Peptides 30 (2009) 1854–1860

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

β-Casomorphins-7 in infants on different type of feeding and different levels of psychomotor development

Natalya V. Kost [a,*], Oleg Yu. Sokolov [a], Oksana B. Kurasova [b], Alexander D. Dmitriev [a], Julia N. Tarakanova [a], Marina V. Gabaeva [a], Yuriy A. Zolotarev [c], Alexander K. Dadayan [c], Sergei A. Grachev [d], Ekaterina V. Korneeva [b], Inna G. Mikheeva [b], Andrey A. Zozulya [a]

[a] National Research Center for Mental Health RAMS, 113152 Moscow, Zagorodnoe shosse 2/2, Russia
[b] Russian State Medical University, 117997 Moscow, Ostrovityanova ul. 1, Russia
[c] Institute of Molecular Genetics RAS, 123182 Moscow, Kurchatova pl. 2, Russia
[d] Ovchinnikov-Shemyakin Institute of Bioorganical Chemistry RAS, 117997 Moscow, Miklukho-Maklay ul 16/10, Russia

ARTICLE INFO

Article history:
Received 7 April 2009
Received in revised form 19 June 2009
Accepted 24 June 2009
Available online 1 July 2009

Keywords:
β-Casomorphins-7
Infants
Breast feeding
Formula feeding
Psychomotor development
Muscle tone

ABSTRACT

Casomorphins are the most important during the first year of life, when postnatal formation is most active and milk is the main source of both nutritive and biologically active material for infants. This study was conducted on a total of 90 infants, of which 37 were fed with breast milk and 53 were fed with formula containing cow milk. The study has firstly indicated substances with immunoreactivity of human (irHCM) and bovine (irBCM) β-casomorphins-7 in blood plasma of naturally and artificially fed infants, respectively. irHCM and irBCM were detected both in the morning before feeding (basal level), and 3 h after feeding. Elevation of irHCM and irBCM levels after feeding was detected mainly in infants in the first 3 months of life. Chromatographic characterization of the material with irBCM has demonstrated that it has the same molecular mass and polarity as synthetic bovine β-casomorphin-7. The highest basal irHCM was observed in breast-fed infants with normal psychomotor development and muscle tone. In contrast, elevated basal irBCM was found in formula-fed infants showing delay in psychomotor development and heightened muscle tone. Among formula-fed infants with normal development, the rate of this parameter directly correlated to basal irBCM. The data indicate that breast feeding has an advantage over artificial feeding for infants' development during the first year of life and support the hypothesis for deterioration of bovine casomorphin elimination as a risk factor for delay in psychomotor development and other diseases such as autism.

1. Introduction

Originally, more than 30 years ago, casomorphins (CMs) were identified in enzymatic bovine casein digest as a substance with defined opioid activity [4]. Later, opioid peptides were shown to be derived either from caseins or other proteins contained in mammalian milk, including human and cow milk [41]. Multiple studies in animal models reported that these peptides and CMs first of all decrease pain sensitivity [3,25,32], possess anxiolytic [13,14], antihypertensive [33] and cardiotropic [23] effects, improve learning and memory [12,35], regulate maternal motivation [10] and mother-oriented ("child's") behavior in rats [11], induce apnea and irregular breathing in adult rats and newborn rabbits [16], increase prolactin release in rats [27], and expression and secretion of gastrointestinal mucins and regulatory peptides in intestinal cells [44,45]. Most of these effects were opioid-dependent, i.e. reversible by the opioid receptors antagonists [3,10,16,25,27,32,35,44]. However, some effects of CMs were opioid-independent [23,33], suggesting that there are additional receptors and signaling pathways for CMs. In fact, β-CM-9 and some other peptides derived from bovine milk casein have been shown to inhibit angiotensine-converting enzyme [33], while bovine and human β-CM-7 appeared to act as antagonists of 5-HT_2-serotonin receptors [38,39]. Non-opioid influence of CM-5 on the β-adrenoceptor complex was detected in guinea pig heart membranes [23]. (D-Pro4)-β-CM-5 was shown to regulate synaptic transmission of acetylcholine in hippocampus and dopamine in striatum, presumably by interaction with the dopamine and serotonin receptors [20].

The wide spectrum of the biological activity of CMs predetermines their role in human health and disease states. A number of studies have data suggested that bovine β-CM-7 can act as a

* Corresponding author at: National Research Center for Mental Health RAMS, Laboratory of Pathophysiology, Zagorodnoe shosse 2/2, 113152 Moscow, Russia. Tel.: +7 495 952 9090.
E-mail address: nat-kost@yandex.ru (N.V. Kost).

0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.06.025

causative agent of cardiovascular disease, type 1 diabetes, sudden infant death syndrome, autism and schizophrenia [6,19]. However, it appears highly unlikely that evolutional (natural) selection retained a harmful product as the main nutritional component for mammalian infants. Most experimental data indicate the positive action of milk-derived peptides [33], and it can be inferred that these exogenous peptides are most effective in early infancy, when permeability of the intestinal mucosa is increased for small peptides and large molecules of proteins [42]. The only direct identification of bovine CM-immunoreactive (irBCM) materials in the blood was done in beagle puppies. The plasma level of irBCM was shown to rise after feeding with cow milk based formula in newborns, but did not in adult dogs [37]. Immunoreactivity of human CM (irHCM) has been detected in cerebrospinal fluid [24,28], blood plasma [15,24,28], urine [15], and milk [17,28] of pregnant, puerperal and lactating women. However, so far neither human nor bovine CMs have been directly identified in the blood of human infants.

The purpose of our study was to detect and characterize materials with immunoreactivity of human and bovine β-CM-7 in the blood plasma of naturally and artificially fed infants, and to investigate the relationship between the level of β-CM-7-immunoreactivity and the rate of the infants' psychomotor development.

