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Autistic children display elevated urine levels of bovine casomorphin-7 immunoreactivity

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ABSTRACT

Elevated concentrations of circulating casomorphins (CM), the exogenous opioid peptides from milk casein, may contribute to the pathogenesis of autism in children. Because several mass spectrometry studies failed to detect casomorphins in autistic children, it was questioned whether these peptides can be detected in body fluids by mass spec. Here we demonstrated, using a novel high sensitivity ELISA method, that autistic children have significantly higher levels of urine CM-7 than control children. The severity of autistic symptoms correlated with concentrations of CM-7 in the urine. Because CMs interact with opioid and serotonin receptors, the known modulators of synaptogenesis, we suggest that chronic exposure to elevated levels of bovine CMs may impair early child development, setting the stage for autistic disorders.

1. Introduction

Autism is a complex neurodevelopmental disorder associated with impaired social interactions, poor communication skills, and repetitive behaviors in children. Autistic children can also display abnormal motor skills, speech and language impairment, and the stereotyped patterns of activities [31]. The prevalence of autistic disorders is about 4.5 per 10,000 children [13], while some studies reported much higher frequency of autism (40–90 per 10,000 children [2]). These discrepancies are likely the reflection of different diagnostic criteria and/or clinical and genetic heterogeneity of children with autism spectrum disorders (ASD) [8]. ASD is a general term introduced by the 10th revision of International Classification of Diseases (ICD-10) that describes a group of complex disorders that include autism (Kanner syndrome, F84.0), Asperger syndrome (highly functional autism, F84.5), Rett syndrome (F84.2), and atypical autism (F84.1). Although more than half of the cases of autism are related to the congenital dysfunction of the brain, suggesting a strong genetic basis, various environmental factors, including prenatal and birth defects, infectious diseases, heavy metal and vaccine poisoning, may also play a significant role in disease progression [24]. While several candidate genes that may contribute to the development of autism have been identified, the role of individual genes remains uncertain [1]. Many researchers now agree that "Autism is a result of the influence of many factors acting on the stage of development" [15,16].

The earlier theory, formerly known as the Panksepp's opioid theory of autism, proposed that both the genetic and environmental factors contribute to the origin of autism. Panksepp described the similarities between the symptoms of autism and the symptoms of long-term morphine use, including decreased pain sensitivity, developmental delays, and reduced social interactions [18]. Following biochemical studies confirmed that autistic children and, in some case, their mothers [33] displayed elevated levels of endogenous opioid peptides in their serum [3,12], blood cells [4], and the cerebrospinal fluid [7,17]. The opioid theory of autism has been further supported by pharmacological studies that demonstrated that the opioid receptor antagonist naltrexone can improve the clinical signs of autism [11].

The discovery of a naturally occurring opioid peptide from milk, exorphins [37] led to the development of the opioid-excess hypothesis of autism. According to this hypothesis, genetic predisposition

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and/or early exposure to environmental stressors may lead to functional alterations in the gut, reduced proteolytic activity, and the increased permeability of the gut mucosa. These factors, possibly in combination with low levels of circulating peptidases and increased blood–brain barrier permeability, may cause hyperpeptidemia and accumulation of opioid peptides such as CM in the blood and the brain. Thus, chronically elevated levels of exorphins in the brain may directly modulate the opioid and other neurotransmitter systems, leading to the development of ASD [26]. Previous studies demonstrated the presence of human and bovine CM-7 in the blood of infants that were breast-fed or received cow's milk formulas. Children with delayed psychomotor development (PMD) had higher levels of bovine CM than children with normal PMD. This study suggested a causative relationship between the levels of circulating CM and PMD in infants [10]. Here we further confirmed this observation by measuring bovine CM-7 content in the urine of children with ASD, using a novel high-sensitivity ELISA method.

2. Materials and methods

2.1. Patients

Patients enrolled in the present study included 4–8 year-old children with ASD ($n = 10$) and healthy control children ($n = 10$). The severity of specific autistic symptoms was evaluated using the Childhood Autism Rating Scale (CARS). Five patients met the ICD-10 criteria for autism (F84.0) with catatonic-regressive symptoms, two of whom had dysontogenetic deviations, and three other patients had normal ontogeny. Five patients met the ICD-10 criteria for Asperger's syndrome (F84.5), two of whom displayed affective symptoms. The intellectual capacity of children in Asperger's group was tested using the Wechsler Preschool and Primary Scale of Intelligence (WPPSI). Three children had normal intelligence, and two other children had reduced intelligence. Control group and the group of children with autism received a standard diet that contained cow milk products. The study was conducted with parental consent.

2.2. Methods

2.2.1. Sample preparation and purification

First morning urine samples were collected, diluted (1:10) into ice-cold acetic acid, and stored at −70 °C for up to one month. Urine samples (30 ml) were thawed prior to ELISA, and applied onto the Sep-pack C-18 cartridges (Peninsula, USA), followed by washing with 30% methanol, 0.04% TFA, and elution with 100% methanol/0.04% TFA. Collected eluates were lyophilized, and bovine CM-7 was analyzed using a previously developed ELISA. Extraction of bovine CM-7 (Sigma, USA) added to casomorphin-free human urine using Sep-pack C-18 cartridges resulted in high recovery rate of about 95%.

