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IMMUNOMODULATION INDUCED BY INTRAPERITONEAL AND INTRACEREBROVENTRICULAR INJECTIONS OF ENKEPHALINASE INHIBITOR DES-TYROSINE[1]–METHIONINE–ENKEPHALIN, AND ABROGATION OF THE EFFECT BY OPIOID ANTAGONISTS NALOXONE AND NALTREXONE

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We have previously shown that exogenous methionine-enkephalin, applied peripherally and centrally, induced a dose-dependent immunomodulation. The present study deals with the interaction of the enkephalinase-inhibitor des-tyrosine[1]-methionine-enkephalin and opioid antagonists naloxone and naltrexone, in relation to a humoral immune response, the plaque-forming cell response in the rat. For this purpose rats were treated intraperitoneally only with 0.2 and 1 mg/kg of des-tyrosine[1] -methionine-enkephalin, or with des-tyrosine[1]-methionine-enkephalin in combination with subcutaneous injections of 5 mg/kg of naloxone, before and after immunization with sheep red blood cells. Other groups of animals were given intracerebroventricularly 20 and 200 μg/kg of des-tyrosine[1]-methionine-enkephalin alone, or des-tyrosine[1]-methionine-enkephalin combined with intracerebroventricular treatment with 10 μg/kg of quaternary naltrexone methylbromide. The last group was treated on the day of immunization and during 3 consecutive days. Experimental and control groups were tested for plaque-forming cell response. The results showed that (a) in rats treated intraperitoneally with des-tyrosine[1]-methionine-enkephalin there was a dose-dependent increase of humoral immune response, and this increase was abrogated by naloxone; and (b) in animals repeatedly injected intracerebroventricularly with des-tyrosine[1]-methionine-enkephalin, the low dose (20 μg/kg) potentiated while the high dose (200 μg/kg) suppressed the plaque-forming cell response, but these effects were completely abolished by i. c. v. administration of quaternary naltrexone. Thus, these results revealed the immunomodulatory activity of des-tyrosine[1]-methionine-enkephalin when given peripherally or centrally, and a negative function of naloxone and quaternary naltrexone on this activity. The finding that quaternary naltrexone, an opioid antagonist that does not cross the blood-brain barrier,

abrogates the central des-tyrosine[1]-methionine-enkephalin-induced immune modulation, suggests the involvement of the CNS opioid system in immunoregulation.

Key Words: enkephalins – enkephalinase – naloxone – naltrexone – immune response – central nervous system.

INTRODUCTION

Extensive studies *in vitro (Plotnikoff et al., 1986) and in vivo* (Janković and Marić, 1986; Janković et al., 1990) have provided evidence that enkephalins modulate a variety of cellular and humoral immune reactions. Thus, methionine-enkephalin (Met-Enk) introduced either peripherally (e.g., intraperitoneally) or centrally (e. g., intracerebroventricularly), was found to increase and decrease immune reactivity, depending on the dose. Namely, lower doses act immunostimulatorily, whereas higher doses are immunosuppressive (Janković and Marić, 1986; Veljić et al., 1990).

Under *in vivo* conditions, both endogenous and exogenous enkephalins are exposed to the degrading activity of specific enkephalinases and nonspecific peptidases (pseudoenkephalinases) (Dua et al., 1985; Roda et al., 1986). Specific and well characterized enzymes, aminopeptidase and enkephalinase (dipeptidyl carboxypeptidase) were shown to be of critical importance in the degradation of endogenous enkephalins (De La Baume et al., 1983). Besides enkephalins, natural enkephalinase substrates *in vivo* encompass also bradykinin, substance P, neurotensin, chemotactic peptide, gastrin and atrial natriuretic factor (Erdös and Skidgel, 1989). Tissue localization of enkephalinases in the vicinity of opioid receptors, particularly in the brain (Malfroy et al., 1979), strongly implies that the enzymes are specifically involved in enkephalin hydrolysis.

Since the function of enkephalins is associated with enkephalin-degrading enzymes, the question arises whether the *in vivo* inhibition of enkephalinases may affect immune responsiveness.In a previous study we demonstrated that intraperitoneally (i.p.) and intracerebroventricularly (i. c. v.) administered specific and nonspecific inhibitors of enkephalin-degrading enzymes differentially affect the humoral immune response in the rat (Janković et al., 1991). One of the enkephalinase inhibitors, des-tyrosine[1]-Met-Enk (DT-Met-Enk), seems to be of particular interest since it represents an inactive tetrapeptide that appears endogenously following cleavage of the N-terminal tyrosine from the Met-Enk molecule (Hambrook et al., 1976). Due to its striking similarity with Met-Enk, this peptide competitively binds to enkephalinases, and thus inhibits Met-Enk hydrolysis. DT-Met-Enk was found to be more active against enkephalinase than against aminopeptidase (Hudgin et al., 1981).

