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Evidence for μ opioid receptor on mouse spleen lymphocytes

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KEY WORDS fentanyl; naloxone; mu opioid receptors; T-lymphocytes; B-lymphocytes; radioligand assay; concanavalin A; lipopolysaccharides; thymidine; spleen

ABSTRACT

AIM: Using ohmefentanyl (Ohm), a high affinity μ opioid receptor agonist, to find whether μ opioid receptor exists on mouse spleen lymphocytes. **METHODS**: The proliferation rates of T-lymphocytes and B-lymphocytes were determined under various concentrations of Ohm with or without naloxone (Nal) *in vitro*. Binding characteristics of [^{3}H]Ohm with mice spleen lymphocytes were studied by radioligand assays. **RESULTS**: Ohm 0.1 pmol · L^{-1} – 1 nmol·L^{-1} enhanced Con A-induced spleen T-cell proliferation *in vitro*. Nal 50 μmol·L^{-1}, which *per se* enhanced the T-cell proliferation, blocked the enhancing effects of Ohm. However, Ohm had no effect on B-cell proliferation. Furthermore, a satisfactory saturable, specific, and reversible binding was demonstrated with K_d of (6.9 ± 0.6) nmol·L^{-1}, B_{max} of (74 ± 6) fmol/10^7 cells. The binding of [^{3}H]Ohm was blocked by unlabeled Ohm or Nal. **CONCLUSION**: Stimulating effects of Ohm on lymphocytes were mediated by opioid receptors. Mouse spleen lymphocytes present μ opioid receptors.

INTRODUCTION

Whether opioid receptor sites present on cells of the immune system attracted many attentions since Wybran first proposed in 1979 that human peripheral blood T-cells might contain opioid receptors[1]. Our previous studies also showed that the opioid peptide (Met-enk) and opioid alkaloid (morphine) could influence several immune functions such as antibody formation, lymphocyte proliferation, and cytokine production through opioid receptors[2-4]. The aim of the present work was to demonstrate the existence of μ opioid receptors on mouse immune cells by radioligand assay, as well as the effect of Ohm on proliferation of mouse spleen lymphocytes *in vitro*.

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MATERIALS AND METHODS

Reagents Ohmefentanyl hydrochloride (Ohm, a white crystal, melting point 206 ℃, purity > 99 %) was synthesized by Prof ZHU You-Cheng from Shanghai Insititute of Materia Medica, Chinese Academy of Sciences. Trypan blue, produced by Shanghai Third Reagent Factory, was dissolved in 0.5 % saline. Naloxone (Nal), concanavalin A (Con A), and lipopolysaccharides (LPS) from *E Coli* were from Sigma Co. [^{3}H]Ohm and [^{3}H]TdR were labeled by Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. The RPMI-1640 medium (Gibco Co) was supplemented with HEPES buffer 1 mmol · L^{-1}, benzylpenicillin 100 kU · L^{-1}, streptomycin 0.1 g · L^{-1}, *L*-glutamine 2 mmol · L^{-1}, 2-mercaptoethanol 50 mol·L^{-1}, and 10 % fetal bovine serum (FBS), pH 7.2.

Mice Inbred ICR mice (♀, Grade Ⅱ, Certificate number 02-49-2, 20 g ± *s* 2 g) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences.

Cells for radioligand assay Spleen was torn and passed through a stainless mesh (40 μm pores). The red blood cells were removed by hyposmotic shock. Following 2 washes with RPMI-1640, the cell suspension was divided into 3 groups. One is directly used for radioligand assay. Other 2 group suspensions

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were separately incubated with Con A 5 mg·L^{-1}, LPS 6.25 mg·L^{-1} at 37 ℃ under 5 % CO_2 for 72 h.

Radioligand binding assay The binding experiments were performed at 30 ℃ in Tris-HCl buffer (pH 7.7) 0.05 mol·L^{-1}. Each assay mixture 250 μL contained 100 μL spleen cells (final concentration 1 × 10^{10} cells/L) and [^{3}H]Ohm 50 μL with increasing concentrations of 0.2 – 6 nmol·L^{-1}. Nonspecific binding was determined by adding unlabeled Ohm 100 μL 0.5 μmol·L^{-1}(final concentration). After 30-min incubation, the samples were immediately cooled in an ice-bath and filtered through glass fiber filters (Whatman GF/C) followed by washing with 10 times of ice-cold Tris-HCl buffer. The fiber filters were dried and transferred to counting vials with scintillation cocktail. The radioactivity was determined by liquid scintillation counting. Each concentration of ligand binding was performed in triplicate. Scatchard analysis was used with a computerized programs (GPIP) for the analysis of binding.