2. Materials and methods

2.1. Patients

Ninety infants younger than 1 year were enrolled in the current study, and written informed consent forms were obtained from the parents of all participants. All infants were patients of the Moscow Izmailovo Children's Clinical Hospital, where they received treatment for acute respiratory viral infection. All procedures were performed right before the patients' discharge, when they had completely recovered from their infections. Pediatric examination of the infants included assessment of psychomotor development using the 30-point scale method developed by Jurba and Mastukova [18]. This method differentially evaluates the development of motor, language and mental functions in infants. According to this test, 27–30 points on the scale corresponds to normal psychomotor development, while 23–26 points are the range for a risk of delayed development, and points below 26 correspond to a definite delay in development. Muscle tone was referred as one of the dynamic functions of age development, and measured in 4-point scale by deviation from physiological one according to Jurba's test. In addition, in general clinical investigation the patient's muscle tone was evaluated as normal, heightened, or lowered.

Blood samples were first obtained as a part of routine diagnostic procedure in the morning before feeding. Ages for the patients were as follows: among 53 artificially fed infants, 13 patients were from 1- to 3-month old, 23 from 4- to 6-month old, and 17 from 7- to 12-month old. Among 37 breast-fed infants, 26 patients were from 1- to 3-month old, 6 from 4- to 6-month old, and 5 older than 6 months. The 4–10-month old naturally fed patients were combined into one group to increase the sampling size. Not all the mothers consented to having blood taken twice from their children. So blood samples were obtained from 39 infants 3 h after feeding with formula based on cow milk with albumin predominance, and from 20 infants 3 h after breast feeding. Plasma samples from 5 healthy adult volunteers were used as a negative control.

Blood samples were collected into tubes containing EDTA (3 mg/ml) as an anticoagulant and peptidase inhibitor bacitracin (150 μg/ml). Plasma was separated immediately by centrifugation at 1000 × *g*, at 4 °C for 10 min and stored at −20 °C until analysis.

2.2. Methods

2.2.1. Extraction of β-casomorphins-7 from plasma

Peptides were extracted from plasma by boiling in a 10-fold excess of 0.25 M acetic acid in a water bath for 15 min. The extracts were frozen, lyophilized, and kept at −70 °C. Just before the analysis, all probes were dissolved in an appropriate buffer and centrifuged at 1000 × *g*, at 4 °C for 10 min, and the supernatant was collected. The extraction recovery rate was about 70% as assessed by the addition of [^{125}I] β-CM-7 (about 50,000 cpm, 100 fmol) to 1 ml of human plasma. The recovery rate of radioactivity after extraction was 82% for bovine and 76% for human [^{125}I] β-CM-7, while immunoreactivity of extracted radioactive materials remained intact up to 90% of reference.

2.2.2. Radioimmunoassay

β-CM-7 antisera were prepared by immunization of rabbits according to method described by Dmitriev et al. [9]. Human (Tyr-Pro-Phe-Val-Glu-Pro-Ile) and bovine (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) β-CM-7 (Sigma, USA) were coupled to a number of proteins: bovine serum albumin (BSA), RNAase, chymotrypsin, thyroglobulin, ovalbumin, cattle immunoglobulins, catalase, using glutaric aldehyde as a cross-linking reagent. The coupling reaction was performed overnight at 0 °C (ice bath) in the mixture contained 0.02% glutaric aldehyde, 0.4 mg/ml carrier protein and 0.2 mg/ml either of casomorphins in 50 mM Na_2HPO_4 (pH 8.0). Rabbits were immunized with makeup conjugates subcutaneously in the back (300 μg per rabbit in 0.5 ml of Friend's adjuvant). The immunization was performed in 7 rounds every 2–3 weeks, and each time the peptides were conjugated to different proteins to enhance the specificity of the serum to CM. The serum was obtained by centrifugation of the blood collected from the auricular vein and stored at −20 °C.

Radioactively labeled peptides were obtained by radioiodination (^{125}I: Amersham, U.K.) of β-CM-7 by a modified method developed by Miller et al. [26] using chloramine T (Merck, Germany) for the iodination and mercaptoethanol (Sigma, USA) to terminate the reaction. The iodinated peptides were purified from Na^{125}I by ion exchange chromatography on QAE-Sephadex A-25 column using 50 mM acetic acid with 0.5% BSA as an eluent. β-CM-7-containing fractions were neutralized by ammonia to pH 7.5 and kept in 25% glycerin at −10 °C for less than 1 month. The purification efficiency of both labeled peptides was more than 99%. The specific radioactivity was 361 and 410 Ci/mmol for human and bovine β-CM-7, respectively.

RIA was carried out in the buffer consisting of 0.15 M NaCl, 25 mM Na_2HPO_4 (pH 7.5), 0.5% BSA, and 0.1% Tween 20, at 4 °C. For the assay, the samples, chromatography fractions or β-CM-7 standards, were dissolved or further diluted by the buffer to the volume of 600 μl and incubated for 24 h with 100 μl of anti-β-CM-7 antiserum (diluted 750-fold for bovine and 500-fold for human β-CM-7 assay). One day later, 100 μl of [^{125}I] β-CM-7 (about 20,000 cpm, 30 pg) was introduced into the mixture and incubation continued for 4 days. On day 4, 100 μl of goat anti-rabbit serum was added for 2 h following by 100 μl of 50-fold diluted neutral rabbit serum and 800 μl of 15% polyethyleneglycol 6000. After thorough mixing, the tubes were centrifuged at 2000 × *g*, 4 °C for 30 min. The supernatant was aspirated and the precipitate was counted in a gamma counter (Gamma Trac, efficiency 70%). Nonspecific binding (about 20%) was determined in the absence of anti-β-CM-7 antiserum.