However, the mechanism underlying the immunomodulatory action of enkephalinase-inhibitors is still unclear. For example, it is unknown whether mu, delta and kappa opioid receptors are involved in the immunomodifying processes induced by enkephalinase-inhibition, and what is the relationship

between enkephalinase-inhibition and opioid antagonists such as naloxone and naltrexone. These classical opioid antagonists, which exert broad specificity for multiple receptor subtypes (Lord et al., 1977), are widely employed in studies devoted to the phenomena mediated by the opioid receptor system.

On this ground, a series of experiments was undertaken to test: (a) the activity of DT-Met-Enk on a humoral immune response, and (b) the antagonizing action of naloxone (Nx) and guaternary naltrexone (QNtx) on DT-Met-Enk. In adition, since QNtx used in this study does not cross the blood-brain barrier (Valentino et al., 1981), its i. c. v. application may contribute to the delineation between central and peripheral effects of the enkephalinase inhibitor.

MATERIALS AND METHODS

Animals. The experiments were carried out in male Wistar rats (200-250 g), obtained from the animal farm of the Military Medical Academy, Belgrade. The animals were maintained in groups of four or individually, with free access to food and water. Experimental and control groups were composed of 15–30 rats.

Drugs. Des-tyrosine[1]-methionine-enkephalin (DT-Met-Enk; Serva, Heidelberg), naloxone (Nx; Sigma, St. Louis) and naltrexone methyl bromide (QNtx; Boehringer Ingelheim, Ingelheim) were employed in the treatment of the animals. All drugs were dissolved in sterile saline, and 1 ml was used for intraperitoneal (i.p.) and subcutaneous (s. c.) injections, and 10 μl for intracerebroventricular (i. c. v.) administration.

Surgery. Cannulation of the lateral ventricles of the rat brain was performed under nembutal (40 mg/kg) anesthesia. Rats were fixed on a stereotaxic instrument (La Precision Cinematographique, Paris), a midline incision was made in the region of bregma, and the cranium was cleaned off and dried. Polyethylene cannulae (0,6 x 35 mm) were bilaterally inserted into the lateral brain ventricles 2 mm behind the coronal suture, 2 mm lateral to the sagital suture and 4 mm in depth. The cannulae were fixed to the skull with dental cement. Following the operation, rats were put in individual cages and allowed to recover for a week.

Antigen and immunization. Animals of all groups were i. p. immunized with 5 x 10^9sheep red blood cells (SRBC) in 1 ml saline. After 4 days, the rats were sacrificed, and their spleens processed for plaque-forming cell (PFC) assay. Splenocytes were washed, their number adjusted to 10^7 cells/ml, and they were mixed 1:2 with 5% suspension of SRBC containing 10% normal guinea pig serum as a source of complement. Suspensions were incubated at 37^0C for 45 min in Cunningham's microchambers and the number of PFC determined microscopically. Values were expressed as PFC/10^6 spleen cells. Student's t-test was employed for statistical evaluation of differences between experimental and control groups of rats.

Treatment. Rats were divided into groups as follows. The first group received one i. p. injection of 0.2 or 1 mg/kg of DT-Met-Enk every 24 h, for 2 days before and 4 days after immunization with SRBC. The second group was

first injected i. p. with 0.2 or 1 mg/kg of DT-Met-Enk and 15 min later with 5 mg/kg of Nx administered s. c. Such a daily treatment lasted for 6 days: 2 days before and 4 days after immunization. The third group was i. c.v. given 20 or 200 μg/kg of DT-Met-Enk every day, starting from the day of immunization and for 3 consecutive days. And, the fourth group of rats was first inoculated with 20 or 200 μg/kg of DT-Met-Enk, and 5 min later with 10 μg/kg of QNtx, in an identical manner as the third group. Appropriate saline-treated controls were set up. It should be mentioned that on the day of immunization the animals received the injection of a drug 1 h before injection of SRBC.

