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Enhancement of T lymphocyte proliferation and suppression of antibody producing cell formation by methionine–enkephalin

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ABSTRACT Methionine–enkephalin (met–enk) 0.1–100 nmol / L significantly enhanced lymphocyte proliferation induced by T cell mitogens. On the other hand, the peptide markedly inhibited splenocyte blastogenesis induced by B cell mitogen lipopolysaccharides (LPS) and sheep red blood cell (SRBC)–driven plaque–forming cell (PFC) formation *in vitro*. Met–enk alone had no effect on immune responses. However, naloxone 50 nmol / L had also a stimulating effect on Con A–induced splenocyte proliferation. The similar results were also observed *in vivo*. The results also indicated that the enhancement of T cell function by met–enk was stronger in immunosuppressed mice than in the normal mice.

KEY WORDS methionine enkephalin; antibody–producing cells; lymphocyte transformation; naloxone; cyclosporins

Experimental evidences in the last few years indicate that endogenous opioid peptides are involved in the modulation of several immune functions. Normal human blood T lymphocytes bear binding sites for opioids[1]. The enkephalins and endorphins enhanced lymphocyte proliferation induced by T cell mitogens in mice and rats, and increased active T cell rosettes in humans[2]. But β–endorphin potently inhibited PHA–induced proliferation of normal human peripheral blood lymphocytes, and met–enkephalin had no effects on mitogen–induced lymphocyte proliferation[3,4]. Besides, the opioid peptides were shown to inhibit antibody synthesis *in vitro*[5]. Both enkephalin and endorphin have no effect on lymphocyte transformation induced by LPS, a specific B cell stimulator.

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Since there is a conflict of opinions about the effects of opioids on immune system, and the mechanisms of actions of opioids on immune system are now unclear, both *in vitro* and *in vivo* studies on the effects of met–enkephalin on mitogen–induced splenocyte proliferation and antibody synthesis driven by SRBC are being carried out.

MATERIALS AND METHODS

Mice Inbred strain mice C57BL (18 ± SD 2 g, ♀) and 615 (4–5 months old, ♀) were obtained from Shanghai Animal Center, Shanghai Branch, Chinese Academy of Sciences.

Chemicals Methionine–enkephalin (met–enk), concanavalin A (Con A), and lipopolysaccharides (LPS) were purchased from Sigma Chemical Company . Cyclosporin A (Cy A) and phytohemagglutinin P (PHA) were obtained from Sandoz Co. and Difico Laboratories, respectively.

Medium RPMI 1640 (Gibco Laboratories) containing media were supplemented with 10 mM HEPES buffer, 100 IU / ml penicillin, 100 μg / ml streptomycin, 2 mM L–glutamine, 50 μM 2–mercaptoethanol, and 10% newborn bovine serum and was adjusted to pH 7.2.

Experimental design The effects of met–enk on lymphocyte blastogenesis and plaque–forming cells (PFC) were evaluated by ip met–enk (in some cases plus Cys A) into sheep red blood cells (SRBC, 3×10^8 cells / mouse in 0.2 ml) sensitized female mice. On d + 4, the spleens were removed for lymphocyte proliferation and PFC tests.

Lymphocyte proliferation assay The

spleens were removed from mice after cervical dislocation. Single cell suspensions were prepared by grinding gently against sterile stainless steel mesh. The red blood cells were removed by hyposmosis shock and the single cell suspensions were washed 3 times by centrifugation in RPMI 1640 media. The cells were then resuspended at 1.5×10^6 and 3×10^6 cells / ml in RPMI 1640 media, respectively. Cell viability exceeded 95% as determined by trypan blue exclusion technique. For *in vitro* experiments, cells (0.1 ml) were dispensed into each well of 96 well flat bottom plates and the cultures included mitogen, met-enk and / or naloxone. For *in vivo* studies, cells (0.2 ml) were distributed into wells with Con A (5 μg / ml) or LPS(10 μg / ml). Total volume of each well was 0.2 ml and the final cell density was 1.5×10^6 cells / ml. The cultures were incubated in a humidified 5% CO_2 enviroment at 37℃. Following a 54 h incubation period, 46.25 kBq of tritiated thymidine was added to per well and the cultures were incubated for an additional 18 h. The cells were harvested on glass fiber filters and the incorporated radioactivity was determined by liquid scintillation counting. All experiments were performed at least twice.

