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The influence of μ-opioid receptor agonist and antagonist peptides on peripheral blood mononuclear cells (PBMCs)

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ABSTRACT

Milk is one of the main source of biologically-active peptides that may function as regulatory substances called food hormones. After passing the gut–blood barrier, the μ-opioid receptor agonist and antagonist peptides may become the new factors influencing various functions of the human organism. The aim of the conducted research was to determine the influence of μ-opioid receptor agonist peptides: human and bovine β-casomorphin-7 (h/bBCM-7) and antagonistic peptides: casoxin-6 and- D (CXN-6/D) on proliferation and cytokine secretion of human peripheral blood mononuclear cells (PBMCs). The PBMCs proliferation was measured by the use of the BrdU test, which assesses the DNA synthesis activity and the WST-1 test which assesses the activity of mitochondrial dehydrogenase enzymes. The influence of all the investigated peptides on secretion of IL-4, IL-8, IL-13 and IFN-γ was determined by the use of the ELISA tests. Incubating the cells with the peptides has not caused any changes to their enzymatic activity, which has been proved by a WST-1 test. When using a BrdU test, however, it has been observed that there appear changes to proliferation of PBMCs correlated to amounts of bromodeoxyuridine incorporated into the cellular DNA. Moreover, changes to secretion of IL-4 and IL-13 by the cells under the influence of agonists were detected, as well as changes to secretion of IFN-gamma under the influence of all the examined substances. The obtained results provide information on immunomodulatory effects of food-derived opioid peptides, which may be of clinical significance especially in the case of allergic diseases in newborns.

1. Introduction

Milk proteins, both bovine and human, make a rich source of biologically active peptides. The μ-opioid receptor agonist peptides, called β-casomorphins (BCMs), are released from the β-casein [37,13] and the antagonist peptides, called casoxins (CXNs), are released from the κ-casein and α_{s1}-casein [39,40]. Those peptides have been identified both in cow [36] and human's milk [30,10] as well as in numerous dairy products [4,31]. They may be liberated from native milk proteins as a result of enzymatic hydrolysis occurring during technological processing of food [20] or in the digestive tract [5,3]. A possibility of crossing the intestinal barrier by the opioid peptides has also been reported [9,32] which has been confirmed by clinical research by Kost et al. [12].

The cells of the digestive, immune, nervous, and endocrine systems are equipped with opioid receptors that modulate functioning of the organism by the agency of endogenous opioids [19]. It has been suggested that upon getting into the bloodstream the exogenous opioid peptides may modulate functioning of the mentioned systems by interactions with μ-opioid receptors [34,8]. Some authors suggest the regulatory influence of β-casomorphins on the neonates' development [38], yet on the other hand, β-casomorphins liberated from the cow's β-casein are accused of participating in etiology of such conditions as: autism [24,33], SIDS [34], diabetes type I, postpartum psychosis [17], circulatory disorders, or food allergies [15,16]. Due to those facts, an increasing number of organizations, including the European Economic Community, the European Union, and the European Food Safety Authority have become involved in regulations connected with the safety of consumption of biologically active peptides in food [22].

Therefore, it is important to define the dose-dependent influence of opioid peptides consumed by people on the functioning of the human body. Moreover, the fact that both peptides, the μ-opioid receptor agonists and its antagonists, coexist in consumed food motivates to search for dependencies resulting from the possibility of simultaneous influence of both those compounds on the human organism.

The aim of this research was to examine the influence of μ-opioid agonist (bovine and human β-casomorphin-7) and antag-

Abbreviations: PBMCs, peripheral blood mononuclear cells; hBCM-7, human β-casomorphin-7; bBCM-7, bovine β-casomorphin-7; CXN-6, casoxin-6; CXN-D, casoxin-D; MOR, morphine; NLX, naloxone; IL, interleukin; IFN, interferon.

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onist peptides (casoxin-6 and casoxin-D) derived from bovine and human milk proteins on proliferation and cytokine's secretion by PBMCs.