RESULTS

Peripheral administration of the enkephalinase-inhibitor DT-Met-Enk produced changes in the PFC response (Figure 1 a) The dose of 0.2 mg/kg showed a moderate enhancing effect, whereas the dose of 1 mg/kg exerted a striking immunostimulatory action. On the other hand, i. c. v. administration of the inhibitor (Figure 1b) produced different dose-dependent immunomodulatory effects: the low dose (20 μg/kg) potentiated, but the high dose (200 μg/kg suppressed the PFC response.

Both the peripheral and central immunomodulatory effects of DT-Met-Enk were abrogated by the opioid antagonists Nx and QNtx (Figures. 1a and 1b). In animals treated s. c. with 5 mg/kg of Nx (Fig. 1a), the antagonist completely inhibited the immunostimulatory activity of 0.2 and 1 mg/kg of DT-Met-Enk

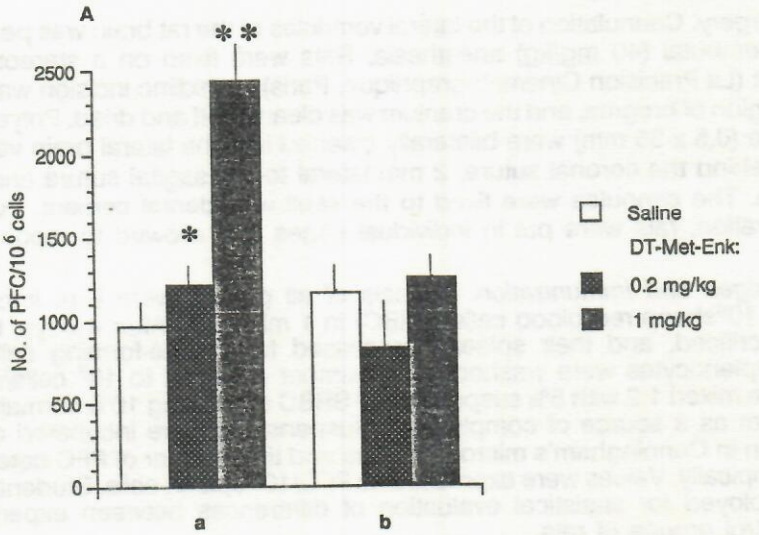

Figure 1. A: peripheral immunomodulating effects of enkephalinase-inhibitor DT-Met-Enk on plaque-forming cell response, and abrogation of these effects by the peripherally applied opioid antagonist naloxone. a, groups i. p. treated with DT-Met-Enk alone, and b, groups first treated i. p. with DT-Met-Enk and then injected s. c. with 5 mg/kg of Nx.

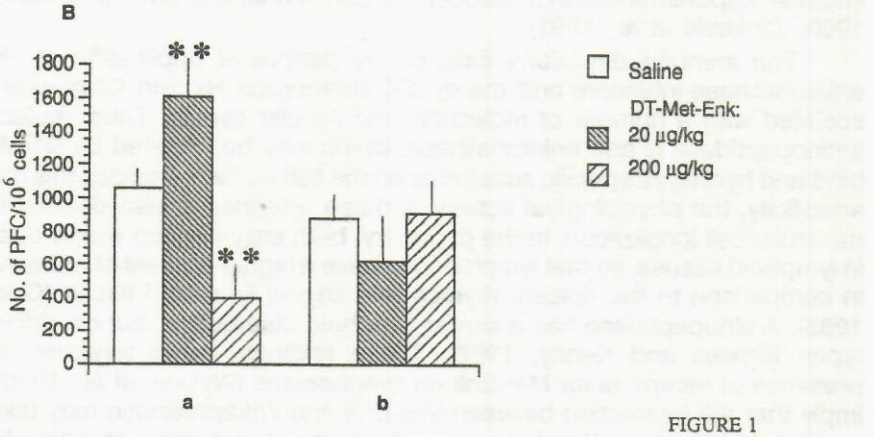

FIGURE 1

B: central immunomodifying effects of enkephalinase-inhibitor DT-Met-Enk on plaque-forming cell response and abolition of these effects by the centrally applied opioid antagonist quaternary naltrexone methylbromide. a, groups i. c. v. treated with DT-Met-Enk and then injected i. c. v. with 10 mg/kg of QNtx.
Statistically significant differences: * $p<0.05$; ** $p<$ (experimental groups versus saline controls).

(Figure 1b). In animals i. c. v. treated with 10 μg/kg of QNtx the immunopotentiation induced by the small dose (20 μ/kg) and immunosuppression produced by large dose of DT-Met-Enk were abolished by QNtx (Figure 1b).