In vivo **PFC assay** The spleens were removed from met-enk-treated mice, which were sensitized with SRBC 4 d ago, and the PFC were tested by using the method described by Cunningham[6].

In vitro **PFC assay** *In vitro* assay for antibody synthesis was carried out. Both spleen cells (from female 615 strain) and SRBC were distributed into 96 well cone-shaped plates at the final concentrations of 5×10^6 cells / ml, respectively. Having been incubated at 37℃ in 5% CO_2 for 5 d, the cultures were harvested and the number of direct PFC (IgM) was evaluated by using Cunningham's method[6].

RESULTS

Effects of met-enk ip to C57BL mice sensitized with SRBC Met-enk 0.1 mg / kg.d ip to SRBC (3×10^8 cells / mouse) sensitized mice significantly increased Con A-induced splenocyte blastogenesis and markedly suppressed PFC formation and LPS-stimulated lymphocyte proliferation (Tab 1). Besides, met-enk could reverse immune suppression caused by Cy A (Tab 1). However, this peptide has no effect on clearance rate of iv charcoal particles in mice (data not shown).

Effects of met-enk on immune function in mice *in vitro*

1 Enhancement of met-enk on Con A- and PHA-induced lymphocyte proliferation Met-enk 0.1-100 nmol / L significantly increased both Con A- and PHA-induced splenocyte proliferation (Tab 2). Met-enk alone had no effect on lymphocyte proliferation (data not shown).

Tab 1. Effects of ip met-enkephalin (met-Enk) on PFC and splenocyte proliferation induced by LPS (10 μg / ml) and Con A (5 μg / ml) in mice and its antagonism to cyclosporin A (Cy A). $^{**}P<0.05$, $^{***}P<0.01$ *vs* **control;** $^{++}P<0.05$ *vs* **Cy A group.** $\bar{x} \pm SD$. $n=8$.

	Dose mg / kg	Treated schedule	PFC / 10^6 cells	ConA	LPS
				$10^{-2} \times$ Radioactivity (dpm)	
Control	—	—	460±61	218±60	188±14
Met-Enk	0.1	-1 - +1d	298±21 ***	370±19 ***	167±21
		+1 - +5d	275±60 ***	365±88 **	107±78 ***
Cy A	50		162±26 ***	137±26 ***	—
		-1 - +1d			
Cy A +met-Enk	50 0.1		282±49 ++	291±112 ++	—

Tab 2. Enhancement of met–enkephalin (met–Enk) on splenocyte proliferation induced by concanavalin A (Con A) and phytohemagglutinin P (PHA), and the influence of naloxone (50 nmol / L) on the enhancing effect *in vitro*. Splenocyte proliferation was expressed as $10^{-2} \times$ dpm / 3 $\times 10^5$ cells. $\bar{x} \pm$ SD. $n = 4$. $^{**}P < 0.05$, $^{***}P < 0.01$ *vs* control; $^{+++}P < 0.01$ *vs* without naloxone control.

Met–Enk (pmol / L)	PHA (10 μg / ml)	Concanavalin A (5 μg / ml) without naloxone	with naloxone
0	433 ± 116	275 ± 9	340 ± 14^{+++}
10^0	470 ± 23	265 ± 9	332 ± 5^{+++}
10^2	495 ± 25	314 ± 26**	330 ± 14
10^4	689 ± 18**	325 ± 18***	328 ± 28
10^6	686 ± 42**	325 ± 19***	330 ± 14
10^8	693 ± 100**	325 ± 7***	349 ± 30

2. Effect of an antagonist naloxone on the enhancement of met–enk–mediated lymphocyte blastogenesis Met–enk 0.1–100 nmol / L and naloxone 50 nmol / L significantly enhanced Con A(5 μg / ml) induced lymphoproliferation (Tab 2). There were no antagonistic or additive effects of naloxone on lymphocyte proliferation by adding met–enk and naloxone into the same culture wells (Tab 2).