Cross-reactivity of human and bovine β-CM-7 to the obtained antisera was negligible. Standard curves proved to be linear in a logit/log plot between 10% and 90% inhibition of [^{125}I] labeled peptides binding to the antibodies over the range of concentrations of 0.03–5.0 pmol/assay tube for human β-CM-7 and 0.01–

2.0 pmol/assay tube for bovine β-CM-7. The detection limit of human and bovine β-CM-7 was 50 and 15 fmol, respectively, which is similar to the literature data [37,40].

2.2.3. Enzyme linked immunosorbent assay (ELISA)

ELISA was performed using purified antibodies from the same antisera that were used for the RIA. Purification was carried out by affine chromatography at BrCN-sepharose-4B conjugated with bovine β-CM-7. Antisera (3 ml) were introduced to a 5 mm × 15 mm column in PBS, the column was washed with PBS to the disappearance of the optical density at 280 nm in the eluate, followed by antibody elution with 0.1 M acetic acid (pH 2.2). The effluent was neutralized with ammonia to pH 7.0. Protein concentration was measured by absorbance at 280 nm. The protein output was about 0.3 mg.

Bovine β-CM-7 was biotinylated with NHS-LC-LC-biotin (succinimidyl-6-(biotinamido)-6-hexanamido hexanoate, Pierce, USA) in equimolar concentrations by overnight incubation of the substances in 0.1 M $NaHCO_3$ (pH 8.0) at 4 °C with subsequent purification by HPLC at ProntoSIL-120-5-C18 column using gradient elution. Acetonitrile with 0.1% TFA was used as a mobile phase. The concentration of the product was evaluated by optical density at 280 nm. For the assay, purified antibodies were diluted in 0.05 M Na-carbonate buffer (pH 9.5) to 4 μg/ml, added to 96-well plates (Nunc, Denmark) (0.25 ml/well), and the plates were incubated for 3 days at 4 °C in a humidified atmosphere. The plates were then washed 5 times with distilled water. The samples, chromatography fractions or β-CM-7 standards were dissolved in a buffer consisting of 0.15 M NaCl, 25 mM Na_2HPO_4 (pH 7.5), 0.2% BSA, and 0.05% Tween 20 (ELISA buffer), added (200 μl/well) to the washed antibody-coated wells together with 50 μl of biotin-β-CM-7 (10 fmol/well) in the same buffer, and incubated for 1.5 h at 37 °C. After subsequent rinsing with water, 250 μl of conjugate of streptavidin with peroxidase (100 ng/ml, Imtek Ltd., Russia) diluted in ELISA buffer were added into each well for 30 min (37 °C). Plates were rinsed again with distilled water 8 times and 250 μl of 2.5% tetramethylbenzidine (Bioservice, Russia) in 0.2 M Na_2HPO_4–citrate buffer (pH 4.0) with 0.01% H_2O_2 and 0.05% ProClin were added. The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4 after 15 min at room temperature and absorbance was immediately measured at 450 nm using plate reader (Uniplan, Russia). Nonspecific binding (about 10%) was determined in wells not coated with antibodies.

Standard curves were linear in a logit/log plot between 10% and 90% inhibition of biotin-labeled bovine β-CM-7 binding to the antibody over the range of concentrations of 0.015–1.2 pmol/well for bovine β-CM-7, IC_{50} was about 0.1 pmol/well. The detection limit of bovine β-CM-7 was 25 fmol. Human β-CM-7 and β-casein from bovine milk (Sigma, USA) in 100-fold excessive concentrations did not displace labeled peptide in this assay.

2.2.4. Chromatography

The molecular weight of β-CM-7 immunoreactive material in infants' plasma was evaluated by gel filtration at HPLC system (Gilson, USA). Lyophilized extracts of plasma samples prepared as above were reconstituted in 25 mM Na_2HPO_4 buffer pH 6.0, centrifuged, and the dissolved material was separated on 7.8 mm × 300 mm Protein Pak 60 column. The elution was carried out with the same buffer at a flow rate of 0.7 ml/min with absorbance detection at 226 nm. Seven fractions (about 2 ml each) were collected, lyophilized and reconstituted in ELISA buffer. Control trials were performed to evaluate the rate of elution of BSA, β-casein from bovine milk, bovine β-CM-7, and synthetic hexapeptide dalargin (Tyr-D-Ala-Gly-Phe-Leu-Arg).

Chromatographic characterization of β-CM-7 immunoreactive material was also done by reverse phase chromatography. Extracts were reconstituted in 1.5 ml of 50% acetonitrile with 0.1% TFA, centrifuged, evaporated, dissolved in 100 μl of 0.1% TFA and separated on 2.0 mm × 75 mm ProntoSIL-120-5-C18 column (flow rate of 0.2 ml/min, 35 °C) using gradient elution in acetonitrile (0–60%) with 0.1% TFA as a mobile phase. Four fractions (about 1.5 ml each) were collected in the range of β-CM-7 elution (identified by control runs of reference substances), evaporated and applied to ELISA.

2.2.5. Statistics

The data analyses were performed using Statistica program (version 6.0). The results were expressed as mean values ± SE. The mean values in different groups were compared using Student's *t*-test. Non-parametric correlations were calculated by Spearman's rank correlation coefficient.

3. Results

3.1. Immunoreactivity of bovine and human β-CM-7 in the blood of infants

The consequent immunization of the rabbits with bovine and human β-CM-7 conjugated with different proteins resulted in antisera with a high titer to the peptides. As measured by half-maximum binding with iodinated peptides (about 20,000 cpm/ml) the titer was 1:7500 and 1:5000 for bovine and human β-CM-7, respectively. Using the antisera, ELISA and RIA of the peptides were created. The immunoreactivity of both bovine and human β-CM-7 in all the infants' blood plasma was measured by RIA.

