BIOCHEMISTRY AND BIOPHYSICS

Additive Synthesis of Regulatory Peptide *in Vivo*: the Introduction of the Vaccine Strain of *Francisella tularensis* Producing β-Endorphin

V. M. Borzenkov, A. P. Pomerantsev, and I. P. Ashmarin

UDC 615.371:579.841.95].012.6.07

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 116, № 8, pp. 151-153, August, 1993
Original article submitted March 26, 1993

Key Words: *producer strain; synthesis, β-endorphin; biological activity*

Among the most important bioregulators of the functional state of the human and animal organism are endogenous peptides [1-3]. Released into the blood, they enter organs and tissues, where they can regulate the psychoemotional state, the immune status, the pain threshold, and many other physiological processes. These natural bioregulators are effective at low concentrations, and their effect is highly specific and selective. However, the range and duration of their effect in the body are considerably limited by the action of proteolytic enzymes [8].

At present liposomes, microcapsules, miniosmotic pumps, and other devices are being used to prolong the effect of peptides in the body [14]. A promising idea is that of creating a system of long-term additive, parallel synthesis of peptides in the body. This can be done by using harmless living microbes that can exist for a prolonged period of time in a macroorganism and produce the peptide in which the researcher is interested. Such microbes can include vaccine strains of bacteria obtained on the basis of pathogens having a predominantly intracellular development, as these strains are known to be able to survive for a long time in the cells of a macroorganism, periodically secreting into the surrounding medium the products of its metabolism [10,11]. One such microbe is the vaccine strain of tularemic microbe, which quickly becomes adapted in the macroorganism and provides stable immunity to the pathogen [12]. The means of transmitting genetic information into cells of the tularemic microbe have been described, and the stability of its inheritance and expression in the recipient cells has been assessed [4].

One of the most widely studied peptides from the point of view of its physiological effects is β-endorphin. Its gene has now been cloned, and the physiological activity of the recombinant peptide has been shown to correspond to the activity of natural β-endorphin [9]. In view of this, and since the methods of assessing the physiological properties of this peptide have been thoroughly worked out, we chose β-endorphin as the subject of our investigation.

MATERIALS AND METHODS

The biological activity of native and recombinant β-endorphin was assessed on CBA mice weighing

Department of Physiology, Biology faculty, M. V. Lomonosov University, Moscow; Research Institute of Applied Microbiology, Obolensk, Moscow Region

0007-4888/93/0008-0942$12.50

18-20 g. The physiological effect of the preparations under investigation was judged according to their ability to inhibit the mice's response to heat stimulation. The analgesic effect of the substances was assessed according to the time it took mice placed on a hot, thermostatically controlled surface (57±0.5°C) to respond to the heat by licking their front paws (the hot-plate test) [6]. We took a two-fold increase in the average time of response to the pain stimulation in the control animals as the quantitative index of inhibition [13]. Two series of independent experiments were carried out in which no fewer than 12 animals were used in each test group. As the positive control we used pure β-endorphin (Serva) in doses of 5, 8, and 10 mg/kg. As the negative control we used strain 15 of *F. tularensis* (supplied by the Research Institute of Applied Microbiology, Obolensk). The experimental preparation was the strain of *F. tularensis* 15E, which inherits the β-endorphin gene. All the samples investigated were prepared in physiological solution, and 0.2 ml of this solution was injected into the lateral caudal vain of the animals. The number of cells in the samples was 10^2 colony-forming units (CFU).

F. tularensis 15E, the producer of β-endorphin, was obtained by transforming the initial strain with plasmid pSKFT5-End, which inherits the β-endorphin gene in the composition of plasmid pSKFT5 [5]. The β-endorphin gene was provided by O. I. Serpinskii and V. V. Kravchenko (of the Research Institute of Molecular Biology, Koltsovo, Novosibirsk Region). The expression of the gene in the cells of the tularemic microbe was controlled by a homologous promoter flanking the fragment of chromosomal DNA of *F. tularensis* within the plasmid pSKFT5 [5]. The amount of β-endorphin synthesized by the cells was determined by the radioimmunoassay method with the aid of an INC kit of reagents (USA). The development dynamics of the culture in the parenchymatous organs was determined on the basis of homogenates formed from isolated liver and spleen according to a method described in the literature [7].