3. Inhibitory effect of met–enk on PFC formation driven by SRBC *in vitro* After incubated with both splenocytes and SRBC (both densities were 5 × 10^6 cells / ml), met–enk was found to inhibit PFC formation markedly *in vitro* (Tab 3).

Tab 3. Suppressive effect of met–enkephalin (met–Enk) on SRBC induced PFC formation *in vitro*. $\bar{x} \pm$ SD. $n = 3$. $^{***}P < 0.01$ *vs* control.

met–Enk (pmol / L)	PFC / 10^6 cultured cells
0	724 ± 70
10^{-2}	423 ± 49***
10^0	247 ± 19***
10^2	448 ± 60***
10^4	405 ± 51***
10^6	303 ± 16***

4. Inhibitory effect of met–enk on LPS induced splenocyte blastogenesis and influence of naloxone on this effect Met–enk 0.1–100 nmol / L inhibited LPS induced lymphocyte proliferation *in vitro*. The effect of met–enk could be completely blocked by naloxone (50 nmol / L). Naloxone per se had no effect on LPS–induced lymphocyte blastogenesis (Tab 4).

Tab 4. Inhibitory effect of met–enkephalin (met–Enk) on LPS (6.25 μg / ml) induced splenocyte proliferation and influence of naloxone (50 nmol / L) on this inhibitory effect *in vitro*. $\bar{x} \pm$ SD. $n = 4$. $^{**}P < 0.05$ *vs* control.

met–Enk (pmol / L)	10^{-2} × Radioactivity (dpm) without naloxone	with naloxone
0	440 ± 75	378 ± 42
10^0	301 ± 57	348 ± 33
10^2	280 ± 33**	344 ± 45
10^4	308 ± 27**	372 ± 38
10^6	276 ± 26**	327 ± 56
10^8	252 ± 30**	348 ± 70

DISCUSSION

Previous studies have shown that there are opioid binding sites on many immune active cells. On the basis of this idea, we investigated the effect of a specific opioid antagonist naloxone on the immunoregulating actions of met–enk. As shown in Tab 2, both a specific opioid delta agonist met–enk and a specific opioid antagonist naloxone had stimulative effects on Con A–induced lymphocyte proliferation. Although met–enk increased T lymphocyte mitogen–induced lymphocyte proliferation, it did not activate the resting lymphocytes to transform. Furthermore, the simultaneous addition of met–enk and naloxone to the same culture wells had neither additive nor antagonistic effects on Con A–induced lymphocyte proliferation, suggesting that the peptide might be acting through the same receptors on T lymphocytes. The phenomena were similar to the observations that naloxone as an agonist in its actions was like β–endorphin and met–enk in inhibiting T lymphocyte

chemotactic factor[7]. That is, no antagonistic properties were seen with naloxone. In this paper, we can not explain why opioid receptor blocker, naloxone, has a stimulating effect on activated T cells.

As shown in Tab 4, met–enk significantly inhibited splenocyte blastogenesis stimulated by LPS, a specific B lymphocyte mitogen, which did not agree with that met–enk had no effect on B cell proliferation[8]. This result suggested that the inhibitory effect of met–enk was through opioid receptors on B cells, because the suppressive effect of the peptide was completely blocked by naloxone. The enhancement of T lymphocyte proliferation by met–enk indicated that the inhibitory effects of met–enk on B lymphocytes were directly at B cell levels but not at T cell levels. However, another opioid, α–endorphin inhibiting PFC formation *in vitro*, was thought to act at the T cell level as well as at the B cell level[9].

According to this paper and other reports[1, 10,11], the influences of opioid peptides on immune functions were possibly through different sites on different immune competent cells, and the final outcome of immunomodulation by opioid peptides is by virtue of these peptides binding to mu and kappa receptors (downregulation) and / or delta and epsilon receptors (upregulation)[12]. The conflicting results among investigators might be due to the different immune status of animals, peptides, or methods used in their experiments. Our *in vivo* results also indicated that the enhancement of T lymphocyte proliferation by met–enk was stronger in cyclosporin A–induced immunosuppressed mice than in the normal mice. These also suggest that the immunomodulation of opioids is related to the immune status of the animals.

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