2. Materials and methods

2.1. Study participants

The examined material consisted of the blood obtained from five selected healthy male volunteers aged 25–30 years old with no symptoms of allergies. The study was conducted with an approval of the local Ethical Committee and all of its volunteers signed an informed consent and filled in a questionnaire on their health condition, which assessed such factors as their prevalence of allergic disease, past diseases and pharmacologic treatment. In the sera of all the five participants the IgE against casein, α-lactoalbumin and β-lactoglobulin were in class 0.

2.2. Peptides

Four opioid peptides: human β-casomorphin-7 (YPFVEPI), bovine β-casomorphin-7 (YPFPGPI), casoxin-D (YVPFPPF) and casoxin-6 (SRYPSY) have been studied. All of them were synthesized at the Department of Bioorganic Chemistry, at Faculty of Chemistry of the University of Gdańsk. According to the producer's recommendation, the peptide purity is 99.4%.

2.3. Isolation of human peripheral blood mononuclear cells (PBMCs)

The peripheral blood was gathered into collecting tubes coated with K_3EDTA (BD Biosciences). The tubes were immediately transported to a laboratory where the isolation of PBMCs was started. The PBMCs were isolated in the gradient of Ficol (Histopaque 1077, Sigma). The PBMC layer was washed twice with RPMI-1640 (Sigma–Aldrich), the cells were counted, and finally diluted with RPMI-1640 containing 1% of a heat inactivated human AB serum as well as 1% of gentamycin and 0.25% of phytohemagglutinin-L (PHA-L, Roche Diagnostics). The cells were seeded on 96-well plates at concentration of 0.25×10^5 cells/well. After 48 h of growing, stimulatory substances were added. The influence of five different concentrations (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12} and 10^{-15} mol/L) of each opioid peptide (human β-casomorphin-7, bovine β-casomorphin-7, casoxin-D, casoxin-6) as well as two standards: morphine (μ-opioid receptor agonist, MOR) and the naloxone (μ-opioid receptor antagonist, NLX) on the human PBMC proliferation has been examined. The whole analysis was performed in triplicate. The level of PBMCs proliferation in the presence of the pure culture medium has been accepted as 100%.

2.3.1. Assessment of the PBMC proliferation using the WST-1 proliferation test

After 12 h of PBMC incubation with the opioids, the WST-1 proliferation test (Roche Diagnostic) was added in the ratio 1:10 and the incubation was continued for 90 min. The cell incubation time with all the investigated substances and with the WST-1 test was determined in previous experiments. The absorbance was measured at the wavelength of λ = 450 nm using an ELISA reader (Biogenet Asys UVM 340).

2.3.2. Assessment of the PBMC proliferation using the BrdU proliferation test

After 2 h of PBMC incubation with the opioids, a BrdU labeling reagent (Roche Diagnostic) was added in the volume of 10 μl per well and the incubation was continued for 10 h. After the incubation, the cell suspension was centrifuged at $300 \times g$ for 10 min and the labeling medium was removed by flicking it off and the rest was dried with a hair-dryer for 15 min. Then, the Fix Denat solution was added and the whole was incubated for 30 min at the room temperature. After that, the Fix Denat was removed and an anti-BrdU-POD solution was added in the amount of 100 μl per well. After 90 min of incubation, the solution was removed and the plate was rinsed 3 times with the 1% PBS. The absorbance measurement was taken at the wavelength of λ = 492 nm after 30 min of incubation with the solution of substrate.

2.3.3. Determination of cytokines

The cells were seeded at the concentration of 2×10^6 cells/ml of the medium containing 1% of a heat inactivated human AB serum as well as 1% of gentamycin and 0.25% of PHA. After 24 h of incubations, the stimulatory substances were added in three concentrations (10^{-6}; 10^{-9} and 10^{-12} mol/L). Morphine and pure medium were used as the controls. After seven days of incubation with the stimulatory substances, the cells were harvested and the supernatant was collected and stored at −70 °C. Commercially available ELISA kits were applied to determine the content of the following interleukins: IL-4, IL-8 (BD Bioscience), IL-13, IFN-γ (Mabtech, USA). The analysis was performed according to the manufacture's instructions. The results of different runs were equalised by a comparison of their standard curves and expressed as pg/ml. The analysis was performed in triplicate.