RESULTS

Data on the effect of commercial β-endorphin on the pain threshold in CBA mice, obtained by the hot-plate test method, are given in Fig. 1. As can be seen, the animals' response to heat stimulation depended on the dose of the preparation administered. The effect of commercial β-endorphin was of short duration, being observed only in the first 30 minutes after the preparation was injected. It

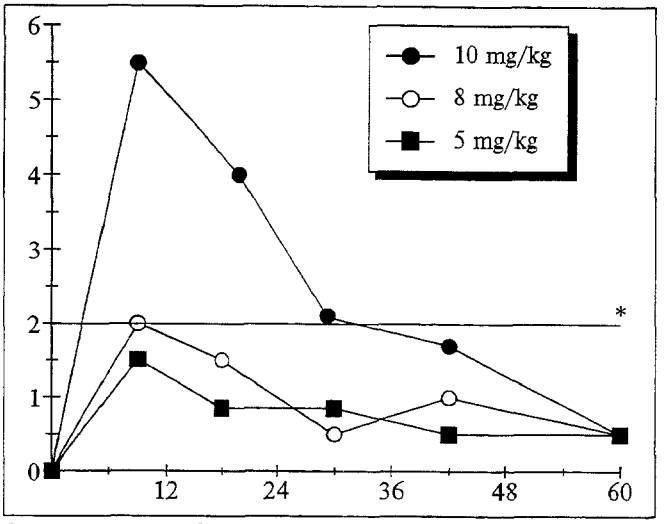

Fig. 1. Effect of β-endorphin on the pain threshold. T: mean (for the two groups of animals used) time of response to pain stimulus before the introduction of β-endorphin; T: mean (for the two groups) time of response to the pain stimulus after the introduction of β-endorphin:*) statistical index of inhibition according to published data [13]; t: test time, min; $t_1 = 14 \pm 3$, $t_2 = 11 \pm 2$, and $t_3 = 17 \pm 3$ sec.

should be noted that the change in the pain threshold under the influence of β-endorphin was accompanied by pronounced muscular rigidity and catatonia. The data obtained are in accord with the results of investigations on the physiological effect of β-endorphin [3], while the short duration of the effect matched other published data [8].

The strain of *F. tularensis* 15E was obtained during the transformation of cells of the vaccine strain of the tularemic microbe with plasmid pSKFT5-End, into which the β-endorphin gene was inserted at the EcoRI site directly next to the promoter of the tularemic microbe. The gene was mapped by us earlier on a fragment of chromosomal DNA of *F. tularensis* and is inherited by plasmid pSKFT5 [5]. The selection of clones inheriting the β-endorphin gene was made on the basis of resistance to tetracycline, since the tet gene of plasmid pSKFT5 was located immediately behind the β-endorphin gene in the same reading frame. The processing of β-endorphin was carried out with the aid of a TAG translation terminator, which separates the structural genes of β-endorphin and tetracycline resistance. The clones of *F. tularensis* 15E inherited plasmid pSKFT5-End in a state in which it was integrated with the chromosome. A comparison of the expression of the β-endorphin gene in cells of the strain *F. tularensis* 15E with that of the initial strain showed that the cells which inherited this gene produced the peptide, while in the cells without the gene there was no β-endorphin. The level of expression of the peptide in the cells of *F.*