Of particular interest is the central antagonizing effect of QNtx applied i. c. v. on the enkephalinase-inhibitor. Since QNtx is unable to cross the blood-brain barrier, it acts within the CNS. Therefore, QNtx-induced effects on DT-Met-Enk and the PFC response are mediated through the mechanisms of the CNS opioid system.

DISCUSSION

It is well documented that endogenous activity of enkephalins is hampered by their rapid degradation in plasma (Hambrook et al., 1976) and cerebrospinal fluid (Dupont et al., 1977; Stine et al., 1980) by enkephalinases. In experiments, this obstacle can be overcome by the application of enkephalinase-inhibitors, such as DT-Met-Enk. Our earlier studies established the relationship between methionine-enkephalin (Met-Enk- and enkephalinase-inhibitors in modulation of the immune response in the rat (Janković et al., 1991). In the present work, DT-Met-Enk modified the PFC response in a dose-dependent fashion, and this induced immunomodulation was abrogated by the opioid receptor antagonists Nx and QNtx. These observations are in substantial agreement with the evidence that exogenous Met-Enk administered either peripherally or centrally affected

immune responsiveness in a dosedependent manner (Janković and Marić, 1986, 1990; Janković et al., 1991).

The immune deviations induced by peripheral application of Met-Enk, enkephalinase-inhibitors and the opioid antagonists Nx and QNtx, can be associated with a number of molecular and cellular events. Thus, molecules of aminopeptidase N and enkephalinase, which may be inhibited by DT-Met-Enk, bind and hydrolyze specific substrates on the cell surface. Besides this substrate specificity, the physiological activity of these enzymes is also defined by their particular cell localization. In the periphery, both enzymes are widely distributed in lymphoid tissues, so that lymph nodes have a higher content of enkephalinase in comparison to the spleen, thymus and lingual lymphoid tissue (Gee et al., 1985). Aminopeptidase has a similar lymphoid distribution, but on different cell types (Bowes and Kenny, 1987). These findings, taken together with the presence of receptors for Met-Enk on lymphocytes (Wybran et al., 1979) would imply that the interaction between Met-Enk and enkephalinase may take place on the lymphocyte membrane. Furthermore, expression of enkephalinase molecules (CALLA antigen) on pre-B cells during early ontogenesis, and their presence on malignant B cells in acute lymphocytic leukemia (Letarte et al., 1988) suggest the importance of the opioid peptide system in normal immune functioning and the development of immune malignancies. The correlation of enkephalinase-inhibitor and opioid receptor antagonists, as described here, seems to provide further support to the contention that the opioid peptide system is of critical importance in the regulation of immune functions.

As for the central action of Met-Enk, enkephalinases and the opioid receptor antagonist QNtx, the pronounced capacity of QNtx to abrogate the enkephalinase-inhibitor activity at the brain level, as demonstrated in this study, may be related to mechanisms other than direct reaction of the opioid system constituents with immune cells *(vide supra)*. In addition to participation of different brain structures in the regulation of immune reactions (reviewed by Janković and Spector, 1986), there is a widespread distribution of opioids and opioid receptors throughout the CNS (Mansour and Khachaturian, 1987). On the other hand, enkephalinases were detected in high concentrations in structures, such as the chorioid plexus, pial membranes and ependymal cells (Erdös and Skidgel, 1989) which are in contact with cerebrospinal fluid and specifically implicated in the transport and distribution of opioids and other peptides (Banks and Kastin, 1987). The physiological coexistence of opioid receptors and enkephalin-degrading enzymes on neuronal membranes probably contributes to quick termination of enkephalin activity, but such a topography makes difficult experimental delineation between the role of enkephalins and that of corresponding enzymes in immune processes. For that reason, the central application of an enkephalinase-inhibitor, e. g. DT-Met-Enk, is expected to prolong the opioid action within the brain by differentially blocking the CNS enkephalinase activity. Indeed, inhibition of brain peptidases by i. c. v. administration of DT-Met-Enk produced a dose-dependent potentiation and inhibition of a humoral immune response. This effect of DT-Met-Enk was

naltrexone-reversible. Since QNtx does not cross the blood-brain barrier, this induced central immunomodulation is most probably restricted to the CNS. However, when considering the central interaction of DT-Met-Enk and QNtx, it should be taken into account that peptides other than opioids contribute to the immunomodulatory phenomena, i. e. DT-Met-Enk may also act on substance P, neurotensin, and a variety of other mediators (Erdös and Skiegel, 1989).