RIA detected immunoreactivity of bovine β-CM-7 (irBCM) in plasma specimens of infants fed with formula containing the proteins of cow milk, with no difference in this parameter before feeding (basal level) in the 1–3-month-old infants (67 ± 19 fmol/ml, $n = 13$) and 4–6-month-old patients (59 ± 16 fmol/ml, $n = 23$) (Fig. 1). However, after the age of sixth month, the basal level of plasma irBCM increased up to 90 ± 14 ($n = 17$) (Fig. 1). The mean level of irBCM rose significantly in 3 h after feeding in the groups of 1–3 and 7–12-month-old infants, but not for the group of 4–6-month-old patients ($p < 0.05$, Fig. 1). There was no detectable irBCM in the plasma of most of the adult volunteers, while in the breast-fed infants it was in the range of the cross-reactivity between human and bovine β-CM-7 (about 3% from the same parameter tested for human CM).

Immunoreactivity of human β-CM-7 (irHCM) was detected in the plasma specimens of breast-fed infants. Prior to the feeding, the average value was also the same in the 1–3-month old (208 ± 23 fmol/ml, $n = 26$) and 4–10-month-old patients (197 ± 30 fmol/ml, $n = 11$). Similarly to irBCM, irHCM significantly rose after feeding in the case of 1–3-month-old infants, but not in the older ones (Fig. 2). As a result, the level of irHCM in blood plasma after feeding the 1–3-month-old infants was significantly higher than in the oldest ones ($p < 0.05$,

important

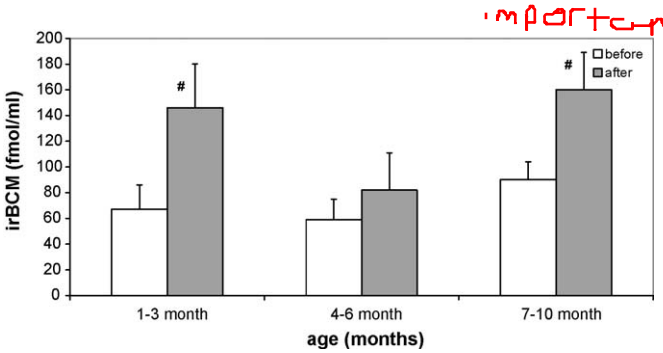

Fig. 1. Immunoreactivity of bovine β-casomorphin-7 in the blood plasma of different age infants. Bars are the mean ± SE. $^{\#}p < 0.05$ refers to comparison of baseline values with values 3 h after feeding.

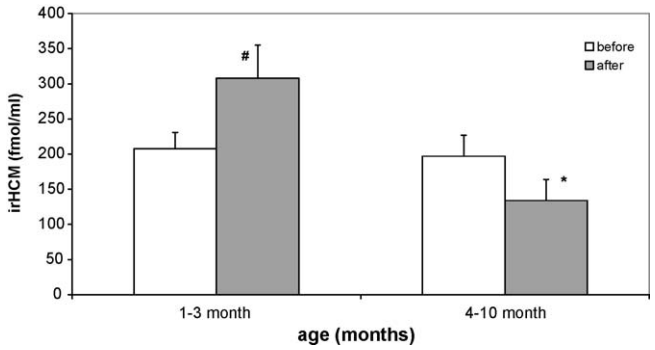

Fig. 2. Immunoreactivity of human β-casomorphin-7 in the blood plasma of different age infants. Bars are mean ± SE. $^{\#}p < 0.05$ refers to comparison of base-line values with values 3 h after feeding, $^{*}p < 0.05$ refers to comparison of post-feding values in the group of different age infants.

Fig. 2). The plasma irHCM of adult volunteers was below the detection limit, but a significant amount of human CM-immunoreactive materials was shown in the plasma of several infants who were fed artificially.

Thus the immunoreactivity of bovine and human β-CM-7 was detected in the blood of first year life infants. It increased after feeding mainly in 1–3-month-old patients.

3.2. Immunoreactivity of β-CM-7 in the blood and psychomotor development of infants

We found no difference in the mean values of psychomotor development of infants who were on breast feeding versus those on artificial feeding. However, there was only one case of developmental delay in the group of breast-fed infants, while 16 infants in the artificially fed group displayed delay in development. The rate of psychomotor development tested on a 30-point scale positively correlated with the basal level of irHCM in the total group of breastfed infants ($R = 0.35$, $p < 0.05$). This correlation was more pronounced and was revealed both before ($R = 0.45$, $p < 0.05$) and after ($R = 0.66$, $p < 0.05$) feeding in the group of 1–3-month-old breast-fed infants. The basal level of irHCM in the blood of infants with normal development was twice as high as that in infants with a risk of developmental delay (Table 1).

One of the parameters tested using Jurba's method was muscle tone. A statistical analysis revealed a direct correlation between the values of this parameter and the basal irHCM in breast-fed infants ($R = 0.51$, $p < 0.01$). This means that the higher the level of irHCM is in the blood, the closer muscle tone is to a normal (physiological) level. The same correlation was detected in both 1–3 ($R = 0.51$, $p < 0.05$) and 4–10 ($R = 0.61$, $p < 0.05$) month old breast-fed infants. There are two polar deviations of muscle tone from normal values. Our study has shown that the level of irHCM in the infants with heightened muscle tone was significantly lower than in those with normal muscle tone (Table 2).

Table 2
Basal immunoreactivity of human and bovine β-casomorphins-7 in the blood plasma of infants with different state of muscle tone.

Muscle tone	irHCM (fmol/ml)	irBCM (fmol/ml)
Physiological	225 ± 25 ($n = 17$)	60 ± 12 ($n = 18$)
Lowered	207 ± 27 ($n = 9$)	99 ± 34 ($n = 17$)
Heightened	127 ± 30 ($n = 12$)$^{\#}$	132 ± 33 ($n = 18$)$^{\#}$

Values are mean ± SE.
$^{\#}$ $p < 0.05$ refers to comparison of base-line values in the group of infants showing physiological muscle tone with values in other groups.