2.3.4. Statistical analysis

For the BrdU proliferation test, a nonparametric test (the Mann–Whitney *U* test) was used to compare the proliferation values for the test cultures with the ones for the control cultures. For WST-1 proliferation test, a parametric test (ANOVA) was used to compare the proliferation values for the test cultures with the ones for the control cultures. The level of significance was $p < 0.05$. Significant results of pairwise comparisons were presented as suppression or stimulation according to the decrease or the increase of the values in the test culture.

3. Results

3.1. Assessment of proliferation changes for human peripherial blood mononuclear cells under the influence of opioid peptides derived from the bovine and human milk

The PBMCs proliferation after 12 h incubation together with the examined substances has been determined using two tests with different working spectra. The WST-1 test was used to assess the level of the mitochondrial dehydrogenase activity which is correlated with the general number of cells and their ability to proliferate. The BrdU-test-based determination of the cellular proliferation made it possible to assess the proliferation changes in time by measuring the incorporation of bromodeoxyuridine (BrdU) in the DNA of the cells during the 10 h incubation with the examined substances.

3.1.1. Results of the WST-1 test

No significant increase of the cellular proliferation resulting from the PBMCs incubation with any of the examined opioids was observed when measured by the activity level of mitochondrial dehydrogenases (Figs. 1 and 2). The PBMC incubation with morphine and naloxone as well as human and bovine β-casomorphin in the femtomolar concentration (10^{-15} mol/L) did not entail any significant changes in relation to the control made of cells cultured in the pure medium. The PBMC incubation with those substances in concentrations higher than a femtomolar one resulted in a decrease of mitochondrial dehydrogenases activity. Casoxin-6 and casoxin-D significantly decreased ($p < 0.05$) the proliferation of the cells in the femtomolar concentration (10^{-15} mol/L) and higher ones. It has

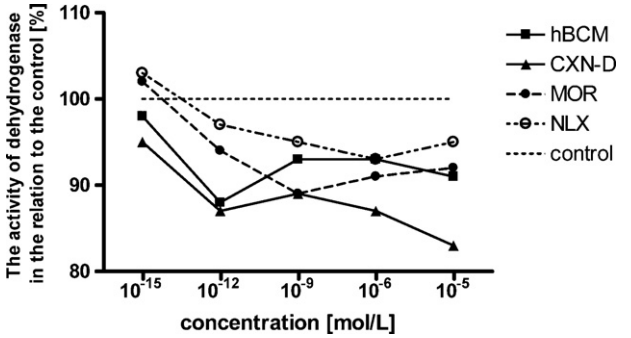

Fig. 1. The influence of human opioid peptides on the proliferation of the human peripheral blood mononuclear cells (PBMCs) measured with the WST-1 test ($n = 20$). The level of PBMCs proliferation cultured in the pure culture medium has been presented as the control (100%). hBCM, human β-casomorphin-7; CXN-D, casoxin-D; MOR, morphine; NLX, naloxone. Significant changes: CXN-D (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12}; 10^{-15} mol/L) vs control $p < 0.05$

been observed that the picomolar concentration (10^{-12} mol/L) was the most effective one in the case of human and bovine BCM-7 and CXN-D (Figs. 1 and 2).

3.1.2. Results of the BrdU test

The level of the BrdU incorporated into the structure of the cellular DNA during the 10 h incubation with the examined substances indicated a stimulation of the PBMCs proliferation by bovine and human β-casomorphin-7 in all the examined concentrations except for the femtomolar concentration (10^{-15} mol/L) in the case of human BCM. As a result of the incubation of human and bovine BCM in the picomolar (10^{-12} mol/L) concentration with the human lymphocytes, the highest and statistically significant increase of cell proliferation was observed when compared to the control (Figs. 3 and 4) ($p < 0.05$). The cell incubation in the presence of casoxin-6 did not entail any statistically significant changes measured by the level of the BrdU incorporation into the cellular DNA (Fig. 4). However, casoxin-D caused a statistically significant decrease of the cell proliferation measured by the lowered BrdU incorporation into the cellular DNA in the case of all the concentrations higher that the femtomolar one (Fig. 3) ($p < 0.05$). The incubation of the cells in the presence of morphine and naloxone resulted in a significant decrease in the cell proliferation when related to the control culture ($p < 0.01$); however, no statistically significant differences between the influences of those two substances were observed.

