	0.5 ± 0.4
Immunorphin (1)	84.1 ± 3.2**	1.3 ± 0.9**	7.6 ± 2.0**
	88.7 ± 2.8**	1.6 ± 1.3**	9.1 ± 0.6
	64.6 ± 3.9	9.1 ± 1.9**	6.1 ± 0.6**
	27.4 ± 5.5	10.3 ± 1.4**	1.3 ± 1.0**
	3.6 ± 1.7**	2.3 ± 0.8**	0.7 ± 0.4
Tuftsin (100)	68.2 ± 4.7	1.9 ± 1.1**	5.6 ± 0.4**
	73.4 ± 2.5	8.0 ± 1.1**	7.4 ± 0.5**
	60.8 ± 3.1	31.2 ± 3.4	6.1 ± 0.3**
	36.8 ± 2.7**	32.4 ± 2.0**	3.0 ± 0.2**
	9.9 ± 2.5**	7.7 ± 1.5**	1.4 ± 0.4**

* Lines for each compound sequentially (top down) correspond to data obtained at 1, 2, 4, 7, and 12 h.
** Reliable difference from control ($p \le 0.01$).

cytes was observed already in 7 h of phagocytosis (CPE 69.6 ± 4.1%), and the whole monolayer of macrophages was destroyed by hour 12 (CPE 99.7 ± 4.4%). Thus, the interaction between salmonellas and macrophages in the control resulted in the death of the latter. In the presence of octarphin or immunorphin in the concentration of 1 nM, the character of interaction between salmonellas and macrophages drastically changed: the digestive ability of macrophages increased so much that by hour 12 of phagocytosis the monolayer contained no destroyed cells at all, and the macrophages contained no undigested microbes (Table 3). Comparison of phagocytosis parameters in the presence of octarphin, immunorphin, and tuftsin showed that octarphin and immunorphin had more than 100-fold higher activity than tuftsin (Table 3).

Thus, octarphin in the concentration of 1 nM activates mouse peritoneal macrophages *in vitro*.

Effect of octarphin on activity of mouse peritoneal macrophages *in vivo*. Investigations showed that the ability of macrophages to adhere to plastic was higher by 63.4% in mice receiving the peptide compared to control animals ($p \le 0.01$) receiving normal saline solution. Besides, the number of spread cells in the experiment was greater by 80.3% than in the control ($p \le 0.01$). Thus, octarphin enhances the ability of peritoneal macrophages for adhesion and spreading when injected intraperitoneally into mice.

Effect of octarphin on activity of T- and B-lymphocytes of mouse spleen *in vivo*. Experimental results (Fig. 2) show that the proliferative response of splenocytes stimulated with Con A or LPS in mice receiving octarphin was 1.7- and 1.5-fold higher, respectively ($p \le 0.01$), than in animals not receiving the peptide. Consequently, intraperitoneally injected octarphin activated both T-lymphocytes and B-cells of mouse spleen.

DISCUSSION

Previously we synthesized 30 fragments of β-endorphin and studied their ability to inhibit specific binding of [^{3}H]immunorphin to mouse peritoneal macrophages. As a result, the shortest peptide possessing actually the same inhibitory activity as β-endorphin proved to be its fragment 12-19 TPLVTLFK (K_i = 3.1 ± 0.3 nM, we dubbed the respective synthetic peptide octarphin) [20]. In this work we have studied the effect of octarphin on immunocompetent mouse cells *in vitro* and *in vivo*. Comparison of the amino acid sequences of human, mouse, and rat β-endorphins [27] has shown their complete coincidence in the region 12-19 (Fig. 3), which is evidence of high conservativeness of the latter. Besides, it allows us to study the biological activity of octarphin on mouse models.

