

## Methionine-enkephalin alteration of mitogenic and mixed lymphocyte culture responses in zinc-deficient mice

LI Gang<sup>1</sup>, Pamela J FRAKER

(Department of Biochemistry, Michigan State University, E Lansing, MI 48824, USA)

**Abstract** The effects of methionine-enkephalin (Met-Enk) on mitogenic and mixed lymphocyte culture (MLC) proliferation of splenocytes from Zn-deficient, restricted and control mice were evaluated. The data from this experiment show that Met-Enk can suppress the responses of splenocyte from the 3 groups to concanavalin A (Con A), but less inhibition was observed in the Zn-deficient group. Met-Enk can also enhance the responses to pokeweed mitogen (PWM) and decrease the response to lipopolysaccharide (LPS) in all groups. Alteration of proliferative responses to Con A and PWM were reversible in the presence of naloxone 10  $\mu\text{mol/L}$  indicating that the effect of Met-Enk on cellular proliferation was mediated by the opioid receptor. In the proliferation of MLC, the response of lymphocytes from Zn-deficient mice was increased in the absence of Met-Enk and Met-Enk can suppress this increased response. It is therefore concluded that Met-Enk can modify the pattern of mitogenic responses and the alteration in Con A and MLC responses can be influenced by zinc deficiency.

**Key words** methionine enkephalin; zinc deficiency; lymphocytes; mitogens; cultured cells

Zinc deficiency in the body is a common cause of stress, leading to immune function impairment<sup>(1)</sup>. On the other hand, the mechanisms of the altered immune responses in Zn-deficient animals are still nebulous even though some roles of zinc on the immune system have been elucidated<sup>(2)</sup>. One attractive trend has emerged in the past decade indicating that some neuropeptides may be secreted and released

from the central nervous system and peripheral organs during stress and play an important role in the regulation of the immune system<sup>(3,4)</sup>.  $\beta$ -Endorphin ( $\beta$ -End) and methionine-enkephalin (Met-Enk) can be released from the pituitary and adrenal glands respectively when the animal is exposed to stress; they are considered to be important immune regulators.

The present experiments focused on the effects of Met-Enk, the pentapeptide, on the mitogenic reaction and MLC response of splenocytes from zinc deficient mice. From these studies, the immunomodulatory mechanisms of Met-Enk during stress may be further clarified.

### Materials and methods

**Mice and diets** A/J mice, ♀, 8 wk (from Jackson Lab, Bar Harbor, ME) were used and divided into 2 groups: control and Zn-deficient groups. Two different 20% egg white diets were used for the 2 groups of either Zn (0.1  $\mu\text{g/g}$ ) deficient or Zn (30  $\mu\text{g/g}$ ) adequate. A 3rd (restricted) group was used to exclude the possible effect of decreased food consumption by inanition in the Zn-deficient group. The zinc adequate diet were fed in amounts limited to the average daily intake of the Zn-deficient group. Diet consumption was measured daily and the mice were weighed at least once a week. All mice had full access to deionized, distilled water (<0.2  $\mu\text{g Zn/g}$ ). To prevent retaking of zinc from body wastes, the mice were housed in stainless steel cages with mesh bottoms. Feed jars and water bottles were washed with HCl 4

mol/L and rinsed with deionized water to remove all residual zinc.

**Reagents** Met-Enk and opioid antagonist naloxone purchased from Sigma were resuspended in 1 mg/ml phosphate buffer saline solution (pH 7.2). Concanavalin A (Con A) and lipopolysaccharide (LPS, *Escherichia coli* 0.55; B5) were purchased from Sigma, pokeweed mitogen (PWM) from Gibco Laboratories. All mitogens were resuspended and diluted in a RPMI 1640 medium before use.

**Medium** The cell culture medium was prepared with RPMI 1640 incomplete medium supplemented with 0.5% delipidated bovine serum albumin (BSA) and 0.5% autologous mouse sera from each dietary group.