Fig. 5

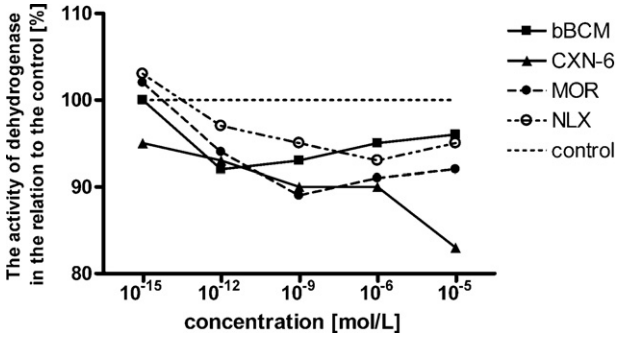

Fig. 2. The influence of bovine opioid peptides on the proliferation of the human peripheral blood mononuclear cells (PBMCs) measured with the WST-1 test ($n = 20$). The level of PBMCs proliferation cultured in the pure culture medium has been presented as the control (100%). bBCM, bovine β-casomorphin-7; CXN-6, casoxin-6; MOR, morphine; NLX, naloxone. Significant changes: CXN-6 (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12}; 10^{-15} mol/L) vs control. $p < 0.05$.

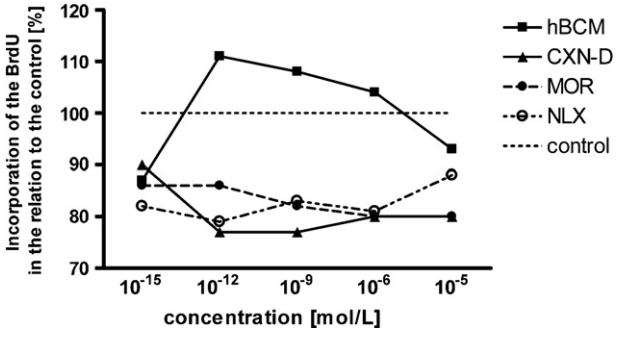

Fig. 3. The influence of human opioid peptides on the proliferation of the human peripheral blood mononuclear cells (PBMCs) measured with the BrdU test ($n = 20$). The level of PBMCs proliferation cultured in the pure culture medium has been presented as the control (100%). hBCM, human β-casomorphin-7; CXN-D, casoxin-D; MOR, morphine; NLX, naloxone. Significant changes: hBCM (10^{-12} mol/L) vs control, $p < 0.05$; CXN-D (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control, $p < 0.05$; MOR (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12}; 10^{-15} mol/L) vs control, $p < 0.01$; NLX (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12}; 10^{-15} mol/L) vs control, $p < 0.01$.

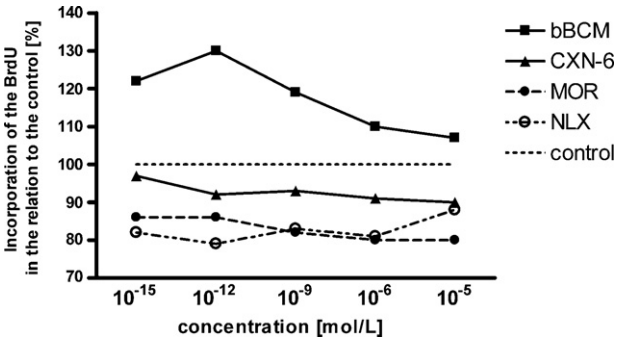

Fig. 4. The influence of bovine opioid peptides on the proliferation of the human peripheral blood mononuclear cells (PBMCs) measured with the BrdU test ($n = 20$). The level of PBMCs proliferation cultured in the pure culture medium has been presented as the control (100%). bBCM, bovine β-casomorphin-7; CXN-6, casoxin-6; MOR, morphine; NLX, naloxone. Significant changes: bBCM (10^{-12} mol/L) vs control, $p < 0.05$; MOR (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12}; 10^{-15} mol/L) vs control, $p < 0.01$; NLX (10^{-5}; 10^{-6}; 10^{-9}; 10^{-12}; 10^{-15} mol/L) vs control, $p < 0.01$.