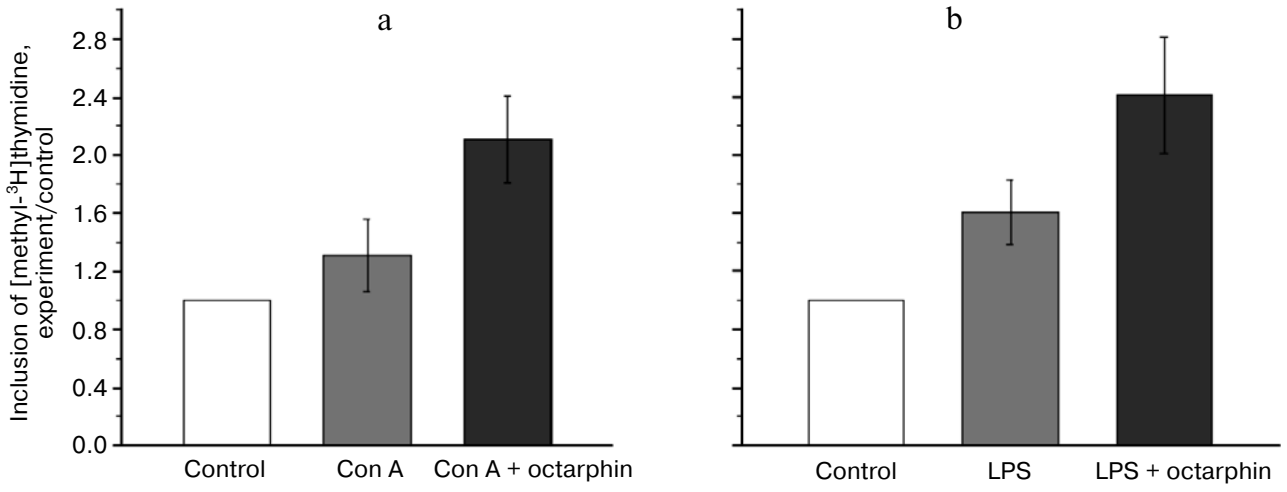

Fig. 2. Effect of octarphin on activity of T-lymphocytes (a) and B-lymphocytes (b) of mouse spleen *in vivo*. The peptide was introduced intraperitoneally in a dose of 20 μg/mouse on day 7, 3, and 1 before isolation of splenocytes. T-Lymphocytes were stimulated with Con A (5 μg/ml), and B-cells were stimulated with LPS of *S. typhi* (10 μg/ml). The mean values of two independent experiments ± standard deviation are shown.

Tritium-labeled octarphin ([^{3}H]octarphin) was obtained and its interaction with mouse peritoneal macrophages was studied to characterize octarphin reception. The experimental results show that the binding of [^{3}H]octarphin to macrophages is characterized by high affinity ($K_d = 2.3 \pm 0.2$ nM; Fig. 1b), specificity (Table 2), and insensitivity to naloxone. The ability of unlabeled octarphin analogs (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL) to inhibit the specific binding of [^{3}H]octarphin was more than 100 times less than in unlabeled octarphin. This means that even a single amino acid substitution in the octarphin molecule results in an abrupt decrease in affinity to the receptor. Henceforth, only octarphin was studied in the tests with immunocompetent mouse cells *in vitro* and *in vivo*. It was not expedient to test the analogs because of their low affinity to the receptor.

First of all, we studied the influence of octarphin on adhesion, spreading, and phagocytic activity of mouse peritoneal macrophages.

As is known, inflammation intensifies the directed migration of leukocytes. Activated cells acquire the ability to adhere to vesicular endothelium and migrate to the region of infection and inflammation, changing their rounded shape for stellation. Therefore, the properties of phagocytes such as adhesion and spreading adequately reflect their functional status. Besides, the adhesion and spreading of macrophages to some extent can be considered as the initial stages of phagocytosis: adhesion and circumvallation of a particle by pseudopodia [28]. The results of this work show that octarphin enhances the ability of mouse peritoneal macrophages for adhesion and spreading *in vitro* and *vivo*.

	1	10	20	30
Human β-End	YGGFMTSEKSQ**TPLVTLFK**NAIIKNAYKKGE			
	1	10	20	30
Murine β-End	YGGFMTSEKSQ**TPLVTLFK**NAIIKNVHKKGQ			
	1	10	20	30
Rat β-End	YGGFMTSEKSQ**TPLVTLFK**NAIIKNVHKKGQ			

Fig. 3. Comparison of β-endorphin amino acid sequences of man, mouse, and rat [27]. Fragment 12-19 (octarphin sequence) is designated with bold.

Phagocytosis of bacteria of the virulent strain *S. typhimurium* 415 *in vitro* by mouse peritoneal macrophages was used as a model system in the study of the influence of the peptides on the phagocytic activity of macrophages. Positive controls were also the tetrapeptide tuftsin (Thr-Lys-Pro-Arg, fragment 289-292 of the IgG C_{H2}-domain) and the selective agonist of nonopioid β-endorphin receptor, decapeptide immunorphin (SLTCLVKGFY). According to the literature, tuftsin stimulates the bactericidal action of macrophages during phagocytosis of *Staphylococcus aureus*, *Staphylococcus londres*, *Listeria monocytogenes*, *Leishmania parasites*, *Escherichia coli*, and *Serratia marcescens* [29]. Table 3 shows the results of investigation of the effect of tuftsin on phagocytosis of the bacterium *S. typhimurium* 415 by mouse peritoneal macrophages. Comparison of phagocytosis parameters in the presence of 100 nM tuftsin with the control values shows that the peptide stimulates the ability of macrophages to digest the consumed microorganisms. In the presence of tuftsin, the monolayer of macrophages contained ~10% of cells with one or two undigested bacteria after 12 h of phagocytosis; the share of destroyed phagocytes was no more than 8%. Octarphin and immunorphin tested in parallel with tuftsin also stimulated the ability of macrophages to digest virulent sal-