**Preparation of cell suspension** Spleens were removed from each group of mice aseptically and pressed through stainless steel screen to separate into single cells. Each group of splenocytes was washed once and resuspended to a final density of  $2.5 \times 10^6$  cells/ml. In the MCL experiments, splenocytes from C57BL/6 were used as stimulator cells and obtained in the method described above. All splenocytes from C57BL/6 mice were mixed, washed once and treated with mitomycin C. After 3 washings, stimulator cells were resuspended in the medium at densities of 2, 5, 7.5 and  $10 \times 10^6$  cells/ml.

**Measurement of mitogenic proliferation** For cell culture 96-well flat bottomed microtiter plates were used. To each well 0.1 ml of splenocytes suspension was added. Mitogens were assayed in triplicate at the following concentrations: Con A 0.25, 0.5, 1 and 2.5  $\mu\text{g/ml}$ ; LPS 25, 50 and 100  $\mu\text{g/ml}$  and PWM 25, 50 and 100  $\mu\text{l/ml}$ .

**Mixed lymphocyte culture** Autologous and allogenic mixed lymphocyte culture experiments were also performed in 96-well flat-bottom microtiter plates. A/J spleno-

cytes were separately mixed with 2, 5, 7.5 or  $10 \times 10^6$  mitomycin C-treated C57BL/6 spleen stimulator cells at triplicate.

Met-Enk was used at 0.1, 1 and 10  $\mu\text{mol/L}$  in each experiment. Culture plates were incubated at 37°C in a humidified atmosphere of 7%  $\text{CO}_2$ . After 24 and 48 (for Con A and LPS), 72 (for PWM) and 96 (for MCL) h, the culture were pulsed with 1 GBq of [*methyl*- $^3\text{H}$ ]thymidine. Cells were harvested 18 h later onto glass microfiber filter using a multiple sample harvester. The incorporation of [*methyl*- $^3\text{H}$ ]thymidine was measured by using a Beckman gamma counter).

To determine whether the effect of Met-Enk on the mitogenic assay was mediated by the opioid receptor, splenocytes from Zn-deficient mice were also cultured with naloxone 0.1, 1 or 10  $\mu\text{mol/L}$  in each experiment.

**Data treatment** The results from assays of [*methyl*- $^3\text{H}$ ]thymidine incorporation were presented as  $\bar{x} \pm \text{SD}$ . Corresponding background has been subtracted from all data in this paper.

## Results

**Met-Enk suppresses the proliferation response to LPS** Splenocytes from either Zn-deficient or control mice were cultured with Met-Enk and LPS for 2 and 3 d. Significant suppression by Met-Enk on responsiveness was obtained in both groups on incubation d 2 (Fig 1). Optimal concentration of LPS was shown to be 50  $\mu\text{g/ml}$ . The highest inhibition were 19% in the Zn-deficient group, 16% in restricted and 21% in control group, and no significant difference was seen among the 3 groups. Since LPS is a kind of B cell mitogen, it seems likely that Met-Enk can inhibit the proliferation of the B cell. The fact that the suppressive effects of Met-Enk can be reversed by naloxone 10  $\mu\text{mol/L}$  indicated that opioid receptors were involved (Tab 1).

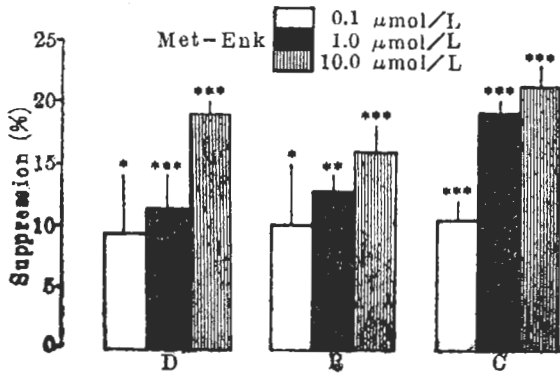


Fig 1. Suppressive effects of Met-Enk on lipopolysaccharide (LPS) response were obtained by incubation for 2 d of splenocytes  $2.5 \times 10^6/\text{ml}$  from Zn-deficient (D), restricted (R) and control (C) groups with LPS  $50 \mu\text{g}/\text{ml}$  and different concentrations of Met-Enk,  $n=3$ ,  $\bar{x} \pm \text{SD}$ . \* $P > 0.05$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ .

Tab 1. Blocking of Met-Enk-induced change of mitogenic response by naloxone. Splenocytes from Zn-deficient mice were stimulated by LPS, Con A and pokeweed mitogen (PWM).  $n=3$ ,  $\bar{x} \pm \text{SD}$ . \* $P > 0.05$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ .

Met-Enk ( $\mu\text{mol}/\text{L}$ )	Naloxone ( $\mu\text{mol}/\text{L}$ )	$10^{-3} \times$ Uptake of [Methyl- $^3\text{H}$ ]TdR (cpm)
LPS		
0	0	$102 \pm 5$
0	10	$95 \pm 1$
1	0	$79 \pm 4^{***}$
1	10	$95 \pm 5^*$
Con A		
0	0	$79 \pm 5$
0	10	$82 \pm 16^*$
0.1	0	$63 \pm 7^{**}$
0.1	10	$73 \pm 18^*$
1	0	$59 \pm 8^{**}$
1	10	$78 \pm 10^*$
PWM		
0	0	$71 \pm 1$
0	10	$72 \pm 31^*$
1	0	$97 \pm 4^{**}$
1	10	$78 \pm 25^*$

**Met-Enk suppresses the proliferation responses to Con A** When splenocytes were cultured with Met-Enk and Con A for 2 d, an inhibition was seen in all 3 groups. There was considerable variation between groups in the degree of suppression of [methyl- $^3\text{H}$ ]thymidine uptake obtained in the presence of different concentrations of Met-Enk and Con A  $0.5 \mu\text{g}/\text{ml}$  (Fig 2). Less inhibition was observed in Zn-deficient group as compared to the control and restricted groups. The suppressive effects of Met-Enk was reversed by naloxone  $10 \mu\text{mol}/\text{L}$  (Tab 1). These results indicate that Met-Enk can suppress the Con A responses, and zinc deficiency can result in a lower susceptibility to Met-Enk-induced inhibition. Since Con A mainly stimulates helper T cells, the lower sensitivity in the case of zinc deficiency was probably due to a decreased number of helper T cells or opioid receptors on the surface of helper T cells. The suppressive effects of Met-Enk on the Con A response were mediated by opioid receptors.

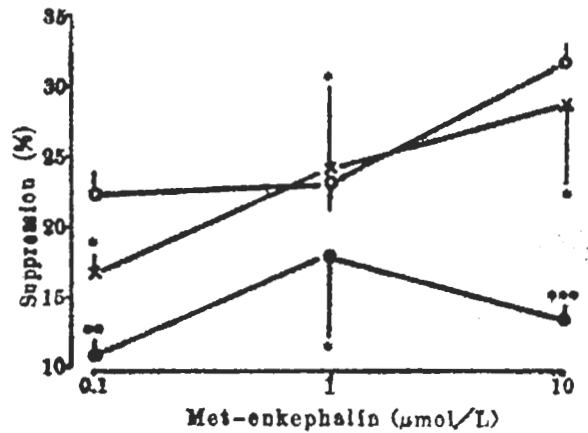


Fig 2. Suppressive effects of Met-Enk on Con A response were obtained by incubation for 2 d of splenocytes  $2.5 \times 10^6/\text{ml}$  from Zn-deficient (●), restricted (×) and control (○) groups with Con A  $0.5 \mu\text{g}/\text{ml}$  and different concentrations of Met-Enk,  $n=3$ ,  $\bar{x} \pm \text{SD}$ . \* $P > 0.05$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ .

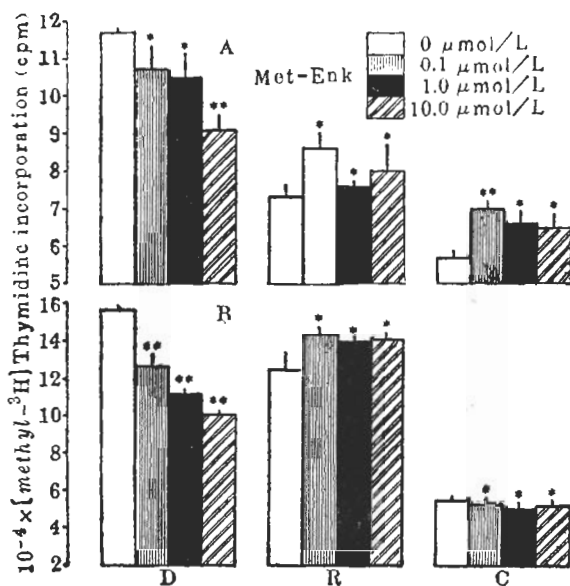
**Met-Enk enhances the proliferation responses to PWM** PWM is a mitogen for

both T and B cells. When cells were cultured on d 4, Met-Enk increased responses of splenocytes to PWM from all 3 groups (Tab 2). The enhancement was seen in either PWM 25 or 50  $\mu\text{g}/\text{ml}$ , and the enhanced% were as high as 30% in the Zn-deficient group, 15% in the restricted group and 24% in the control group. Increased responses were reversed by naloxone 10  $\mu\text{mol}/\text{L}$ , indicating an opioid mechanism was involved in the responses (Tab 1).

**Tab 2.** Effect of Met-Enk on PWM response. Splenocytes from Zn-deficient, restricted and control mice were cultured with PWM 25 and 50  $\mu\text{l}/\text{ml}$  and different concentrations of Met-Enk for 4 d.  $n=3$ ,  $\bar{x}\pm\text{SD}$ . \* $P>0.05$ , \*\* $P<0.05$ , \*\*\* $P<0.01$ .

Dietary group	Met-Enk ( $\mu\text{mol}/\text{L}$ )	$10^{-3} \times$ Uptake of [Methyl- $^3\text{H}$ ]TdR	
		(cpm)	Change (%)
PWM 25 $\mu\text{l}/\text{ml}$			
Control	0	$66\pm 1$	
	0.1	$78\pm 7^*$	+18
	1.0	$80\pm 8^*$	+21
	10.0	$82\pm 3^{**}$	+24
Zn-deficient	0	$56\pm 1$	
	0.1	$66\pm 2^{**}$	+16
	1.0	$74\pm 10^{**}$	+30
	10.0	$64\pm 8^*$	+12
Restricted	0	$77\pm 27$	
	0.1	$86\pm 5^*$	+12
	1.0	$89\pm 4^*$	+16
	10.0	$88\pm 12^*$	+15
PWM 50 $\mu\text{l}/\text{ml}$			
Control	0	$89\pm 3$	
	0.1	$107\pm 1^*$	+19
	1.0	$105\pm 9^*$	+18
	10.0	$111\pm 7^*$	+24
Zn-deficient	0	$74\pm 5$	
	0.1	$88\pm 3^{**}$	+19
	1.0	$91\pm 5^{**}$	+22
	10.0	$87\pm 2^{**}$	+17
Restricted	0	$61\pm 2$	
	0.1	$64\pm 2^*$	+5
	1.0	$66\pm 2^{***}$	+9
	10.0	$60\pm 3^*$	-2

**Met-Enk altered MLC response** Different concentrations of stimulator cells were used to measure the proliferative phase of the MLC response. The results of this assay in the case of 2 and  $7.5 \times 10^6$  allogenic cells are shown in Fig 3. Different responsive directions were seen in the Zn-deficient, restricted and control groups. As the dose of Met-Enk rose, an increased suppressive effect was shown on the MLC response in Zn-deficient mice. By contrast, a slightly higher enhancement or no significant alteration could be demonstrated in both restricted and control groups. Additionally, lymphocytes from the Zn-deficient group gave a higher proliferative response than the other 2 groups in the absence of Met-Enk; this finding is consistent with the results of similar work<sup>(5)</sup>. Since the mixed lymphocyte culture is the proliferative phase of immature T-cells, the suppression of MLC by Met-Enk in Zn-deficient mice