In conclusion, the results showed that peripheral and central blocking of enkephalinase activity by enkephalinase-inhibitor produced immunomodulating effects which are similar to those induced by administration of exogenous Met-Enk. These effects may be abolished by classical antagonists of opioid receptors, such as naloxone and naltrexone. Thus, this study substantiates the contention that the endogenous opioid system circuits are important for the functioning of the immune system.

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REFERENCES

1. *Bank, W. A. and Kastin, A. J.* 1987. Saturable transport of peptides across the blood-brainbarrier. *Life Sci. 41,* 1319–1338.
2. *Bowen, M. A. & Kenny A. J.* 1987. An immunohistochemical study of endopeptidase- 24.11 and aminopeptidase N in lymphoid tissues. *Immunology, 60,* 247–253.
3. *De La Baume, S., Yi, C. C., Schawartz, J. -C., Chaillet, P., Marcai-Collado, H. and Contentin, J.* 1983. Participation of both "enkephalinase" and aminopeptidase activities in the metabolism of endogenous enkephalins. *Neuroscience 8,* 143–151.
4. *Dua, A. K., Pinsky, C. and LaBella F. S.* 1985. Peptidases that terminate the action of enkephalins. Consideration of physiological importance for amino-, carboxy, endo-, and pseudo-enkephalinase, *Life Sci. 37,* 985–992.
5. *Dupont, A., Cusan, L., Garon, M., Alvarado-Urbina, G. and Labrie, F.* 1977. Extremely rapid degradation of ^{3}H-Methionine-enkephalin by various rat tissues in vivo and in vitro. *Life Sci. 21,* 907–914.
6. *Erdö, E. G. & Skidgel, R. A.* 1989. Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. *FASEB J. 3,* 145–151.
7. *Gee, N. S., Bowen, M. A., Buck, P. and Kenny, A. J.* 1985. An immunoradiometric assay for endopeptidase-24.11 shows it to be a widely distributed enzyme in pig tissues. *Biochem. J. 228, 119–125.*
8. Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, C. F. C. 1976. Mode of deactivation of enkephalins by rat and human plasma and rat brain homogenates. *Nature 262,* 783–783.
9. *Hudgin, R. L., Charleson, S. E., Zimmerman, M., Mumford, R. and Wood, P. L.* 1981. Enkephalinase: selective peptide inhibitors. *Life Sci. 29,* 2593–2601.
10. *Janković, B. D. and Marić, D.* 1986. Modulation of in vivo immune response by enkephalins. *Clin. Neuropharmacol (Suppl.) 9,* 467–468.
11. *Janković, B. D. and Marić, D.* 1990. In vivo modulation of the immune system by enkephalins. *Int. J. Neurosci. 51,* 167–169.
12. *Janković, B. D., Marić, D. and Veljić, J.* 1990. Cerebrally mediated modulation of anaphylactic shock by methionine-enkephalin. *Int J. Neurosci. 51,* 193–194.

