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Immunomodulating effects of morphine microinjected into periaqueductal gray[1]

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AIM: To study the effects of morphine on immune system through rat brain periaqueductal gray (PAG). **METHODS**: Three hours after microinjection of morphine through the implanted steel tubes to PAG, splenic cytokines interleukin 2 (IL-2), interleukin 6 (IL-6), tumor necrosis factor (TNF), and natural killer cells (NK) activity were measured. **RESULTS**: Microinjection of morphine (0.5 μL, 3672 ng) into PAG region had no influence on IL-6 and TNF-α (production of splenic macrophages, suppressed the natural killer cell (NK) activity and enhanced T-lymphocyte functions, including concanavalin A (Con A)-induced T-cell proliferation, IL-2 and TNF-β production. Both the suppressive and stimulating actions were blocked by PAG preinjection of the μ opioid receptor antagonist naloxone (0.5 μL, 1 μg), which alone showed the contrary effect to morphine. **CONCLUSION**: Morphine affected immunofunctions through opioid receptors in PAG, and the influences on various immunocompetent cells were different.

KEY WORDS morphine; naloxone; periaqueductal gray; T-lymphocytes; macrophages; natural killer cells

Opiates play a key role in the neuroimmunomodulating network. Opiate addicts reduce host immuno-defence functions, which increase susceptibility to the infections of bacteria and fungi as well as virus. In addition to the direct effects of opiates on the immunocytes, these effects on immune function are mediated indirectly by the central nervous system (CNS). Morphine binds specifically to μ opioid receptors. In CNS, periaqueductal gray (PAG) is one of the regions containing μ opioid receptors. In this paper, the effects of microinjection of morphine into rat brain PAG on the functions of T-cells, splenic macrophages, and NK cells were studied to show the neuroimmunomodulating effects of morphine mediated by the CNS.

MATERIALS AND METHODS

Reagents Morphine was produced by Qinhai Pharmaceutics and recrystallized by Dr YIN Dun-Xiang, Department of Organic Synthesis of our Institute. Naloxone, concanavalin A (Con A), lipopolysaccharides (LPS), dactinomycin (Dac), aod deoxyribonucleases (DNase) were from Sigma Co. Methyl-α-*d*-mannoside was from Bucha Co. 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was from Fluka Co. RPMI 1640 medium (Gibco Co) containing media were supplemented with HEPES buffer 10 mmol·L^{-1}, penicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1}, *L*-glutamine 2 mmol·L^{-1}, 2-mercaptoethanol 50 μmol·L^{-1}, and 10 % newborn bovine serum, pH 7.2. Human recombinant interleukin 2 (hrIL-2) was kindly supplied by Prof LIU Xing-Yuan, Shanghai Institute of Biochemistry. Trypsin was from Shanghai Institute of Biological Products, Chinese Ministry of Public Health. Crystal violet (AR) was from Shanghai Chongming Chemical Reagent Factory. [^{3}H]TdR was from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences.

Animals ICR mice, ♀, 3-month old, weighing

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20±s 2 g, Sprague-Dawley (S-D) rats, ♀, weighing 215±s 15 g, were from Shanghai Animal Center, Chinese Academy of Sciences.

Cell line YAC-1 cell line (a Moloney leukemia virus-induced mouse T-lymphoma cell) and L929 cell line (tumorigenic murine fibroblast) were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The two cell lines were subcultured with 10 % newborn bovine serum RPMI 1640 medium. MH60.BSF2 cell line was kindly supplied by Dr TIAN Zhi—Gang, Shandong Medical Academy of Sciences, and cultured with 10 % newborn bovine serum RPMI 1640 medium containing human recombinant interleukin 2 (hrIL-2) 20 $kU \cdot L^{-1}$.

Implantation into PAG S-D rats were implanted under barbiturate anesthesia with Φ 0.7 mm stainless steel tubes (anterior-posterior 2.7 mm, medial-lateral ±0.8 mm, dorsal-ventral 5.5 mm). One week later, drugs were injected: control group saline (0.5 μL + 0.5 μL); morphine group saline (0.5 μL) + morphine (0.5 μL, 3672 ng); naloxone group naloxone (0.5 μL, 1 μg) + saline (0.5 μL); naloxone blocked group naloxone (0.5 μL, 1 μg) + morphine (0.5 μL, 3672 ng). After 3 h, spleen was mechanically dissociated into cells, washed twice, and suspended in RPMI 1640. The histologic examination of implantation was confirmed by amino black 10B injected into the site after experiment.