The opposite results were obtained in infants fed with formula. The basal level of irBCM increased 2-fold in the blood plasma of the infants who displayed delays in development (estimated by the Jurba's test to be less than 23 points) (Table 1). At the same time, the rate of psychomotor development positively correlated with post-feeding irBCM ($R = 0.49$, $p < 0.05$) in the common group of infants with normal development and those with a risk of developmental delay, and this correlation was more pronounced at 4–12 months of age ($R = 0.57$, $p < 0.05$). The increase in irBCM 3 h after feeding was detected only in infants with normal rate of psychomotor development (Table 1). There was no correlation between the points of muscle tone and irBCM levels in artificially fed infants. However, the average irBCM level in plasma of the infants with heightened muscle tone was twice as high as in patients with normal muscle tone (Table 2).

3.3. Chromatographic analysis of extracted material with irBCM

Chromatographic study of the material with irBCM extracted from plasma was performed by HPLC, using specially elaborated ELISA to detect the immunoreactivity in fractions. The assay was found to be as sensitive to bovine β-CM-7 as RIA, but ELISA is a more efficient and environmentally appropriate method than RIA.

The molecular weight of bovine β-CM-7 immunoreactive material from infants' plasma was evaluated by HPLC gel filtration. Three samples of blood plasma extracts from the artificially fed infants eluted on Protein Pak 60 column as multiple peaks as detected by UV absorption. A typical elution profile (Fig. 3) shows that the major protein fraction eluted from the column with a void volume within the first 10 min of elution just as high molecular weight BSA and β-casein did. The retention time of short peptides such as β-CM-7 and dalargin was about 20 min, and their UV absorption at 226 nm was relatively low. irBCM was detected in the fractions eluted very close to the position of synthetic bovine β-CM-7, but not in high molecular weight fractions (Fig. 3).

Reverse phase chromatography performed using ProntoSIL-120-5-C18 column also showed that bovine β-CM-7 immunoreactive material from infants' plasma had the same retention time as the synthetic peptide (Fig. 4). Thus chromatographic analysis of the samples demonstrated that the material with irBCM extracted from the blood plasma of artificially fed infants has molecular mass and polarity similar to synthetic bovine β-CM-7.

Table 1
Immunoreactivity of human and bovine β-casomorphins-7 in the blood plasma of infants with different level of psychomotor development.

Psychomotor development	irHCM (fmol/ml)		irBCM (fmol/ml)	
	Before feeding	After feeding	Before feeding	After feeding
Normal (>26 points)	236 ± 23 ($n = 28$)	208 ± 29 ($n = 15$)	52 ± 10 ($n = 21$)	122 ± 32* ($n = 12$)
Risk of delay (26–23 points)	122 ± 29$^{\#}$ ($n = 8$)	116 ± 42 ($n = 4$)	123 ± 38$^{\#}$ ($n = 16$)	103 ± 29 ($n = 15$)
Delay in development (<23 points)	154 ($n = 1$)	189 ($n = 1$)	108 ± 22$^{\#}$ ($n = 16$)	135 ± 30 ($n = 12$)

Values are mean ± SE.
* $p < 0.05$ refers to comparison of base-line values with values 3 h after feeding.
$^{\#}$ $p < 0.05$ refers to comparison of base-line values in the group of infants showing normal development with values in other groups.

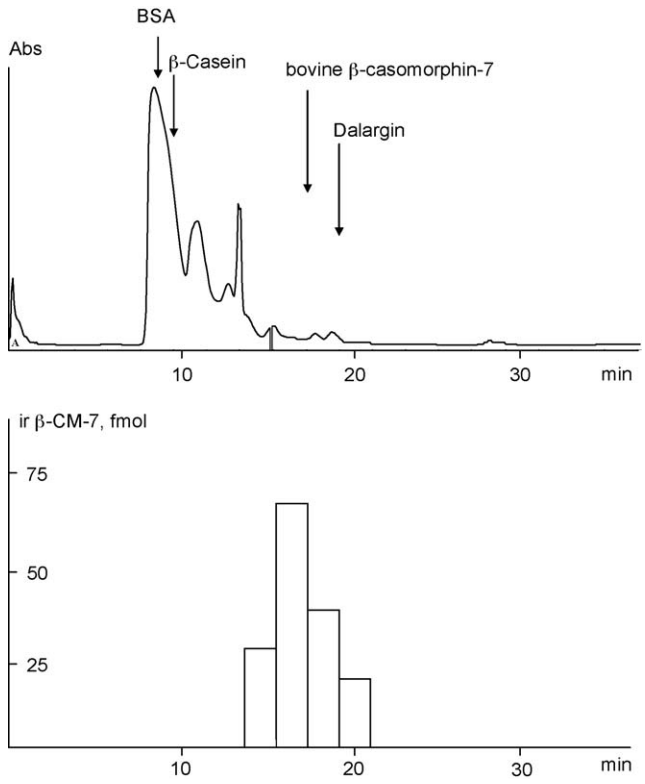

Fig. 3. Gel filtration analysis of bovine β-casomorphin-7 immunoreactive material from blood plasma of formula-fed infants. Elution profile was obtained by HPLC on Protein Pak-60 column and shown as 226 nm absorbance and immunoreactivity of corresponding fractions. Arrows indicate peak elution position for BSA, β-casein, synthetic β-casomorphin-7 and synthetic hexapeptide dalargin.