13. *Janković, B. D., Veljić, J., Pešić, G. and Marić, D.* 1991. Enkephalinase inhibitors modulate immune response. *Int J. Neuro. ci. 59,* 45–51.
14. *Janković, B. D. and Spector, N. H.* 1986. Effects on the immune system of lesioning and stimulation of the nervous system: Neuroimmunomodulation, in *Enkephalin. and Endorphin. Stre. and. the. Immune Sy. tem. (Plotnikoff, N. P., Faith, R. E., Murgo, A. J. and Good, R. A., ed.),* pp. 189–220. *Plenum Pre., New York.*
15. *Letarte, M., Vera, S., Tran, R., Addi., J. B. L., Onizuka, R. J., Quackenbu. h, E. J., Jongeneed, C. V. and McInne., R. R.* 1988. Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase, *J. Exp. Med. 168,* 1247–1253.
16. *Lord, J. A. H., Waterfield, A. A., Hughe., J. and Ko. terlitz, H. W.* 1977. Endogenous opioid peptides: Multiple agonists and receptors. *Nature 267,* 447–451.
17. *Malfroy, B., Swert., J. P., Lloren., C. and Schwartz, J.-C.* 1979. Regional distribution of a high affinity enkephalin-degrading peptidase ("enkephalinase") and effects of lesions suggest localisation in the vicinity of opiate receptors in brain. *Neuro. ci. Lett. 11,* 329–334.
18. *Man. our, A., Khachaturian, H., Lewi., M. E. and Wat. on, S. J.* 1987. Autoradiographic differentiation of mu, delta and kappa opioid receptors in the rat forebrain and midbrain. *J. Neuro. ci. 7,* 2445–2464.
19. *Plotnikoff, N. P., Faith, R. E., Murgo, A. J. and Good, R. A.* 1986. Enkephalins and Endorphins. Stress and the Immune System. *Plenum Pre., New. York.*
20. *Roda, L. G., Ro. cetti, G., Po. enti, R., Venturelli, F. and Vita, F.* 1986. Control mechanisms in the hydrolysis of adrenal-released enkephalins, in Enkephalins and Endorphins. *Stre. and the Immune Sy. tem. (Plotnikoff, N. P., Faith, R. E., Murgo, A. J. and Good, R. A., ed.), pp.* 17–33. *Plenum Pre., New. York.*
21. *Stine, S. M., Yang, H. Y. T. and Co. ta, E.* 1980. Inhibition of in situ metabolism of [^{3}H](met^5)-enkephalin and potentiation of (met^5)-enkephalin analgesia by captopril. *Brain Re. 188,* 295–299.
22. *Valentino, R. J., Herling, S., Wood., J. H., Medzihrad. ky, F. and Merz., H.* 1981. Quaternary naltrexone: Evidence for the central mediation of discriminative stimulus effects of narcotic agonists and antagonists. *J. Pharmacol. Exp. Ther. 217,* 652–659.
23. *Veljić J., Marić, D., and Janković, B. D.* 1990. Effect of intracerebroventricularly injected methionine-enkephalin on humoral immune response in the rat. *Period. Biol. 92,* 73–74.
24. *Wybran, J., Appelboom, T., Famaey, J. P. and Govaert., A.* 1979. Suggestive evidence for morphine and methionine-enkephalin binding sites on human blood T lymphocytes. *J. Immunol. 123,* 1068–1070.

IMUNOMODULACIJA IZAZVANA INTRAPERITONEALNOM I INTRACEREBROVENTRIKULARNOM APLIKACIJOM INHIBITORA ENKEFALINAZE DES-TIROZIN-METIONIN-ENKEFALINA I BLOKADA OVOG EFEKTA OPIOIDNIM ANTAGONISTIMA NALOKSONOM I NALTREKSONOM

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SADRŽAJ

Mi smo pokazali da metionin-enkefalin, aplikovan periferno i centralno, indukuje dozno-zavisnu imunomodulaciju. U ovom radu je izučavana interakcija inhibitora enkefalinaze des-tirozin-metionin-enkefalina, opioidnih antagonista naloksona i naltreksona i humoralnog imunog odgovora. U tu svrhu pacovi su tretirani intraperitonealno (i.p.) sa 0,2 i 1 mg/kg des-tirozin-metionin-enkefalina, ili u kombinaciji sa subkutanim injekcijama naloksona u dozi od 5 mg/kg pre i posle imunizacije sa ovčijim eritrocitima. Druge grupe životinja su dobijale

des-tirozin-metionin-enkefalin intracerebroventrikularno (i. c. v.) u dozi od 20 i 200 μg/kg samog ili u kombinaciji sa i. c. v. injekcijama kvaternernog naltrekson metilbromida u dozi od 10 μg/kg. Poslednja grupa je bila tretirana na dan imunizacije i sledeća tri dana.

U eksperimentalnim i kontrolnim grupama određivan je broj ćelija slezine koje stvaraju hemolitičke plake. Rezultati su pokazali da su pacovi a) tretirani i. p. sa des-tirozin-metionin-enkefalinom imali dozno-zavisno povećanje humoralnog imunog odgovora koje se poništava sa naloksonom; b) životinje tretirane i. c. v. sa des-tirozin-metionin-enkefalinom u maloj dozi od 20 μg/kg potencirale su humoralni imuni odgovor, dok su visoke doze (200 μg/kg) suprimirale ovaj odgovor, ali ovi efekti su bili kompletno poništeni pomoću i. c. v. aplikacije kvaternernog naltreksona. Ovi rezultati otkrivaju imunomodulatornu aktivnost des-tirozin-metionin-enkefalina datog periferno ili centralno, i negativnu funkciju naloksona i kvaternernog naltreksona na ovu aktivnost.

Ovi rezultati ukazuju na uključenost opioidnog sistema CNS-a u imunoregulaciji.