2.2.2. ELISA

Rabbit antiserum to CM-7 has been previously described [10]. Affinity purification of antibodies was performed using bovine CM-7 (Sigma, USA) conjugated to BrCN-sepharose-4B beads. 3 ml of antiserum was diluted in PBS, passed through the column, and extensively washed with PBS. Antibodies were eluted by acetic acid, pH 2.2, and neutralized with ammonia to pH 7.0. Protein concentration was determined at OD_{280}. Total protein yield was 0.3 mg.

Bovine CM-7 (Sigma, USA) was biotinylated overnight at 4 °C by incubation with the equimolar concentration of Succinimidyl-6-(Biotinamido)-6-Hexanamido Hexanoate (NHS-LC-LC-Biotin, Pierce, USA) in 0.1 M $NaHCO_3$, pH 8.0. Biotinylated CM-7 was purified by HPLC using ProntoSIL-120-5-C18 and acetonitrile 0–60%/0.1% TFA.

To prepare immunosorbent, antibodies were diluted in the 0.05 M Na-carbonate buffer, pH 9.5 to a final concentration 4 μg/ml, and 250 μl/well of protein solution were incubated for 3 day at 4 °C in 96-well plates (Nunc, Denmark) under high humidity conditions. Prior to ELISA, wells were washed 5 times with distilled water. Samples and peptide standards were diluted in ELISA buffer (0.15 M NaCl, 25 mM Na_2HPO_4, pH 7.5, 0.2% BSA, and 0.05% Tween-20), and 200 μl were added to each well, followed by addition of 50 μl (10 fmoles/well) of biotinylated CM-7 in ELISA buffer. The plates were incubated for 1.5 h at 37 °C, washed with distillated water, and 250 μl of streptavidin-peroxidase conjugate (100 ng/ml, Imtek Ltd., Russia) in ELISA buffer were added and incubated for 30 min at 37 °C.

Plates were washed 8 times with distilled water and 250 μl 2.5% tetramethylbenzidine (Bioservice, Russia) at in 0.2 M Na_2HPO_4-citrate buffer, pH 4.0, 0.01% H_2O_2, 0.05% inhibitor proxen were added in each well. After 15-min incubation at room temperature, reaction was stopped by 50 μl of 1 M H_2SO_4. Absorption at OD_{450} was immediately determined using the plate reader "Sunrise" (Tecan, Austria). Non-specific adsorption of samples to antibody-free wells was estimated at approximately 10% levels.

The calibration curve for the inhibitory effect of the non-labeled bovine CM-7 on the binding reaction of biotinylated bovine CM-7 with antibodies was linear from 10% to 90% of specific binding, and corresponded to the range of peptide concentration 0.015–1.2 pmoles/well, respectively. The minimal detectable limit of CM-7-immunoreactive material was 25 fmol.

Human CM-7 and bovine beta-casein (Sigma, USA) added in a 100-fold excess did not display biotinylated peptide in these conditions. The identity of the bovine CM-7 detected by ELISA was confirmed by reverse phase and gel-filtration HPLC [10]. The method sensitivity achieved by ELISA (10 pg) exceeds the known mass-spectrometric methods by an order of magnitude [6].

2.2.3. Assessment of renal excretion

Renal excretion was assessed by monitoring the severity of microalbuminuria in urine samples, using a pyrogallol red technique (Unimed, Russia) and a spectrophotometer Ultrospec 1100-Pro (Amersham, USA). Microalbuminuria measurement was used as the equivalent of a commonly used creatinine method.

2.2.4. Statistical analysis

Statistical analysis was performed using Statistica, version 6.0. Statistical significance between experimental groups was determined using the one-way ANOVA. The Spearman correlation coefficient was used to assess the relationship between clinical and biochemical variables. Results are reported as "means ± standard error of means."

3. Results

CM-7 levels were measured in urine samples of healthy control children and children with Asperger's and Kanner's syndromes. The concentration of bovine CM-7 in the urine was significantly higher in Asperger's patients than in healthy children (Fig. 1; 75 ± 10 pg/ml *vs.* 58 ± 7 pg/ml, $p < 0.05$). Patients with Kanner's syndrome had significantly higher levels of CM-7 than children with Asperger's syndrome (Fig. 1; 104 ± 10 *vs.* 75 ± 10 pg/ml, $p < 0.05$). The level of microalbuminuria was significantly higher in patients with Kanner's syndrome than in patients with Asperger's syndrome and healthy children (31 ± 4, 22 ± 3, and 20 ± 3 μg/ml, respectively, $p < 0.05$). This finding agrees with previously published data that showed higher levels of microalbuminuria in patients with Kanner's syndrome, which is known to be associated with the metabolic syndrome [14], in comparison with patients with Asperger's syndrome, which is not associated with the metabolic syndrome [29].