STUDY OF THE SEIZURE THRESHOLD AND INCREASING RESISTANCE OF RATS TO STRESS AFTER IMMUNIZATION TO DIAZEPAM-BINDING INHIBITOR FRAGMENT

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Active immunization to endogenous bioregulators of the physiological and psychoemotional status mainly induces changes in animals in the opposite direction compared with the effects of the bioregulators themselves [1, 6]. In particular, we have found a long (lasting many months) shift in the level of emotional activity and the seizure threshold in albino rats immunized to adrenalin [3] and to the synthetic antidepressant sydnophen, which has elements of structural similarity with catecholamines [8].

The aim of this investigation was to study the resistance of rats, immunized to octadecaneuropeptide (ODN), an endogenous peptide of "conflict-inducing behavior," the active fragment of diazepam-binding inhibitor (DBI), to emotional stress. DBI was isolated from rat brain tissue during a search for endogenous ligands of benzodiazepine receptors (endozepines [13]). DBI and ODN have been shown to be present in brain structures controlling emotional activity, and in blood plasma and peripheral tissues [9, 14]. By blocking binding of ligands with benzodiazepine receptors, DBI counteracts the inhibitory sedative effects of GABA and potentiates the state of discomfort of animals under stress conditions. Injection of DBI and its active fragments into the cerebral ventricles leads to weakening of positive motivation in rats in a conflict test [10, 13], and in a larger dose, may even give rise to clonic convulsions [10]. These findings suggested that immunization to ODN would have the opposite effect compared with the peptide itself on behavior, i.e., it would diminish the feeling of fear and anxiety and would possibly protect against convulsants. The aim of the investigation was to test this hypothesis.

EXPERIMENTAL METHOD

The human ODN used in the work had the following structural formula:

CQATVGDITERPGMLDFTGK (Cys-ODN)

ODN was synthesized by stepwise lengthening of the peptide chain with the aid of activated esters, at the Vektor Research-Production Combine by the method in [5]. A conjugate of ODN with bovine serum albumin (BSA) was obtained with the aid of the N-hydroxysuccinimide ester of 3-(2-pyridyldithio)propionic acid (SPDP). Rat and human ODN differ with respect to five amino acids The possibility of using human ODN to immunize rats was based on the following facts. First, preliminary experiments showed that intranasal administration of the human peptide (40 μg/kg in 10 μl) induced a stronger sense of fear, characteristic of ODN, in rats during the open field test with stress factors: the number of visits to the center of the field was reduced by 2.6 times. Similar results were obtained in a previous study [7] in which injection of

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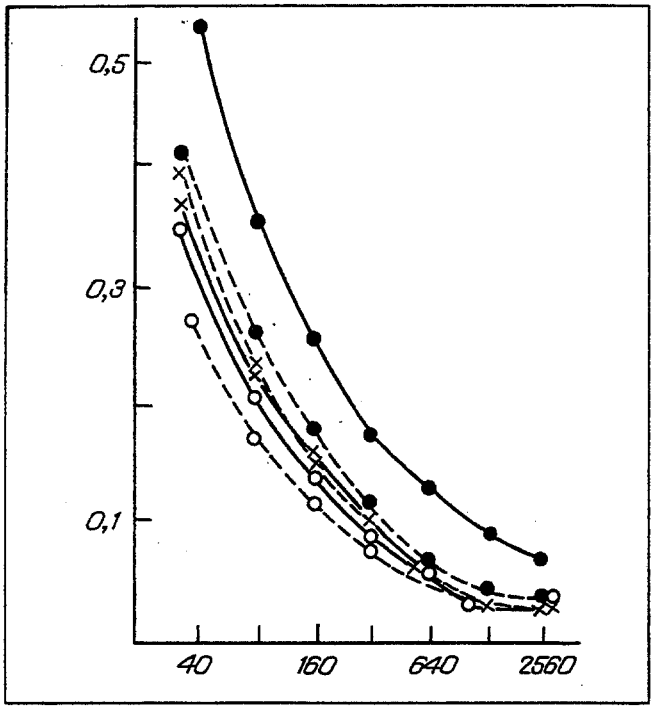

Fig. 1. Titration curves of rats' sera after 4th reimmunization to ODN. Abscissa, log of dilution of sera. Filled circles – experiment (ODN-BSA), empty circles – control 1 (physiological saline), crosses – control 2 (BSA). Broken lines denote binding after preincubation with ODN.