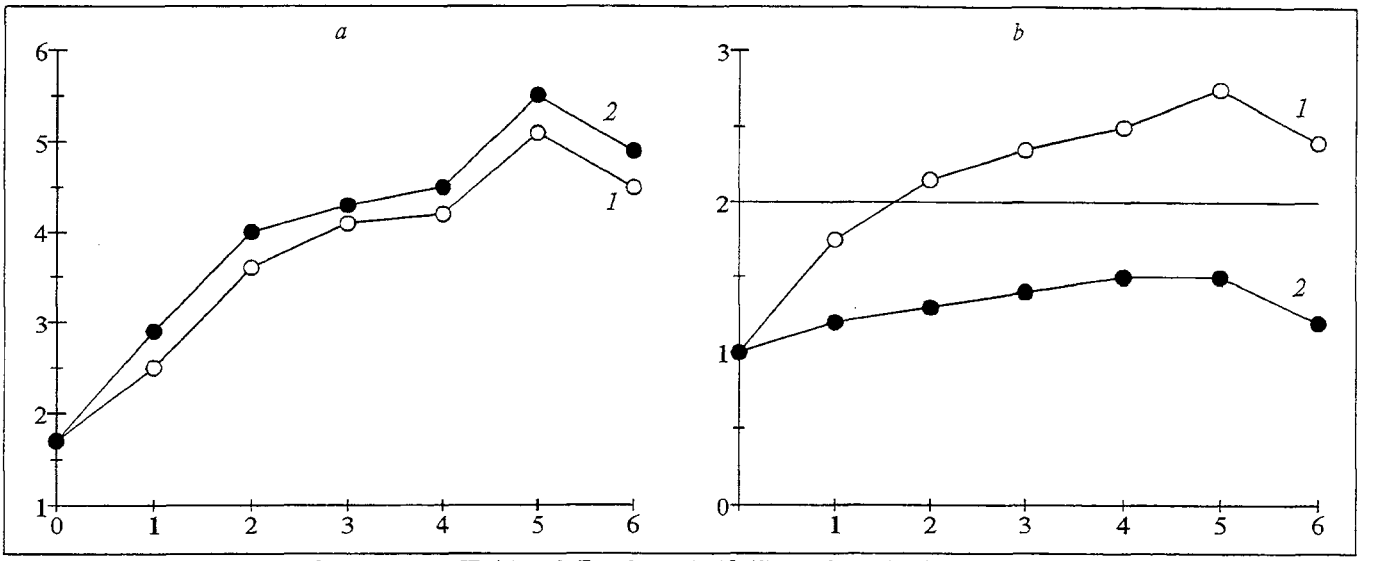

Fig. 2. Effect of strains of *F. tularensis* 15E (*1*) and *F. tularensis* 15 (*2*) on the pain threshold of mice during development of the culture in the animal organism. t: test time, days; $t_1 = 16 \pm 3$ and $t_2 = 12 \pm 2$ sec; *a*) multiplication dynamics of the tularemic microbe in mouse liver, *b*) analgesic effect. Notation as for Fig. 1.

tularensis 15E, grown in a modified Scherer aqueous medium up to a density of 10^9 CFU/ml, was 15 picomole/liter for the cells, and for the culture medium 20 picomole/liter, as determined by the standard method [14].

Since the concentration of β-endorphin in the culture medium and that in the producer cells were of the same order of magnitude, we can assume that the peptide under investigation is released into the environment in the process of cultivation of the strain in question. On this assumption we investigated the physiological activity of the recombinant β-endorphin by carrying out experiments *in vivo*.

A comparison of the physiological effect of the tularemic microbe producing β-endorphin with that of the initial strain is shown in Fig. 2. The physiological effect was determined *in vivo* in CBA mice.

As can be seen, intravenous injection of the strains led to changes in the pain threshold in the experimental animals in both cases (Fig. 2, curves *1* and *2*). However, in the hot-plate test the analgesic effect of the cells of the strain that produced β-endorphin was of a longer duration and significantly exceeded the statistical index of inhibition as compared with that of the initial strain. Besides the analgesic effect, the introduction of the strain that produced β-endorphin brought about a state of general muscular rigidity and catatonia in the experimental animals, as in the case with pure β-endorphin. In the mice given the initial strain no analogous physiological reactions were observed.

A comparison was made of the nature of the change in the pain threshold in the experimental animals as a function of time after the introduction of the strain (Fig. 2, *b*, curve *1*) with the multiplication dynamics of the cells of the strain under investigation (Fig. 2, *a*) in the body. This comparison suggests that there is a correlation between the number of cells in the organism and the amount of synthesized β-endorphin, since the maximum physiological effect coincided with the peak on the curve showing the content of cells in the body. The correlation between the multiplication dynamics of the cells of the initial strain and the observed analgesic effect of this strain can be attributed to the peculiarities of the pathogenesis of the tularemic microbe. On the basis of the data presented above we may conclude that the use of the vaccine strain of the microbe as a source of additive synthesis of β-endorphin produces an analgesic effect that lasts longer than that of commercial β-endorphin and leads to changes in the spectrum of stereotypic behavioral reactions *in vivo* that are typical for opiate peptides [3].

The proposed method of additive synthesis of biologically active substances can be improved by using other vaccine vector microorganisms with a higher level of expression and secretion of synthesized product and a low intrinsic peptidase activity. Moreover, it is possible to aim the regulators directly at the target organs by choosing microbes with tropism for the required organs and tissues.

REFERENCES

1. I. P. Ashmarin, *Zh. Evol. Biokhim.*, № 5, 570 (1977).
2. V. M. Borzenkov, *Abstract of Dissertation for the degree of Candidate of Medical Science*, Moscow State University (1982).