Proliferation assay Spleen cells were prepared as above. The cells were then resuspended at 2 × 10^9 cells/L in RPMI-1640 media. Cells 100 μL were dispensed into each well of 96-well microtiter plates with or without mitogen (Con A 5 mg·L^{-1} or LPS 6.25 mg·L^{-1}). RPMI-1640 medium 200 μL were added into each well. After various concentrations of Ohm were incubated with or without Nal 50 mol·L^{-1} at 37 ℃ under 5 % CO_2 for 66 h, [^{3}H]TdR 9.25 kBq/well was added and the plates were incubated for another 6 h, then harvested onto glass fiber filter. The lymphocyte proliferation was expressed as Bq/2 × 10^5 cells.

Lymphocyte viability assay After lymphocytes were incubated with Ohm 0.1 pmol·L^{-1} – 1 nmol·L^{-1} with or without mitogen for 24, 48, 72 h, trypan blue exclusion was used to count the ratio of cell viability.

RESULTS

Effects of Ohm on activated spleen lymphocyte proliferation Con A-induced spleen T-lymphocyte proliferation was enhanced by Ohm 0.1 pmol·L^{-1} – 1 nmol·L^{-1}. LPS-induced B-lymphocyte proliferation was not changed by Ohm. Ohm 0.1 – 1 μmol·L^{-1} depressed both spleen T- and B-lymphocyte proliferation (Tab 1).

Tab 1. **Effect of Ohm on Con A (5 mg·L^{-1})- and LPS (6.25 mg·L^{-1})-induced splenocyte proliferation *in vitro*. n = 3 wells from 10 mice. $\bar{x} \pm s$.**
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Ohm/nmol·L^{-1}	[^{3}H]TdR uptake, Bq/2 × 10^5 cells	
	LPS	Con A
0	367 ± 17^a	167 ± 33^b
0.0001	317 ± 50^a	283 ± 33^b
0.001	333 ± 50^a	300 ± 33^b
0.01	317 ± 33^a	333 ± 50^b
0.1	350 ± 67^a	267 ± 33^b
1	317 ± 33^a	250 ± 17^b
10	300 ± 33^a	217 ± 33^a
100	183 ± 17^c	50 ± 17^c
1000	200 ± 50^c	50 ± 5^c

Influence of naloxone on Ohm-induced splenocyte proliferation Naloxone 50 μmol·L^{-1} stimulated the Con A-induced T-cell proliferation, antagonized the promotive effect of Ohm on activated T-cell proliferation, but had no influence on the inhibiting effect of Ohm 0.1 – 1 μmol·L^{-1} on the T- and B-cell proliferation (Tab 2).

Tab 2. **Effect of Ohm and Nal 50 μmol·L^{-1} on Con A (5 mg·L^{-1})- and LPS (6.25 mg·L^{-1})-induced splenocyte proliferation *in vitro*. n = 4 wells from 10 mice. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.**

Ohm/nmol·L^{-1}	[^{3}H]TdR uptake, Bq/2 × 10^5 cells			
	LPS		Con A	
	Ohm	Ohm + Nal	Ohm	Ohm + Nal
0	750 ± 83	767 ± 83	333 ± 50	533 ± 67^b
0.001	700 ± 83^a	717 ± 83^a	600 ± 83^b	400 ± 50^a
0.01	667 ± 100^a	783 ± 100^a	617 ± 83^b	367 ± 33^a
0.1	767 ± 67^a	700 ± 83^a	533 ± 67^b	433 ± 50^a
1	683 ± 67^a	750 ± 100^a	500 ± 50^b	367 ± 50^a
10	700 ± 50^a	717 ± 50^a	350 ± 33^a	517 ± 50^b
100	350 ± 50^c	350 ± 33^c	167 ± 33^c	150 ± 17^c
1000	383 ± 67^c	367 ± 50^c	167 ± 17^c	150 ± 17^c

Effect of Ohm on lymphocyte viability The ratios of viability of lymphocytes cultured with Ohm 1 μmol·L^{-1} after 24, 48, and 72 h were all > 95 %.