Additionally, a competitive test was performed consisting of pre-incubation of the cells with a μ-opioid receptor antagonist (casoxin-6 or casoxin-D), a later application of an agonist and a re-incubation. When casoxin was coadministered with

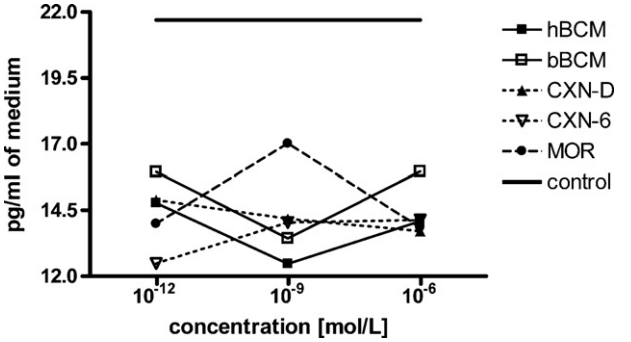

Fig. 5. The influence of bovine and human opioid peptides on the interferon-γ secretion by the human peripheral blood mononuclear cells (PBMCs) ($n = 15$). The level of INF- γ secreted by the PBMCs cultured in the pure culture medium has been presented as the control. hBCM, human β-casomorphin-7; bBCM, bovine β-casomorphin-7; CXN-D, casoxin-D; CXN-6, casoxin-6; MOR, morphine. Significant changes: hBCM (10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control. $p < 0.01$; bBCM (10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control. $p < 0.01$; CXN-D (10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control, $p < 0.01$; CXN-6 (10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control. $p < 0.01$; MOR (10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control, $p < 0.01$.

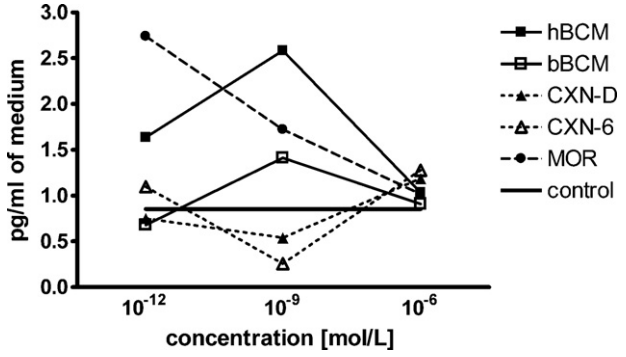

Fig. 6. The influence of bovine and human opioid peptides on the interleukin-4 (IL-4) secretion by the human peripheral blood mononuclear cells (PBMCs) ($n = 15$). The level of IL-4 secreted by the PBMCs cultured in the pure culture medium has been presented as the control. hBCM, human β-casomorphin-7; bBCM, bovine β-casomorphin-7; CXN-D, casoxin-D; CXN-6, casoxin-6; MOR, morphine. Significant changes: hBCM (10^{-9} mol/L) vs control, $p < 0.05$; CXN-D (10^{-9} mol/L) vs control. $p < 0.05$; CXN-6 (10^{-9} mol/L) vs control. $p < 0.05$; MOR (10^{-6}; 10^{-9}; 10^{-12} mol/L) vs control, $p < 0.05$.

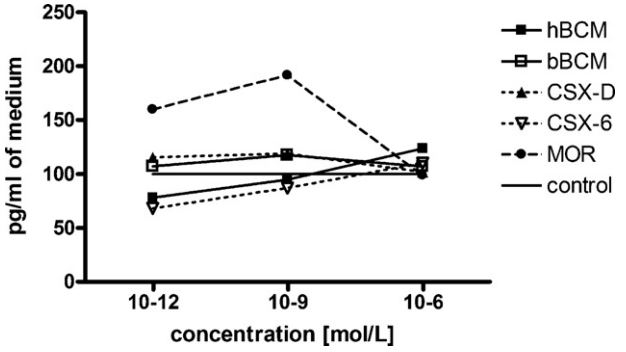

Fig. 7. The influence of bovine and human opioid peptides on the interleukin-8 (IL-8) secretion by the human peripheral blood mononuclear cells (PBMCs) ($n = 15$). The level of IL-8 secreted by the PBMCs cultured in the pure culture medium has been presented as the control. hBCM, human β-casomorphin-7; bBCM, bovine β-casomorphin-7; CXN-D, casoxin-D; CXN-6, casoxin-6; MOR, morphine. Significant changes: bBCM (10^{-9}; 10^{-12} mol/L) vs control, $p < 0.01$; CXN-D (10^{-12} mol/L) vs hBCM (10^{-12} mol/L), $p < 0.05$; MOR (10^{-9}; 10^{-12} mol/L) vs control, $p < 0.01$.