**Fig 3.** Effect of Met-Enk on MLC response. A/J responder splenocytes prepared from Zn-deficient (D), restricted (R) and control (C) mice were cultured with mitomycin C-treated C57BL/6 stimulator cells  $2 \times 10^6/\text{ml}$  (A) or  $7.5 \times 10^6/\text{ml}$  (B) and different concentrations of Met-Enk for 5 d,  $n=3$ ,  $\bar{x}\pm\text{SD}$ . \* $P>0.05$ , \*\* $P<0.05$ .

suggests that immature T cells have a higher sensitivity to Met-Enk during zinc deficiency.

## Discussion

In this experiment, Met-Enk inhibited the proliferation of splenocytes exposed to B cell mitogen, LPS. Suppression of B cells by Met-Enk has been seen in splenocyte antibody production<sup>(8)</sup> and bone marrow immature B cell antibody production (unpublished). In the present studies, suppressed LPS responses by Met-Enk were similar in all 3 groups. It seems that zinc deficiency did not alter the decreased responsiveness of B cells to mitogen in the presence of Met-Enk, and these results further indicate that suppression of Met-Enk to B cells proliferation occurred not only under stress but also under normal conditions.

The same situation occurred in the case of PWM, but the responses were enhanced instead of decreased. Previous work has shown that no significant difference in PWM responses in the absence of neuropeptides was demonstrated between Zn-deficient, restricted and control groups<sup>(2)</sup>. The present results indicate that Met-Enk enhances the PWM response of splenocytes, and that the zinc ion has no effect on this enhancement.

In the case of Con A, however, a notably reduced uptake of [*methyl*-<sup>3</sup>H]thymidine was obtained in the control and restricted groups. Although cellular proliferation in the Zn-deficient group was also inhibited in cultures containing Met-Enk, this suppression was less than that in the control and restricted groups (Fig 2). It has been reported that the target cells for Con A are mainly helper T cells, those with a  $\text{Lyt}-1^+2^-3^-$  phenotype<sup>(7)</sup>. Met-Enk induced less suppression in the culture of Zn-deficient lymphocytes with Con A, suggesting that zinc deficiency might modulate either the expression of Met-Enk receptors of the  $\text{Lyt}-1^+2^-3^-$  population or

a number of the  $\text{Lyt}-1^+2^-3^-$  population. Compared to Con A, Met-Enk enhances the response to PWM in the 3 groups. It is therefore suspected that different T cell subsets were activated by Met-Enk.

It has been reported that the mixed lymphocyte response is the proliferative phase of immature T cells, the  $\text{Lyt}-1^+2^+3^+$  cells<sup>(8)</sup>. The suppressive effect of Met-Enk on the MLC in Zn-deficient mice may suggest an elevated level of expression for the receptor of Met-Enk on the immature T-cells in this group. It seems as if immature cells, no matter whether T cells or B cells, exhibit a difference in expression of receptors for Met-Enk as compared to mature cells. This conclusion agrees with work on bone marrow immature B cells that showed bone marrow immature B cells in Zn-deficient mice were more sensitive to the inhibition of Met-Enk than in the control group (unpublished).

The mechanisms of the effect of Met-Enk on mitogenic and MLC responses in zinc deficiency are still not known fully, because they refer to a complicated relationship among zinc, Met-Enk and the immune system. Current evidence has demonstrated that zinc deficiency can constitute a kind of stress that activates the pituitary-adrenal axis in the body. During stress Met-Enk can be released from the adrenal gland to influence the immune system. Zinc can also influence the effect of Met-Enk directly. For example, the enhancement of active T cell rosette formation by Met-Enk in the presence of  $\text{ZnCl}_2$  is greater than the effects of either of these agents alone<sup>(9)</sup>. However, a contrary report indicates that zinc has a high affinity for thiol groups in opioid receptors and therefore can block opioid binding and the effects of opiates<sup>(10)</sup>. Furthermore, major enkephalin-degrading enzymes are zinc metalloenzymes<sup>(11)</sup>. It is possible that zinc deficiency can lead to a decrease in the enzyme activity

and relative increase of Met-Enk concentration. As indicated above, a mutual relationship between zinc and Met-Enk in mitogenic responses and MLC may exist and the precise mechanisms of the effects of Met-Enk on zinc deficiency remain to be identified.