Saturation of Ohm binding to spleen lymphocytes Specific binding was gradually saturat-

ed with increasing cell concentration (Fig 1A) about 60 % – 70 % of total binding. Nonspecific binding were determined by adding unlabeled Ohm 0.5 μmol · L^{-1} which increased linearly with [^{3}H]Ohm concentration. While, specific binding was gradually saturated with increasing [^{3}H]Ohm concentration (Fig 1B).

Scatchard analysis of [^{3}H]Ohm to μ opioid receptors on spleen lymphocytes Scatchard plot showed the presence of a single site with K_d of (6.9 ± 0.6) nmol · L^{-1}, B_{max} of (74 ± 6) fmol/10^7 cells on spleen lymphocytes (Fig 1C).

Effect of Olm and Nal on [^{3}H]Ohm binding to spleen lymphocytes The specific binding of [^{3}H] Ohm was blocked by unlabeled Ohm or Nal (Fig 1D).

DISCUSSION

The results showed that the specific binding of [^{3}H]Ohm to spleen lymphocytes increased with

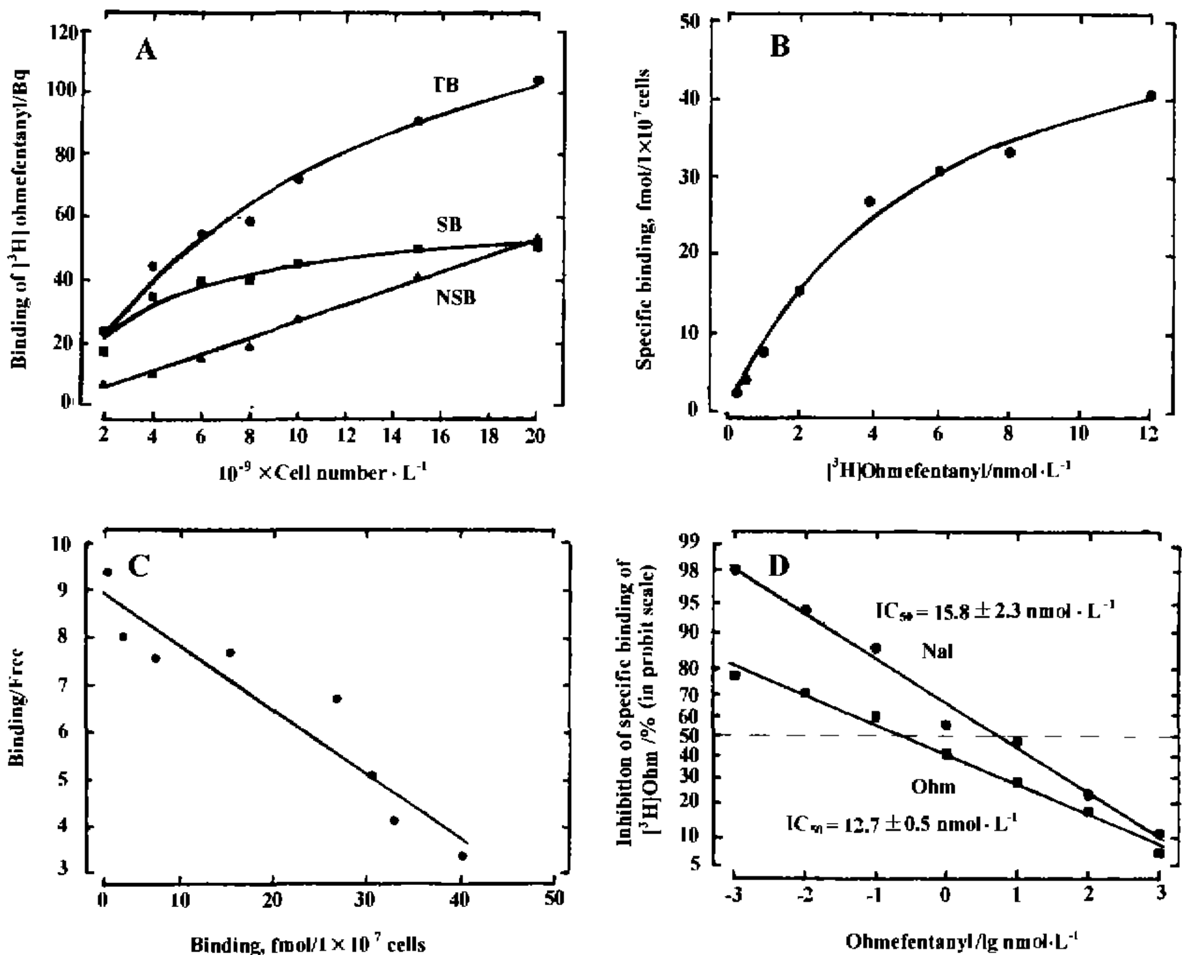

Fig 1. Binding characteristics of [^{3}H] ohmefentanyl on mouse spleen lymphocytes. A) Saturation binding of [^{3}H]Ohm to mouse spleen lymphocytes with increasing concentration of splenocytes. Total binding (TB), nonspecific binding (NSB), specific binding (SB). B) Specific binding of [^{3}H]Ohm. C) Scatchard analysis of [^{3}H]Ohm to μ opioid receptors on mouse spleen lymphocytes. B_{max} = (74 ± 6) fmol/1 × 10^7 cells, K_d = (6.9 ± 0.6) nmol·L^{-1}. D) Effect of Ohm and Nal on the binding of [^{3}H]Ohm.

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both the increasing number of splenocytes and the increasing concentration of [^{3}H]Ohm. The binding revealed equilibrium when the concentration of cells arrived at 2×10^{10} cells/L and the concentration of [^{3}H]Ohm reached 12 $\text{nmol} \cdot \text{L}^{-1}$. Scatchard analysis indicated the presence of a single site. The radioligand bound to spleen lymphocytes could be displaced by the unlabeled Ohm or naloxone. This suggested that the spleen lymphocytes contain μ opioid receptor.