human ODN into the substantia nigra of the rat brain evoked a seizure reaction. Second, cross-reactivity between antibodies to human and rat DBI, although not great, does nevertheless exist [11]. Wistar albino rats were divided into three groups: physiological saline was injected into group 1 (control), BSA treated with SPDP and adjuvants (control) into group 2, and ODN-BSA conjugate with adjuvants (experiment) into group 3. The dose for primary immunization was between 500 and 750 μg protein/kg, and during subsequent immunizations 250-350 μg/kg. The first immunization was given with Freund's complete adjuvant ("Calbiochem"), subsequent immunizations with incomplete adjuvant ("Reakompleks" Research-Production Combine). The substances were injected subcutaneously at 4 points in the dorsal region and the interval between the 1st and 2nd injections was 1 month, between the 2nd and 3rd 2 months, and the 3rd, 4th, and 5th injections 25 days. Blood for determination of the titer of the antisera to ODN was taken from the caudal vein 10-12 days after the 3rd, 4th, and 5th immunizations. The antibody titer was determined by enzyme immunoassay. As antigen ODN was adsorbed to a plateau (100 μg/ml in 0.1 M Na-phosphate buffer, pH 7.5) After washing, dilutions of sera from all groups of rats (1:40-1:5000 in 0.1 M Tris-HCl buffer with Tween-20, pH 7.5), were introduced into the wells, after which secondary antibodies to rat γ-globulin, labeled with peroxidase ("Initsiativa" Moscow Technological Combine), and orthophenylenediamine ("Sigma") as substrate for the enzyme, were next used. In control tests the sera were incubated beforehand with a saturating amount of ODN. The rats' emotional activity was assessed by the following behavioral tests: general motor and orienting activity in a RODEO actometer, open field behavior against the background of stress (bright light and a loud ringing), and in the conflict test by Vogel's method, which has been used by other workers to determine conflict-inducing properties of ODN [10]. In this test, after drink deprivation for 3 days the animal was placed in a conflict situation between an aversive procedure (electric shocks produced by a current of 0.5 A from the drinking bowl, a Col-Born chamber, USA), and positive drinking motivation. The rats were placed in the chamber 24 h before the experiment began to familiarize themselves with the position of the drinking bowl.

The pain threshold was measured by the tail-flick method using focused light.

The sensitivity of the rats to convulsants was determined by giving a single intraperitoneal injection of metrazol in a dose of 40 or 50 mg/kg. The intensity of the seizure syndrome was estimated from the latent period of the reaction, its intensity, and the number of animals giving a generalized convulsion. Under stress conditions, alcohol consumption is

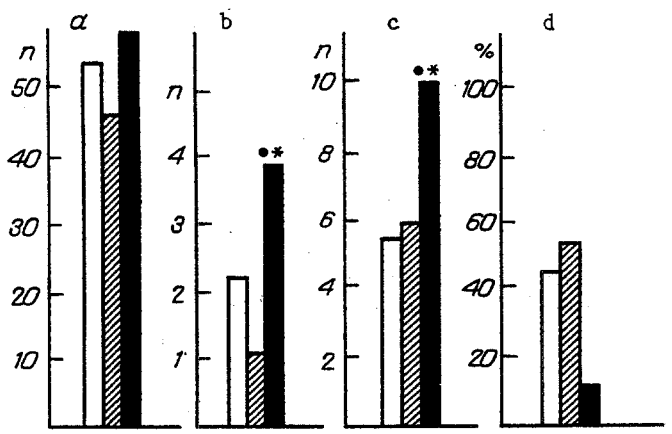

Fig. 2

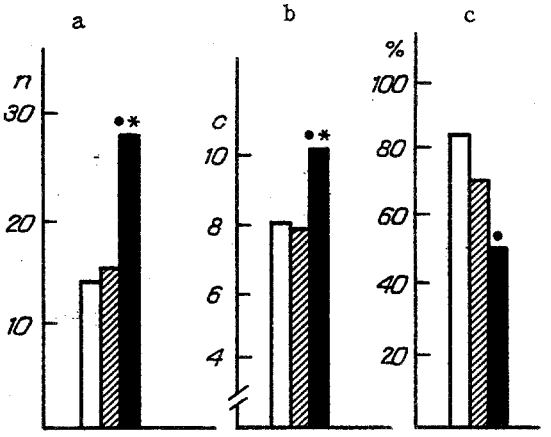

Fig. 3

Fig. 2. Effect of immunization to ODN on open-field behavior of rats: a) horizontal activity, b) visiting center of field, c) vertical activity (standings), d) number of rats with marked panic reaction. Black column – experiment, white column – control 1 (physiological saline), obliquely shaded – control 2 (BSA). Dot indicates significant differences from control 1, asterisk – the same from control 2.

Fig. 3. Effect of immunization to ODN on number of shocks in Vogel test (a), threshold of pain sensitivity (b – time of tail flick, in sec), and number of generalized seizure reactions in response to metrazol (c). Legend as to Fig. 2.

known to increase [2]. Accordingly, we studied the effect of immunization on consumption of 15% ethanol solution under free choice conditions, in normal animals and against the background of daily exposure to stress induced by electric shock. The coefficient of preference for ethanol (f) was calculated as the ratio of the quantity of alcohol drunk to the total volume of water and ethanol drunk. The experiments were carried out 1-2 weeks after the 4th and 5th immunizations. The numerical results were subjected to statistical analysis by Student's t test and by the Wilcoxon–Mann–Whitney nonparametric test.