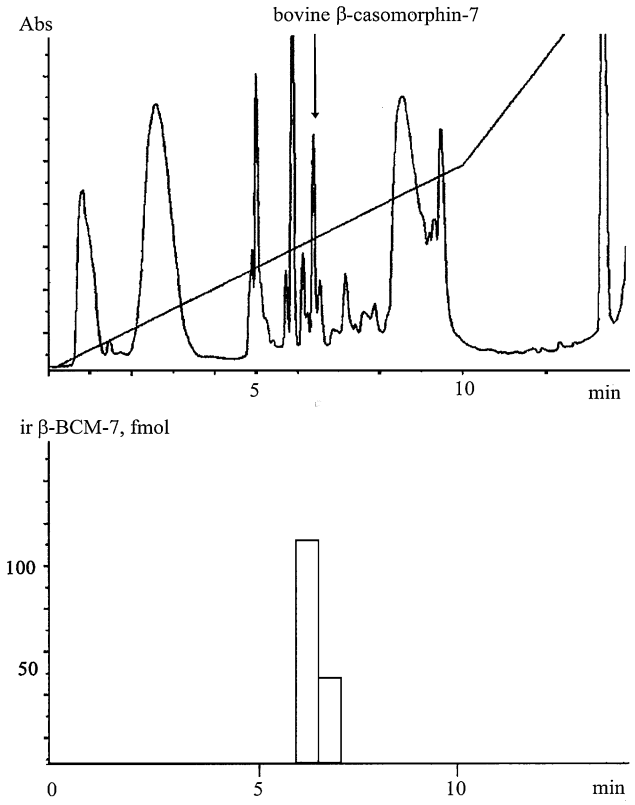

Fig. 4. Reverse phase chromatography of bovine β-casomorphin-7 immunoreactive material from blood plasma of formula-fed infants. Elution profile was obtained by HPLC on ProntoSIL-120-5-C18 column and shown as 226 nm absorbance and immunoreactivity of corresponding fractions. Arrow indicates peak elution position for synthetic β-casomorphin-7.

4. Discussion

The biological activity of materials entering the human organism with food is the main issue in dietology. It is most important during the first year of life when postnatal formation of all organism systems is most active. At this time, milk is the main source of both nutritive and biologically active materials for infants. Thus the aim of our study was to estimate the level of CM-immunoreactive material in blood plasma of infants on different types of feeding and investigate the possible relation between this immunoreactivity and the level of their psychomotor development.

The substances with the immunoreactivity of human and bovine β-CM-7 were detected in the blood plasma of most of the infants fed with breast milk and cow milk based formula, respectively. Chromatographic analysis of blood plasma extract from formula-fed infants demonstrated that the material with irBCM has the same molecular mass and polarity as synthetic bovine β-CM-7. The level of irBCM in infants was similar to that measured earlier in newborn dogs fed with cow milk [37].

Both irBCM and irHCM were detected in infants' plasma samples taken in the morning after 6 h intermission between feeding (basal level) and 3 h after morning nursing. The average basal level of irBCM and irHCM did not significantly vary according to age. While the average value of both bovine and human CM immunoreactivity increased 3 h after feeding mainly in infants of 1–3-month age (Figs. 1 and 2). This is in agreement with the well known fact that the intestinal mucosa of newborns is permeable for peptides and even for relatively large proteins [42]. It is worth noting that irBCM also increased after bottle-feeding in infants 6-month of age and older. This might be due to an increase in protein abundance in formula used for feeding this age group. In fact, we have observed that the greater the protein content was of the formula, the higher was the level of irBCM detected in blood obtained after feeding. However, this relationship was traced only as a tendency.

While getting to the circulatory system, β-casomorphins and their precursors can penetrate the blood–brain barrier [2,5]. This has been confirmed by post-mortal study that detected β-CM-immunoreactivity in a number of brain regions of human infants [29]. As CMs reach the brain, they might affect the development of the infants' central nervous system. Our study has circumstantially confirmed this. In fact, irHCM in breast-fed infants with normal psychomotor development and normal muscle tone was twice as high as in infants with a risk of developmental delay and a heightened tone, respectively (Tables 1 and 2). The rate of psychomotor development directly correlated with irHCM in breast-fed infants, and this correlation was most pronounced in the infants in the first 3 months of life—that very age when permeability of the intestinal mucosa for peptides is most probable.

A similar correlation was detected for the formula-fed group, but only for those whose psychomotor development was close to their age norm. Moreover, a difference between basal and post-feeding irBCM was detected only in normally developing infants (Table 1). So under regular uptake and removal, bovine CM such as human CM is supposed to have a positive action upon infants' development. However, in formula-fed infants with risk and delay in development as well as with heightened muscle tone, basal irBCM was significantly greater than in normal ones (Tables 1 and 2). High basal irBCM in those infants might be because of any disturbances in degradation and elimination of food-born peptides, especially CM. Prolonged circulation of bovine CM in high

concentration could induce pathological reactions that entail a delay in development. For comparison, a study in animal has shown that β-CM-5 at a low dose could ameliorate impaired memory function in mice, while at high dose it induces amnesia [34,35].

It should be noted that hyperpeptidemia is considered to be involved in pathogenesis of autistic spectrum disorders in children [6,30]. Moreover, of other dietary protein-derived peptides bovine CM and gliadorphins are supposed to be the most pathophysiologically important in autism [30,36]. However, the data on direct identification of these peptides in any samples from children with this disease are very inconsistent. Chromatographic analysis of urine samples from the patients with autism spectrum disorders showed apparent peptiduria and presence of material with the same retention time as bovine CM, and irBCM [1,31], while mass spectrometry failed to detect CM in the urine of both healthy and autistic children [7,8].