3. E. Costa and B. Trabucci, *Endorphins* (1956).
4. A. P. Pomerantsev, I. V. Domaradskii, and I. P. Doronin, *Current Topics of Tularemia* [in Russian], Moscow (1991), p. 148.
5. A. P. Pomerantsev, I. V. Domaradskii, *et al.*, *Molek. Genetika*, № 7, 12 (1991).
6. S. I. Ankier, Europ. *J. Pharmacol.*, **27**, 1 (1974).
7. L. S. Antoni, E. Skamene, *et al.*, *Infect. Immunol.*, **56**, 2089 (1988).
8. A. Ermisch, H. Ruhle, et al., J. Cereb. Blood Flow Metab., **5**, № 3, 350 (1985).
9. K. Nagahari, S. Kanaya, *et al.*, *EMBO J.*, **4**, № 132, 3589 (1985).
10. E. Skamene, *Rev. Infect. Dis.*, 5, 823 (1983).
11. J. K. Spitznagel, *Ibid.*, p. 806.
12. A. Tarnvik, *Ibid.*, **11**, № 3, 440 (1989).
13. L. F. Tseng, H. H. Loh, and C. H. Li, *Nature*, **263**, № 16, 239 (1976).
14. S. L. Wardlaw and A. G. Fraute, *J. Clin. Endocr.*, **48**, № 1, 176 (1979).

A Spin-Probe Study of the Effect of Sodium Hypochlorite on Human Blood Lipoproteins

O. M. Panasenko, S. A. Evgina, and V. I. Sergienko

UDC 616.153.963'915-02:615.31:546.33]-07

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 116, № 8, pp. 153-155, August, 1993
Original article submitted March 4, 1993

Key Words: *lipoproteins; sodium hypochlorite; lipid peroxidation; spin probes; lipoprotein structure*

It is well known that lipid peroxidation (LPO), the process generally involving serum lipoproteins (LP), accompanies the development of a variety of pathological processes, in particular atherosclerosis [8,13]. The role of oxidative modifiers can be assigned primarily to reactive oxygen forms such as H_2O_2, $O^{\cdot}_2$, $OCl^{\cdot}$, and $OH^{\cdot}$, which are generated by activated neutrophils and monocyte-derived macrophages [4]. Recently, it was shown that hypochlorite ($OCl^{\cdot}$) intensively oxidizes lipids and damages the serum LP proteins [1,6,7], thus being a possible cause of the accumulation of modified LP in the human organism.

In the present study we investigated the structural alterations induced by sodium hypochlorite (NaOCl) in the surface proteolipid layer of low-density LP (LDL) from human blood.

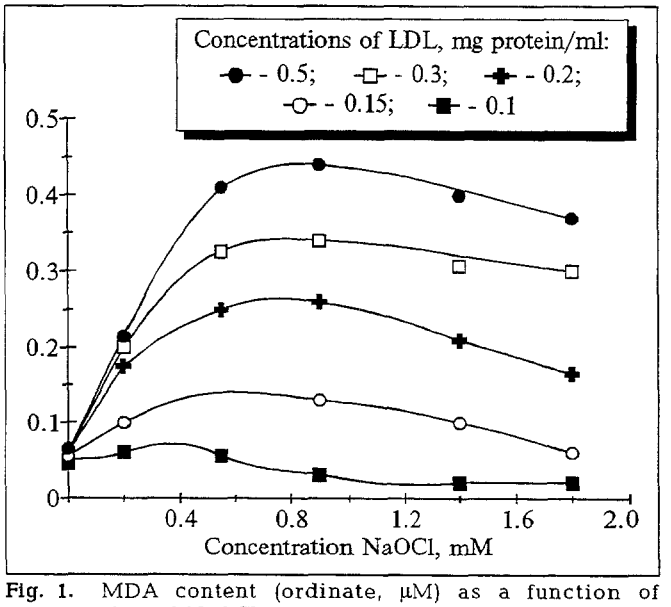

Fig. 1. MDA content (ordinate, μM) as a function of concentration of NaOCl after incubation of LDL with NaOCl at 37°C for 1 h. The incubation medium contains 145 mM NaOCl, 10 mM phosphate buffer saline, pH 7.4.

Research Institute of Physicochemical Medicine, Moscow (Presented by Yu. M. Lopukhin, Member of the Russian Academy of Medical Sciences)

0007-4888/93/0008-0945$12.50