monellas, but at lower concentrations compared to tuftsin. In the presence of octarphin or immunorphin at a concentration of 1 nM, macrophages almost completely digested the consumed microorganisms by hour 7 and 12 of phagocytosis, respectively (Table 3). In the case of tuftsin, the analogous effect was achieved only at a concentration of 100 nM. Thus, the abilities of octarphin and immunorphin to stimulate the digestive activity of phagocytes at least 100-fold exceeded the activity of tuftsin.

According to the literature data, β-endorphin enhances the migration of neutrophils and stimulates the phagocytosis of latex particles and *Candida albicans* by macrophages [30-32]. Ichinose et al. [31] showed that the ability to stimulate latex consumption by phagocytes is inherent, besides β-endorphin, also of its fragments 1-27 and 6-31; the effects of β-endorphin and its fragments was not inhibited by naloxone. However, [Met^5]- and [Leu^5]enkephalins, α- and γ-endorphins, and β-endorphin fragments 18-31 and 28-31 were inactive. These results suggest that realization of this effect involves the central part of the β-endorphin molecule.

In the following stage, we studied the effect of octarphin on the activity of mouse spleen T- and B-lymphocytes *in vivo*.

It should be noted that the data on the effect of β-endorphin on T-lymphocytes are rather contradictory: both stimulating and inhibitory effects of the hormone on mitogen-induced proliferative response of T-cells have been reported [33-35]. As is shown, the stimulating effect of β-endorphin on T-lymphocytes is associated with its ability to enhance IL-2 production by these cells; subsequent interaction between the cytokine and the receptors results in cell division. The effect was not suppressed by naloxone and, consequently, was mediated by the naloxone-insensitive receptor [36]. Further studies showed that the effect of β-endorphin on T-lymphocytes mediated by the nonopioid receptor was determined by the central part of the molecule (tentatively, sequence 6-23) [34, 37, 38]. Previously we showed that β-endorphin and immunorphin in the concentrations of 10^{-11}-10^{-9} M stimulated the Con A-induced proliferation of T-lymphocytes isolated from human peripheral blood [8]. The effects of β-endorphin and immunorphin on proliferation of T-lymphocytes were not inhibited by naloxone. The study of ^{125}I-labeled immunorphin reception by T-lymphocytes has shown that the peptide binds with high affinity to the naloxone-insensitive receptor (K_d = 7 nM). However, the unlabeled immunorphin completely inhibits the specific binding of ^{125}I-labeled β-endorphin to T-lymphocytes (K_i = 6 nM) [7, 8]. Thus, β-endorphin and immunorphin are shown to bind to the common naloxone-insensitive receptor of T-lymphocytes in donor blood.

The results of this work show that the intraperitoneally injected octarphin activates both T- and B-lymphocytes of mouse spleen (Fig. 2). It is known that *in vitro* β-endorphin does not influence splenocyte proliferation induced by specific B-lymphocyte mitogens: LPS or dextran sulfate [37, 39]. Although the *in vitro* studies showed the absence of influence of octarphin on B-lymphocytes (data not shown), LPS-stimulated cells isolated from the spleen of mice receiving intraperitoneal injections of the peptide proliferated 1.5-fold more actively than the control (without the peptide). Such effect is probably associated with octarphin stimulation of T-cells and macrophages, which in turn activate B-cells.

Thus, octarphin stimulates the activity of immunocompetent mouse cells *in vitro* and *in vivo*. It enhances the adhesion and spreading of peritoneal macrophages and their ability to digest bacteria of the virulent strain *S. typhimurium* 415 *in vitro*. Intraperitoneal injection of the peptide increases the activity of peritoneal macrophages and spleen T- and B-lymphocytes. The effect of octarphin is mediated by the nonopioid β-endorphin receptor. Consequently, region 12-19 of the β-endorphin molecule ensures the binding to the nonopioid receptor and signal transduction into the cell.

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