## References

- 1 Hildebrandt KM, Luecke RW, Fraker PJ. Effects of maternal dietary zinc on growth and mitogenic responsiveness in suckling mice. *J Nutr* 1982; 112 : 1921
- 2 Fraker PJ. Zinc deficiency: a common immunodeficiency state. *Surv Immunol Res* 1983; 2 : 155
- 3 Hanbauer I, Kelly GD, Saiani L, Yang HYT. [Met<sup>5</sup>]-enkephalin-like peptides of the adrenal medulla; release by nerve stimulation and functional implication. *Peptides* 1982; 3 : 469
- 4 Plotnikoff NP, Murgu AJ, Miller GC, Corder CN, Faith RE. Enkephalins: immunomodulators. *Fed Proc* 1985; 44 : 118
- 5 DePasquale-Jardieu P, Fraker PJ. Altered B-cell responses induced by a trace element deficiency. *Ibid* 1982; 41 : 218
- 6 Johnson HM, Smith EM, Torres BA, Blalock JE. Regulation of the *in vitro* antibody response by neuroendocrine hormones. *Proc Natl Acad Sci USA* 1982; 79 : 4171
- 7 Nakayama E, Dippold W, Shiku H, Oettgen HF, Old LJ. Alloantigen-induced T-cell proliferation: Lyt phenotype of responding cells and blocking of proliferation by Lyt antisera. *Ibid* 1980; 77 : 2890
- 8 Simon MM, Abenhardt B. Generation of effector cells from T cell subsets II. Lyt 123 T cells contain the precursors for all primary cytotoxic effector cells and for cells involved in the regulation of cytotoxic responses. *Eur J Immunol* 1980; 10 : 334
- 9 Murgu AJ, Plotnikoff NP, Faith RE. Effect of methionine-enkephalin plus ZnCl<sub>2</sub> on active T cell rosettes. *Neuropeptides* 1985; 5 : 367
- 10 Simon EJ, Groth J. Kinetics of opiate receptor inactivation by sulfhydryl reagents: Evidence for conformational change in presence of sodium ions. *Proc Natl Acad Sci USA* 1975; 72 : 2404
- 11 Barclay RK, Phillipps MA. Inhibition of enkephalin-degrading aminopeptidase activity by certain peptides. *Biochem Biophys Res Commun* 1980; 96 : 1732

# 甲硫氨酸-脑啡肽改变缺锌小鼠对促细胞分裂素和混合淋巴细胞培养的反应

李刚<sup>1</sup>, Pamela J FRAKER

(Department of Biochemistry, Michigan State University, E Lansing, MI 48824, USA)

**提要** 本实验测定甲硫氨酸-脑啡肽对缺锌小鼠淋巴细胞在促细胞分裂素反应和混合淋巴细胞培养(MLC)上的效应。结果显示甲硫氨酸-脑啡肽能抑制各组淋巴细胞对 Con A 的反应,但对缺锌组抑制作用较弱,能增进对 PWM 和减少对 LPS 的反应。这些改变均可被纳络酮 10 μmol/L 所逆转。缺锌组的 MLC 反应增高,甲硫氨酸-脑啡肽可抑制这种增加的反应。结果表明蛋氨酸-脑啡肽能改变小鼠淋巴细胞对促细胞分裂素

反应,锌缺乏可影响淋巴细胞对 Con A 和 MLC 的反应。

**关键词** 甲硫氨酸-脑啡肽; 锌缺乏; 淋巴细胞; 促细胞分裂素; 细胞培养

<sup>1</sup>现在: 西安医科大学生物化学教研室, 西安 710061, 中国