β-casomorphin-7, the effect of BCM in stimulation the PBMCs response was partially prevented in the case of all the examined concentrations. The inhibition of BCMs effect was in the ranged from 6% (hBCM, the concentration 10^{-15} mol/L) to 27% (bBCM, the concentration of 10^{-5} mol/L).

3.1.3. The influence of opioid peptides derived from the bovine and human milk proteins on cytokine's secretion by PBMCs

The influence of all the examined peptides on secretion of four cytokines: IL-4, IL-8, IL-13 and IFN-γ has been assessed. The level of each cytokine secreted by the PBMCs cultured in the pure culture medium has been presented as the control. The incubation of PBMCs with μ-opioid receptor agonist peptides caused the increase of IL-4 secretion (Fig. 6). Morphine significantly increased the IL-4 secretion ($p < 0.05$) at all concentrations. Human BCM-7 at the nanomolar concentration (10^{-9} mol/L) caused significant increase from control ($p < 0.05$). The incubation of the cells with the μ-opioid receptor antagonists resulted in a significant decrease in the level of the IL-4 secreted by the cells at the nanomolar concentration ($p < 0.05$). Both, in the case of the μ-opioid receptor agonist and its antagonist peptides, the nanomolar concentration was the most effective one that entailed changes in the IL-4 secretion by the PBMCs (Fig. 6). Morphine and bovine β-casomorphin-7 in the nano- and picomolar concentrations entailed a significant increase in the IL-8 secretion ($p < 0.01$) (Fig. 7). The PBMC incubation with the μ-opioid receptor agonist in the micromolar concentration (10^{-6} mol/L) did not cause an increase in the IL-8 secretion when compared to the control. No differences were observed in the IL-8 secretion by the PBMCs incubated with the bovine μ-opioid receptor agonist and antagonist. However, in the case of human peptides, CXN-D caused a significantly decreased secretion of IL-8 than BCM-7 ($p < 0.05$) (Fig. 7). The PBMC incubation with all the examined opioids entailed an inhibition of the IFN-γ secretion by the PBMCs in relation to the control culture made of cells cultured in the pure medium ($p < 0.01$). No statistically significant differences between the influences of particular examined compound substances were noticed (Fig. 5). The PBMC incubation with the examined opioids did not influence the IL-13 secretion when compared to the cells cultured in the pure medium (Fig. 8). However, significant differences between the IL-13 secretions under the influences of particular opioids were observed. Casoxin-D caused a lower IL-13 secretion than human β-casomorphin-7; yet, those differences were statistically significant only in the case of the nanomolar concentration (10^{-9} mol/L) ($p < 0.01$).

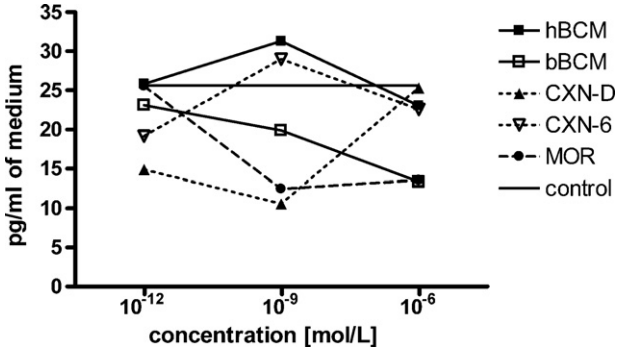

Fig. 8. The influence of bovine and human opioid peptides on the interleukin-13 (IL-13) secretion by the human peripheral blood mononuclear cells (PBMCs) ($n = 15$). The level of IL-13 secreted by the PBMCs cultured in the pure culture medium has been presented as the control. hBCM, Human β-casomorphin-7; bBCM, bovine β-casomorphin-7; CXN-D, casoxin-D; CXN-6, casoxin-6; MOR, morphine. Significant changes: CXN-D (10^{-9} mol/L) vs hBCM, $p < 0.01$.