Lymphocyte proliferation The splenic cell suspension was added to 96-well plate (with Con A 5 mg $\cdot L^{-1}$, final volume 200 μL and cell concentration $2 \times 10^9 \cdot L^{-1}$), 5 wells for each sample. After incubation at 37 C in a 5 % CO_2 incubator for 72 h, the cultures were added with [^{3}H]TdR (24.5 kBq/well) for the final 6-h incubation and were harvested on glass fiber filters. Cell proliferation was expressed as dpm/2×10^6 cells.

IL-2 production[1] The splenic cells ($2 \times 10^9 \cdot L^{-1}$, 1 mL/well) were cultured with Con A (5 mg $\cdot L^{-1}$) in 24-well plate, at 37 C in an incubator with 5 % CO_2 for 24 h, 2 wells per sample. After incubation, all the supernatants containing IL-2 were collected and clarified by centrifugation ($600 \times g$, 10 min). The supernatants were stored at −25 C until assay for IL-2 activity.

IL-2 assay IL-2 activity was measured by proliferation of Con A-activated T-cells. Splenocyte suspension of ICR mice was added to the 25 mL culture with Con A 5 mg $\cdot L^{-1}$. After incubation at 37 C in a 5 % CO_2 incubator for 72 h, the cells were washed three times, and resuspended in RPMI 1640 with methyl-α-*d*-mannoside (25 mmol $\cdot L^{-1}$) to 2×10^6 cells/well. This suspension was distributed over a 96-well plate. To the suspension, 100 μL of cell culture supernatant or various concentrations of standard hrIL-2 were added. The cultures were incubated at 37 C in a 5 % CO_2 incubator for 24 h. These cultures were added with [^{3}H]TdR (9.25 kBq /well) for the final 6-h incubation and were harvested onto glass fiber filters. IL-2 activity was calculated from the standard hrIL-2.

Splenic macrophages The splenic cell suspension (1 mL, 4×10^6 cells/well) was added to 24-well plate, 2 wells for each sample. After incubation at 37 C in a 5 % CO_2 incubator for 2 h, non-adherent cells were removed by washing twice with RPMI 1640 medium. The adherent cells were used as splenic macrophages.

TNF production[2,3] The splenic cells (1 mL, 4×10^6 cells/well), for TNF-β or the splenic macrophages, for TNF-α, were cultured with Con A (5 mg $\cdot L^{-1}$) or LPS (10 mg $\cdot L^{-1}$) in 24-well plate, at 37 C in a 5 % CO_2 incubator for 6 h, 2 wells per sample. After incubation, all the supernatants containing TNF were collected and clarified by centrifugation ($600 \times g$, 10 min). The supernatants were stored at −25 C until assay for TNF activity.

TNF assay The TNF activities in different samples were determined by the cytotoxicity assay against L929 cells (5×10^4 cells/100 μL) seeded in the 96-well plate and incubated at 37 C in 5 % CO_2 atmosphere for 24 h. The culture medium was removed. TNF supernatant (100 μL) and RPMI 1640 medium containing Dac (final concentration 1 mg $\cdot L^{-1}$) were added. Plates were reincubated for 20 h. The cells stained with 0.5 % crystal violet (containing 3.2 % formaldehyde isotonic solution) for 15 min. Plates were rinsed and dried. The residue was dissolved in 47.5 % ethanol (100 μL/well) which was measured at 570 nm on an ELISA autoreader. TNF activity was calculated:

Cytotoxicity (%) = (Control − Test)/Control × 100%.

IL-6 production[4] The splenic cell suspension (1 mL, 1.3×10^6 cells/well) was added to 24-well

plate with LPS 6.25 mg·L^{-1}, 2 wells for each sample. After incubation at 37 ℃ in a 5% CO_2 incubator for 64 h, IL-6 was collected and clarified by centrifugation (600 ×g, 10 min). The supernatants were stored at −25 ℃ until assay for IL-6 activity.

IL-6 assay[5] The proliferation bioassay for IL-6 was performed using an IL-6 dependent B cell hybridoma, MH60.BSF2. The MH60.BSF2 cells (1×10^4 cells/well) were cultured in the presence of macrophage and L929 cell supernatants collected. After 24 h or 48 h, depending on the growth condition of MH60·BSF2, MTT 20 μL (10 g·L^{-1}) were added for 5 h and then 100 μL of 10% SDS-0.01mol·L^{-1} HCl were added to each well after mixing slightly on a plate mixer. The culture plates were incubated at 37 ℃ in a 5% CO_2 incubator for another 12 h. The absorbance (A) at 570 nm was measured by ELISA spectrophotometer.