EXPERIMENTAL RESULTS

Curves of titration of the rats' sera are given in Fig. 1. The titers of antibodies to ODN in the experimental animals was 1:320, 1:1280, and 1:2560 after the 3rd, 4th, and 5th immunizations respectively. The titer was calculated as the greatest dilution of the experimental serum at which its binding differed significantly from that of both control groups. Preincubation with ODN abolished differences between binding of the experimental and control samples. The results thus demonstrate that antibodies to the peptide are formed in rats immunized with the ODN–BSA conjugate.

Testing the animals in the dark chamber of the RODEO apparatus revealed no changes in parameters of motor-investigative activity after immunization to ODN. However, under stress conditions the behavior of the rats immunized with the conjugate differed from the behavior of the control rats. The experimental rats visited the center of the open field more often, and made more vertical standings (Fig. 2b, c). In addition, after 15 and 20 sec of exposure to the loud ringing, half of the control animals developed a marked stress reaction (running without stopping and jumping out of the chamber). In the experimental group only one of 10 rats gave this kind of panic reaction (Fig. 2d). The results are evidence that immunization to ODN increases resistance to audiogenic stress.

The behavior of rats immunized to the peptide in a conflict situation was characterized by an increase in the number of laps, despite the electric shocks (Fig. 3a), indicating weakening of the sensation of fear and strengthening of the positive motivation.

In the experimental rats the threshold of nociceptive sensitivity was raised (Fig. 3b). Intranasal administration of the peptide in a dose of 40 μg/kg, on the other hand, increased sensitivity to pain (by 20% $p < 0.05$). This involvement of

ODN in the regulation of sensitivity to pain was demonstrated by the writers for the first time. Other workers [10] did not observe changes in the pain threshold in response to injection of the peptide into the cerebral ventricles, and it may be, therefore, that the effect is mediated through peripheral systems.

The sensitivity of the rats to the convulsant drug metrazol depended on its dose. If metrazol was injected in a dose of 40 mg/kg the experimental rats were more resistant to the action of the drug as regards the intensity of the seizures and their number (Fig. 3c), but on injection of a larger dose, the effect was seen only on the latent period of the reaction.

In animals immunized with the conjugate, the addiction to ethanol was reduced. The coefficient of preference for ethanol in the two control groups was 0.30 and in the experimental group 0.10. Under stress conditions the quantity of alcohol drunk in the control increased ($f = 0.49$), whereas in the experiment it was almost unchanged ($f = 0.12$). The effect was observed for 3 weeks after reimmunization.

Immunization with the ODN-BSA conjugate thus induces antibody formation to the peptide in rats, but at the same time, behavioral changes and changes in physiological parameters were observed in these animals. The cross-reactivity of antibodies to human and rat DBI [11] and also the general trend of the physiological changes after immunization suggest that the effect of the antibodies formed is manifested on the level and/or activity of endogenous ODN. Animals immunized to the peptide, while not differing in motor activity under conditions of relative rest, possess increased resistance to stressors, and their sense of discomfort in the conflict situation is weakened. Weakening of stress-reactivity can be explained, first, by binding of a conflict-inducing anxiogenic peptide, whose level during stress is increased in brain structures [4], by the antibodies. Immunization also had a protective effect after injection of metrazol. The mechanism of action of metrazol has not been completely established. It has been suggested that it reduces the flow of chloride ions, through its effect on the benzodiazepine site of the $GABA_A$ receptor [12]. ODN is also a convulsant, whose action is inhibited by R0151788, an antagonist of benzodiazepine receptors [7, 10]. It can be postulated on the basis of these results that ODN mediates the effects of metrazol. During activation of the immune response, voluntary consumption of alcohol was reduced and the increase in its consumption during stress was prevented. There are no data in the literature on the role of the peptide in regulating addiction to ethanol [15]. The result can evidently be attributed to the general antistressor effect of immunization to ODN, for we know that substances possessing a sedative, tranquilizing action, abolish addiction to alcohol [2].

Explanation of the mechanisms of the effects obtained in these experiments requires a study of the blood and tissue levels of the peptide and parameters of the GABA-benzodiazepine receptor complex in the immunized animals. Meanwhile, reactions of rats immunized to ODN were largely opposite to those caused by injection of exogenous peptide, as regards behavioral effects and responses to convulsants and pain. It can be concluded that immunization to ODN reduces the negative modulatory effect of the endogenous peptide on the function of GABA-ergic transmission and has a stress-protective and anticonvulsant action.

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