In addition, our work demonstrated that Ohm at low concentration stimulated the proliferation of Con A-activated T-cells (0.1 $\text{pmol} \cdot \text{L}^{-1}$ – 1 $\text{nmol} \cdot \text{L}^{-1}$), which means that the stimulating effect is mediated by the μ opioid receptors on lymphocytes. However, Ohm 1 $\text{pmol} \cdot \text{L}^{-1}$ – 10 $\text{nmol} \cdot \text{L}^{-1}$ had no effect on the proliferation of LPS-activated B-cells. It suggested that μ opioid receptors exist mainly on T-lymphocytes. The inhibition of proliferation by high concentration of Ohm (0.1 – 1 $\mu\text{mol} \cdot \text{L}^{-1}$) could not be blocked by naloxone, showing that it might be a direct action not mediated by μ opioid receptors.

In conclusion, firstly, Ohm directly affected lymphocyte proliferation through opioid receptors *in vitro*. Secondly, the binding characteristics of [^{3}H]Ohm on mouse lymphocytes showed satisfactory saturability, specificity, and reversibility. Both results demonstrate that mouse spleen lymphocytes contain μ opioid receptors.

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小鼠脾脏淋巴细胞上存在 μ 阿片受体的证据

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关键词 芬太尼；纳络酮；μ 阿片受体；T 淋巴细胞；B 淋巴细胞；放射配位体测定；伴刀豆球蛋白 A；脂多糖；胸苷；脾

目的：用高亲和性 μ 阿片受体激动剂羟甲芬太尼研究小鼠脾脏淋巴细胞是否存在阿片受体. **方法**：观察羟甲芬太尼(Ohm)对脾脏淋巴细胞体外增殖的影响及纳络酮的阻断作用；采用[^{3}H]Ohm 放射配体分析法检测小鼠脾脏淋巴细胞上的阿片受体. **结果**：Ohm 0.1 $\text{pmol} \cdot \text{L}^{-1}$ – 1 $\text{nmol} \cdot \text{L}^{-1}$增加 Con A 诱导的 T-淋巴细胞增殖，这种增殖作用可以被纳络酮阻断. Ohm 不能促进 LPS 诱导的 B-细胞增殖. Ohm 0.1 – 1 $\mu\text{mol} \cdot \text{L}^{-1}$能够抑制活化的淋巴细胞增殖. 这种抑制作用不被纳络酮阻断. [^{3}H]Ohm与淋巴细胞阿片受体的结合具有特异性、饱和性、可逆性. **结论**：Ohm 对静止及活化的 T-细胞的促增殖作用是由阿片受体介导的. 小鼠脾脏淋巴细胞上存在阿片受体.

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