NK cell activity assay NK cell activity was assessed in a [^{3}H]TdR release assay with YAC-1, a murine lymphoma cell line as targets. [^{3}H]TdR labeled YAC-1 cells 100 μL, 2×10^6·L^{-1} were added to each well of 96-well plate containing splenic cells (100 μL, 2×10^7 cells/well) with the effector/target ratios of 100:1, 3 wells for each sample. Maximal release of [^{3}H]TdR was determined by incubating targets with 100 μL Triton × 100, spontaneous release was determined as the amount of radioactivity released in the presence of media alone. After incubation at 37 ℃ in 5% CO_2 incubator for 7.5 h, the cultures were added with trypsin (0.15%) and DNase (0.0125%) for 30 min. The cultures were harvested onto glass fiber filters. The specific lysis (%) was calculated:

Specific lysis (%) = (Experimental release − Spontaneous release) / (Maximal release − Spontaneous release) × 100%

RESULTS

T-cell functions, including IL-2, TNF-β production, and the proliferation induced by Con A were enhanced 3 h after injection of morphine into PAG. The enhancements were blocked by preinjection of naloxone, which *per se* inhibited T-cell functions. PAG microinjection of morphine had no influence on IL-6 nor TNF-α production of splenic macrophages. Naloxone had no influence on these cytokines either. No antagonistic effects between morphine and naloxone were detected. The NK cell-inhibiting effect of morphine was blocked by prior microinjection of naloxone into PAG (Tab 1).

DISCUSSION

The present study demonstrated that microinjection of morphine into rat brain PAG had various effects on different immune competent cells, stimulating IL-2, TNF-β production and the proliferation of Con A-induced splenic T-cells, but inhibiting the NK cell activity. The blockage of both the stimulating and inhibiting effects by prior microinjection

Tab 1. Effects of microinjection of morphine and naloxone into rat periaqueductal gray on Con A-induced IL-2, TNF-β production, and splenic T-cell proliferation (expressed as dpm of [^{3}H]TdR uptake), LPS-induced IL-6, TNF-α production of splenic macrophages, and NK cell activity (effector: target = 100:1). $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* saline.

	n	Saline	Morphine	Naloxone	Naloxone + Morphine
T-cell proliferation (dpm×10^{-4})	5	5.1±1.0	10.1±0.7^c	1.6±0.0^c	5.4±0.7
IL-2 activity (kU·L^{-1})	5	256±11	429±4^c	162±36^c	297±59
TNF-β activity (specific lysis %)	5	38.0±4.0	54.0±2.0^c	20.0±4.0^c	32.0±4.0
IL-6 activity (A 570 nm)	4	1.57±0.03	1.62±0.10	1.61±0.01	1.58±0.01
TNF-α activity (specific lysis %)	4	28.9±2.6	28.9±2.6	28.9±2.6	26.3±2.6
NK activity (specific cytotoxity %)	3	63.3±3.8	45.5±2.5^b	71.9±2.2^b	53.4±9.5

of naloxone into PAG indicated that the effects were pharmacologically specific and suggested that the immunomodulating effects of morphine *in vivo* could at least partially mediated by CNS, specially by opioid receptors in PAG.

Foot-shock, during which endogenous opiates are released in PAG[6], suppressed NK cell activity[7]. Microinjection of morphine into PAG suppressed NK cell activity in rats, and this suppression can be blocked by intraperitoneal injection of naltrexone 5 min before PAG microinjections of morphine[8] and preinjection of naloxone into PAG, suggesting that PAG might be one of the primary central nervous sites involved in neuroimmunomodulation of opiates.

Our study also showed that microinjection of morphine into PAG had no effect on splenic macrophages, including the production of IL-6 and TNF-α. In our another experiment (data not shown), direct inhibiting effects of morphine on mouse peritoneal macrophages, including IL-1 and TNF-α production were observed *in vitro* suggested that a direct suppressive effects of morphine on reticuloendothelial system might exist.

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中脑导水管周围灰质微量注射吗啡的免疫调节作用

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目的：研究吗啡经大鼠中脑导水管周围灰质对免疫系统的影响. **方法**：大鼠 PAG 埋植钢管，经此管微量注射吗啡后观察大鼠细胞因子 IL-2，IL-6，TNF 及 NK 活力的变化. **结果**：PAG 微量注射吗啡(0,5 μL，3672 ng)显著促进 IL-2，TNF-β 的产生及 T 细胞的增殖，对 NK 细胞的杀伤活性却有抑制作用，这些促进或抑制作用都能被 PAG 预先微量注射纳洛酮所阻断，对大鼠脾脏巨噬细胞的 IL-6及 TNF-α 的产生都无影响. **结论**：吗啡通过 PAG 的阿片受体影响免疫功能，并且对不同的免疫活性细胞的影响各不相同.

关键词 吗啡；纳洛酮；导水管周围灰质；T-淋巴细胞；巨噬细胞；自然杀伤细胞