4. Discussion

It has been proved that the cells of the nervous and immune systems are capable of communicating with each other by the agency of endogenous opioid peptides. As early as in 1986, Shavit et al. paid their attention at the new function of endogenous opioids as particles carrying information to the cells of the immune system [29]. Those cells are equipped with all three classes of opioid receptors (μ, δ, and κ) involved in transmitting signals by endo- and exogenous opioids [18]. Moreover, lymphocytes, monocytes and macrophages are capable of synthesizing endogenous opioids that participate in a regulation of immune system functioning [23].

Cabot et al. have examined the role of β-endorphins produced by the cells of the immune system in inflammatory processes. The lymphocyte-derived β-endorphin may induce analgesia effect at the inflamed tissue by their interaction with the peripheral sensory nerve terminals and play the role of mediators for the immune system when attracting lymphocytes to the inflamated tissue [2].

The influence of the exogenous opioid peptides, which are provided to the organism together with food, on functioning of the immune system, and by its agency also on the nervous one has not been fully explained so far.

In the conducted experiment, an attempt to describe the role and the influence of the food-originating μ-opioid receptor agonists and its antagonists as well as their concentrations on proliferation and secretory properties of human peripheral blood mononuclear

cells has been undertaken. Morphine - the agonist of the μ-opioid receptor - and naloxone - the antagonist of the μ-opioid receptor - have been used as a control and a reference point during the result interpretation. The previous studies confirm an immunosuppressive influence of morphine and indicate the μ-opioid receptor as the pathway of that reaction [25,7]. The observed inhibitory influence of morphine and naloxone on the human PBMCs proliferation confirms the results of the previous research.

Sütas et al. have observed an inhibitory influence of the pepsin–trypsin milk hydrolysates as well as of β- and α_{s1}-casein on the proliferation of PBMCs isolated from healthy donors. That effect was strengthened by additional hydrolysis with the use of bacterial enzymes from the *Lactobacillus casei* strain [35]. The researchers have not performed analyses (verb)/analyses (noun) aiming at identification of the opioid peptides obtained in the hydrolysates, however the research carried out by De Noni and Cattaneo firmly indicates the presence of those peptides in similar extracts [4]. The results obtained by Sütas et al. [35] may be connected with the presence of opioid peptides in milk hydrolysates; however, other biologically active peptides liberated from the milk protein structure during digestion should also be remembered. A similar experiment has been carried out by Otani and Hata who showed such an inhibitory influence on the proliferation of mouse lymphocytes and rabbit Peyer's patch cells [21]. Those researchers paid their attention at a significant suppression increase after trypsin and pancreatinine had been used. The studies conducted by Elitsur and Luk confirmed the immunosuppressive character of BCM-7 in the experiment with human colonic lamina propria lymphocyte [6].

Kayser and Meisel [11] have examined the influence of biologically active peptides liberated from milk, including bovine BCM-7, on proliferation of human PBMCs using the BrdU test. They observed a stimulation of cells that is concentration-dependent, which is in agreement with the results that we have obtained. The highest increase of proliferation was observed in the case of the cells incubated with BCM-7 in the concentrations of 10^{-6}, 10^{-5} and 10^{-4} mol/L. However, in the experiment that we have conducted, the highest increase of the proliferation was observed when the concentrations were at 10^{-15}, 10^{-12} and 10^{-9} mol/L. The discrepancies in the obtained results are most likely to originate from methodological differences for the aforementioned researchers in their experiment used lymphocytes that had been previously separated from monocytes. In spite of that, both experiments confirm the immunomodulatory nature of bovine BCM-7.