The explanation of opposite effects of human and bovine CM on infants' psychomotor development and muscle tone probably lay in their species-specificity. Indeed, the structure of bovine and human β-casein is matched only at 47% and the sequence of bovine and human CMs differs in 2 amino acids. This likely defines a difference in their biological properties. Both human and bovine CMs are shown to interact with opioid [43] and serotonin [38,39] receptors which are known to be of great importance for CNS maturation [22,43], but bovine CM has higher affinity to μ- and δ-opioid receptors [21] and also to 5-HT_2-serotonin receptors [39] versus human CM. Therefore under durable circulation in organism bovine CM more likely provokes desensitization of those receptors than human CM does. The data on desensitization of guinea pig heart β-adrenoceptors in the presence of β-CM-5 in relatively low concentrations [23] circumstantially support the hypothesis.

5. Conclusions

This is the first study that identified substances with immunoreactivity of human and bovine β-casomorphins-7 in blood plasma of infants under 1 year of age who were fed with breast milk and formula containing cow milk, respectively. Chromatographic analysis of the material with irBCM has demonstrated that it has the same molecular mass and polarity as synthetic bovine β-CM-7. irHCM and irBCM were detected both in the morning before feeding (basal level) and 3 h after feeding. Increase in both irHCM and irBCM levels 3 h after feeding was detected mainly in infants of 3 and less months old. The greatest basal irHCM was revealed in breastfed infants with normal psychomotor development and muscle tone. In contrast, elevated basal irBCM was found in formula-fed infants showing delay in psychomotor development and heightened muscle tone, while among formula-fed infants with normal development the rate of this parameter directly correlated to basal irBCM. The data support known opinion that the breast feeding has an advantage over formula for infants' development and defines a deterioration of bovine CM elimination as a risk factor for delay in psychomotor development.

Acknowledgements

This work was supported in part by grant from Russian Foundation for Basic Research (06-04-08257). We thank Ms. Natalia White and Dr. Valeria Zarkhin for assistance with English translation.

References

[1] Alcorn A, Berney T, Bretherton K, Mills M, Savery D, Shattock P. Urinary compounds in autism. J Intellect Disabil Res 2004;48(March Pt 3):274–8.
[2] Banks WA, Kastin AJ. Saturable transport of peptides across the blood–brain barrier. Life Sci 1987;41:1319–38.
[3] Brantl V, Pfeiffer A, Herz A, Henschen A, Lottspeich F. Antinociceptive potencies of beta-casomorphin analogs as compared to their affinities towards mu and delta opiate receptor sites in brain and periphery. Peptides 1982;3(5):793–7.
[4] Brantl V, Teschemacher H, Henschen A, Lottspeich F. Novel opioid peptides derived from casein (beta-casomorphins). I. Isolation from bovine casein peptone. Hoppe Seylers Z Physiol Chem 1979;360(9):1211–6.
[5] Brantl V, Neubert K. Opioid peptides derived from food proteins. TJPS 1986;7:6–7.
[6] Cade R, Privette M, Fregly M, Rowland N, Sun Z, Zele V. Autism and schizophrenia: intestinal disorders. Nutr Neurosci 2000;3:57–72.
[7] Cass H, Gringras P, March J, McKendrick I, O'Hare AE, Owen L, et al. Absence of urinary opioid peptides in children with autism. Arch Dis Child 2008;93(9):745–50.
[8] Dettmer K, Hanna D, Whetstone P, Hansen R, Hammock BD. Autism and urinary exogenous neuropeptides: development of an on-line SPE-HPLC–tandem mass spectrometry method to test the opioid excess theory. Anal Bioanal Chem 2007;388(August (8)):1643–51.
[9] Dmitriev AD, Golikova YI, Kobilyanskiy AG. Elaboration of specific antiserum to opioid peptides and their employment to radioimmune assay of that materials. Neurochem Russ 1982;1(1):66–74.
[10] Dubynin VA, Ivleva IA, Beliaeva IA, Dobriakova IV, Andreeva LA, Kamenski AA. Influence of acute and chronic administrations of beta-casomorphins on the maternal motivation in albino rats. Ross Fiziol Zh Im I M Sechenova 2005;91(1):80–8.
[11] Dubynin VA, Ivleva YA, Stovolosov IS, Belyaeva YA, Dobryakova YV, Andreeva LA, et al. Effect of beta-casomorphines on mother-oriented (''child's'') behavior of white rats. Dokl Biol Sci 2007;412:1–4.
[12] Dubynin VA, Malinovskaia IV, Beliaeva IA, Stovolosov IS, Bespalova ZD, Andreeva LA, et al. Delayed effect of exorphins on learning of albino rat pups. Izv Akad Nauk Ser Biol 2008;(1):53–60.
[13] Dubynin VA, Malinovskaya IV, Ivleva YA, Andreeva LA, Kamenskii AA, Ashmarin IP. Delayed behavioral effects of beta-casomorphin-7 depend on age and gender of albino rat pups. Bull Exp Biol Med 2000;130(11):1031–4.
[14] Dubynin VA, Zemskaia NI, Ivleva IA, Kamenski AA, Andreeva LA, Miasoedov NF. Behavioural effects of beta-casomorphin-7 in its intranasal administration. Zh Vyssh Nerv Deiat Im I P Pavlova 2004;54(3):373–81.
[15] Erlandsson I, Lindstrom L, Nyberg F. B-casomorphin-8 immunoreactive material in human urine. In: Nyberg F, Brantl V, editors. β-Casomorphins and related peptides. Uppsala, Sweden: Fyris-Tryck AB; 1990. p. 151–5.
[16] Hedner J, Hedner T. β-Casomorphins induce apnea and irregular breathing in adult rats and newborn rabbits. Life Sci 1987;41(November (20)):2303–12.
[17] Jarmołowska B, Sidor K, Iwan M, Bielikowicz K, Kaczmarski M, Kostyra E, et al. Changes of beta-casomorphin content in human milk during lactation. Peptides 2007;28(10):1982–6.
[18] Jurba LT, Mastukova EM. Disturbance of psychomotor development in infants of the first year of life. Moscow: Medicina; 1981.
[19] Kaminski S, Cieslińska A, Kostyra E. Polymorphism of bovine beta-casein and its potential effect on human health. J Appl Genet 2007;48(3):189–98.
[20] Kammerer E, Koch S, Leon Roque DM. Action of (D-Pro4)-beta-casomorphin1–5 on processes of synaptic transmission. Biomed Biochim Acta 1985;44(9):1379–87.
[21] Koh G, Brantl V. Binding of β-casomorphins to opioid receptors. In: Nyberg F, Brantl V, editors. β-Casomorphins and related peptides. Uppsala, Sweden: Fyris-Tryck AB; 1990. p. 43–52.
[22] Leonardo ED, Hen R. Genetics of affective and anxiety disorders. Annu Rev Psychol 2006;57:117–37.
[23] Liebmann C, Mentz P, Schnittler M, Neubert K, Barth A. Non-opioid effects of β-casomorphin-5 in guinea-pig heart: alterations to the β-adrenoceptors-G-protein complex and inhibition of myocardial responses to isoproterenol. Peptides 1991;12(2):265–70.
[24] Lindström LH, Nyberg F, Terenius L, Bauer K, Besev G, Gunne LM, et al. CSF and plasma beta-casomorphin-like opioid peptides in postpartum psychosis. Am J Psychiatry 1984;141(9):1059–66.
[25] Matthies H, Stark H, Hartrodt B, Ruethrich HL, Spieler HT, Barth A, et al. Derivatives of beta-casomorphins with high analgesic potency. Peptides 1984;5(3):463–70.
[26] Miller RG, Chang K, Cooper B, Cuatrecasas P. Radioimmunoassay and characterization of enkephalins in rat tissues. J Biol Chem 1978;253:531–8.
[27] Nedvídková J, Kasafírek E, Dlabac A, Felt V. Effect of beta-casomorphin and its analogue on serum prolactin in the rat. Exp Clin Endocrinol 1985;85(2):249–52.
[28] Nyberg F, Lieberman H, Lindström LH, Lyrenäs S, Koch G, Terenius L. Immunoreactive beta-casomorphin-8 in cerebrospinal fluid from pregnant and lactating women: correlation with plasma levels. J Clin Endocrinol Metab 1989;68(2):283–9.
[29] Pasi A, Mahler H, Lansel N, Bernasconi C, Messiha FS. B-casomorphin-immunoreactivity in the brain stem of the human infant. Res Commun Chem Pathol Pharmacol 1993;80:305–22.
[30] Reichelt KL, Knivsberg AM. Can the pathophysiology of autism be explained by the nature of the discovered urine peptides? Nutr Neurosci 2003;6(1):19–28.
[31] Reichelt KL. Biochemistry and psychophysiology of autistic syndromes. Tidsskr Nor Laegeforen 1994;114(12):1432–4.