The presented results indicate a stimulatory effect of both bovine and human BCM on human PBMCs in the initial period of peptide incubation together with the cells, which is confirmed by the increased level of BrdU incorporated in the cellular DNA. However, after the 12 h exposure of the cells to bovine and human BCM-7, an inhibitory influence of those peptides on the cell proliferation has been observed, which was reflected in their lowered metabolic activities. The remaining examined substances did not stimulate the cell proliferation after 10 h incubation with human PBMCs. Those results suggest the existence of additional mechanisms of β-casomorphin influence on the immune system cells.

The inhibition of stimulatory influence of β-casomorphins by pre-incubation of the PBMCs with the agonist of the μ-opioid receptor proves the existence of an opioid pathway for the influence of those peptides. Their surface enzyme - proline-derived dipeptidyl peptidase-4 (DPPIV, EC 3.4.14.5) - is a crucial factor that may play an important role in modulating the exogenous opioid peptide influence on the immune system cells. That enzyme, capable of inactivating opioid peptides, plays a pivotal role in launching the proliferation and lymphocyte differentiation. That fact confirms the inhibitory influence of DPPIV inhibitors on the lymphocyte proliferation and cytokine production as well as on the B-cells differentiation and antibody production. Moreover, the research carried out by Schön et al. proves that an eruption of the enzyme outside the membrane strengthens the proliferation effect [28,27]. The DPPIV inhibitors consist of peptides that include proline in their third position as well as dipeptides X-Pro that are products of the reaction catalyzed by the discussed enzyme. Casoxin-D is built similarly to the aforementioned peptides. Thus, it may be suspected that the intensive inhibition of the PBMCs proliferation by that peptide could occur by means of CD 26 inhibition. The sequence of bovine β-casomorphin-7 (YPFPGHI) indicates two hypothetic places of cutting the peptide by DPPIV - between amino acid residues 2 and 3 as well as 4 and 5. Such created degradation products may manifest inhibitory actions toward DPPIV, which may be the cause of the decrease in the cell proliferation after 12 h incubation with casomorphin.

Kraus et al. have examined the influence of morphine on interleukin secretion by the immune system cells [14]. That problem has been also deeply examined by Roy et al. [26]. The studies showed an inhibitory influence of morphine on secretion of the interleukins connected with the Th1 pathway (IL-2 and IFN-γ) and stimulatory influence on secretion of the interleukins connected with the Th2 pathway, such as IL-4 and IL-5. No similar effects have been observed in mice deprived of the μ-opioid receptor. That made it possible for the authors to draw the conclusion that morphine, by interaction with the μ-opioid receptor, triggers differentiation of the Th2 lymphocytes and entails an increase of the IL-4 gene expression by the NFAT mechanism. The observed increased level of IL-4 secreted by the PBMCs incubated with morphine at the concentrations of 10^{-12} and 10^{-9} mol/L with the simultaneous lowering of the IFN-γ secretion level fully confirms the results described above. An effect similar to that of morphine has been observed in the case of every concentration of human BCM-7 as well as in the case of the bovine one but only at the concentration of 10^{-9} mol/L. That, in the context of the results obtained by Roy et al. may indicate influence of the food-originated μ-opioid receptor antagonists similar to that of morphine.

That fact becomes particularly important in the case of newborns, whose only food is made of milk. Kost et al. [12] have examined the content of human and bovine BCM in the sera of the children up to the tenth month of age, fed with artificial formulas or their mothers' milk. The average content was 60 fmol/mL for the bovine BCM-7 and 200 fmol/mL for the human BCM-7 and it kept increasing after feeding. Whereas Assargard et al. determined picomolar contents of BCM-5 containing peptides in the sera of pregnant women [1]. Therefore, the concentrations of opioid peptides applied in our study were within the real values for the exogenous opioid peptides in human sera that can have an effect on the immune system.

Within that aspect, the presented results may be of clinical significance. All the examined peptides have manifested a concentration-dependent inhibitory influence on the IFN-γ secretion, and thus on the Th1 lymphocyte differentiation. As it has been suggested by Sütas et al. [35], that fact may contribute to creation of natural immunotoleration in newborns. Thus, the obtained results include information that may be used in drawing up the contents of milk formulas for the newborns suffering from allergies to milk proteins so that their tolerance to those allergens could be developed. Moreover, the obtained results provide information on the immunomodulatory influence of peptides from food on the human immune system, which may be of crucial significance in numerous diseases.

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