[32] Rüthrich HL, Grecksch G, Schmidt R. Phe1-substituted beta-casomorphin-5 analogues with analgesic activity. Peptides 1994;15(3):457–60.
[33] Saito T. Antihypertensive peptides derived from bovine casein and whey proteins. Adv Exp Med Biol 2008;606:295–317.
[34] Sakaguchi M, Koseki M, Wakamatsu M, Matsumura E. Effects of beta-casomorphin-5 on passive avoidance response in mice. Biosci Biotechnol Biochem 2003;67(11):2501–4.
[35] Sakaguchi M, Koseki M, Wakamatsu M, Matsumura E. Effects of systemic administration of beta-casomorphin-5 on learning and memory in mice. Eur J Pharmacol 2006;530(1–2):81–7.
[36] Shattock P, Whiteley P. Biochemical aspects in autism spectrum disorders: updating the opioid-excess theory and presenting new opportunities for biomedical intervention. Expert Opin Ther Targets 2002;6(2):175–83.
[37] Singh M, Rosen CL, Chang KJ, Haddad GG. Plasma beta-casomorphin-7 immunoreactive peptide increases after milk intake in newborn but not in adult dogs. Pediatr Res 1989;26(July (1)):34–8.
[38] Sokolov OY, Kost NV, Zolotarev YA, Ryukert EN, Zozulya AA. Influence of human β-casomorphin-7 on specific binding of 3H-spiperone to the 5-HT2-receptors of rat brain frontal cortex. Protein Pept Lett 2006;13(2):169–70.
[39] Sokolov OY, Pryanikova NA, Kost NV, Zolotarev YA, Ryukert EN, Zozulya AA. Reactions between beta-casomorphins-7 and 5-HT_2-serotonin receptors. Bull Exp Biol Med 2005;140(November (5)):582–4.
[40] Svedberg J, de Haas J, Leimenstoll G, Paul F, Teschemacher H. Demonstration of beta-casomorphin immunoreactive materials in in vitro digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. Peptides 1985;6(September–October (5)):825–30.
[41] Teschemacher H, Koch G, Brantl V. Milk protein-derived opioid receptor ligands. Biopolymers 1997;43(2):99–117.
[42] Thomson AB, Keelan M. The development of the small intestine. Can J Physiol Pharmacol 1986;64(January (1)):13–29.
[43] Volterra A, Restani P, Brunello N, Galli CL, Racagni G. Interaction of β-casomorphins with multiple opioid receptors: in vitro and in vivo studies in the newborn rat brain. Dev Brain Res 1986;30:25–30.
[44] Zoghbi S, Trompette A, Claustre J, El Homsi M, Garzón J, Jourdan G, et al. beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a mu-opioid pathway. Am J Physiol Gastrointest Liver Physiol 2006;290(6):G1105–13.
[45] Zong YF, Chen WH, Zhang YS, Zou SX. Effects of intra-gastric beta-casomorphin-7 on somatostatin and gastrin gene expression in rat gastric mucosa. World J Gastroenterol 2007;13(14):2094–9.