Indian J Microbiol (September 2009) 49:276–282
DOI: 10.1007/s12088-009-0045-6

ORIGINAL ARTICLE

Effect of morphine on *Mycobacterium smegmatis* infection in mice and macrophages

Raman Preet Singh · Sarbjit Singh Jhamb · Prati Pal Singh

Received: 16 July 2008 / Accepted: 18 November 2008
© Association of Microbiologists of India 2009

Abstract The immunomodulatory effects of opioids are known in various infections. However, little is known about the effects of opioids in tuberculosis (TB). In the present study, we report the effects of morphine in *Mycobacterium smegmatis* infection in mice and macrophages. Morphine exerted a dose-dependent suppression of infection in vivo: 50 and 100 mg/kg morphine exerted significant ($P<0.05$) suppression whereas 5 mg/kg morphine showed no effect. Analogous to the in vivo effects, incubation of *M. smegmatis* -infected mouse peritoneal macrophages with morphine (100 μM) showed significant reduction in intramacrophage CFU counts. However, morphine did not show any direct antimycobacterial activity in broth dilution assay upto 100 μM concentration. Further, morphine-induced intramacrophage killing of *M. smegmatis* was abrogated by naloxone and aminoguanidine indicating the involvement of opioid-receptor activation and nitric oxide production in protective effects of morphine. In conclusion, morphine suppressed the progression of experimental TB in both mice and macrophage models.

Keywords Morphine · *Mycobacterium smegmatis* · Mice · Macrophages

R. P. Singh · S. S. Jhamb (✉) · P. P. Singh
National Institute of Pharmaceutical Education and Research, Phase-X, S. A. S Nagar - 160 062,
India

E-mail: sarbjitjhamb@rediffmail.com

Introduction

Opioids comprise an important pharmacological class of analgesics which act by binding to G-protein coupled opioid receptors. Long before opioids were discovered, their addictive potential was identified. However, the effects of opioids on the immune system were realized in the 20th century [1]. Opioids exert immunomodulatory effects by direct and indirect mechanisms. The direct effects of opioids result from activation of opioid receptors expressed by immune cells which lead to alterations in various physiological functions of immune cells. On the other hand, the indirect effects of opioids are mediated through the central nervous system and involve activation of hypothalamus-pituitary-adrenal axis [2–4]. Clinically, patients receiving chronic opioid therapy and opioid addicts exhibit alterations in immune functions predisposing them to infections [1, 4]. Experimental studies suggest that chronic exposure to morphine leads to suppression of normal cellular functions of immune cells, particularly macrophages and lymphocytes [2, 5].

Despite several reports demonstrating the immunosuppressive effects of morphine, the immunostimulatory effects of morphine have also been documented. Morphine can augment protective immune responses, both in vitro and in vivo, leading to suppression of infection [6–8]. The effects observed are dose-dependent and biphasic in nature [8–11].

Clinical studies show that opioid addicts are relatively more susceptible to mycobacterial infections than non-addicts [12] and also show reduced reactivity towards tuberculin test [13]. On the contrary, morphine can also increase the phagocytosis of *Mycobacterium tuberculosis* by microglial cells [14]. Recently, we have reported the protective effects

of morphine in *M. tuberculosis* infection in mice and macrophages [15]. The protective role of opioids may result from macrophage activation at lower concentrations leading to enhanced production of cytokines and nitric oxide (NO) [3, 8–11, 15]. These molecules play a crucial role in intramacrophage and in vivo killing of mycobacteria as well [15–18]. Here, we report the effect of morphine on survival of *M. smegmatis* in murine and macrophage models of TB.

Materials and methods

Animals

Swiss albino mice (20 ± 2 g), obtained from Central Animal Facility of the institute, were maintained in 12 h light/dark cycle and provided food and water ad libitum. All studies were carried out as per protocol approved by Institutional Animal Ethics Committee (IAEC) prior to experimentation.

Bacteria and infection

M. smegmatis was obtained from Tuberculosis Research Centre, Chennai, India. The bacteria were grown in Middlebrook 7H9 medium (HiMedia, India) supplemented with 10% ADC (HiMedia). Log phase cultures were centrifuged and washed twice with sterile saline. The cell suspension was adjusted to McFarland standard no. 5 (1×10^7 bacteria/ml) and sonicated (20 KHz, 10 seconds, 10 cycles; Bandelin, Germany). Mice were infected intravenously with 0.1 ml of the bacterial suspension. The size of inoculum was confirmed by plating 10-fold serial dilutions (prepared in saline) on Middlebrook 7H10 agar medium (HiMedia) with 10% ADC supplement followed by CFU enumeration after 3–4 days of incubation at 37°C in 5% CO_2 atmosphere.

Drugs

Morphine was obtained from Government Opium and Alkaloid Factory, Ghazipur, India. Naloxone and aminoguanidine (AG) were obtained from Sigma, USA. The drugs were dissolved in sterile saline.

Drug treatment

M. smegmatis-infected mice were administered 5, 50 and 100 mg/kg morphine following two dosage regimens. In single dose regimen, morphine was administered on day 0 of infection (3 h post-infection). In the two-dose regimen, morphine was administered on day 0 (3 h post-infection) and day +3 of infection (n=10 per group). Control groups were administered saline on similar schedule.

CFU enumeration

M. smegmatis-infected mice were sacrificed on day +7 of infection by cervical dislocation. Lungs and spleen were homogenized in saline and 10-fold serial dilutions were plated on Middlebrook 7H10 medium with 10% ADC supplement. CFUs were enumerated after 3–4 days of incubation at 37°C in 5% CO_2 atmosphere.

Spleen and lung pathology

On the day of sacrifice, the spleen and lungs were removed. The organ pathologies were determined as described earlier [15].

Intramacrophage killing

Elicited peritoneal macrophages were harvested from mice as described elsewhere [9]. Adherent macrophage monolayers were infected with *M. smegmatis* as described elsewhere [15] and incubated with different concentrations of morphine (1 pM to 100 μM) in presence or absence of 10 μM naloxone and 100 μM AG. Following drug treatment, *M. smegmatis*-infected macrophages were lysed on day 0, day +1 and day +3 as described elsewhere [15]. Tenfold serial dilutions of macrophage lysates were plated on Middlebrook 7H10 medium supplemented with 10% ADC and CFUs were enumerated after 3–4 days of incubation at 37°C in 5% CO_2 atmosphere.

Direct antimycobacterial activity of morphine

The direct antimycobacterial activity of morphine against *M. smegmatis* was assessed by broth dilution method [19].

Statistical analysis

CFU counts were converted to logarithmic scale and evaluated by one-way ANOVA using Sigma Stat program.

Results

Effect of morphine treatment on lung CFU counts

Mice infected with *M. smegmatis* were administered morphine following two regimens. In the single dose regimen (day 0), low dose of morphine (5 mg/kg) had no effect on bacterial load in lungs as compared to saline-treated control. However, treatment with higher doses of 50 and 100 mg/kg morphine resulted in significant ($P < 0.01$) reduction in bacterial load in lungs (data not shown). Similarly, administration of two doses of morphine

(day 0 and day +3) showed a dose-dependent reduction in bacterial load in lungs (Fig. 1). Further, the bacterial load was not significantly different in the two dosage regimens.

Effect of morphine treatment on spleen CFU counts

In the single dose regimen, administration of low dose of morphine (5 mg/kg) had no effect on bacterial load in spleen as compared to saline-treated group. However, treatment with higher doses of morphine (50 and 100 mg/kg) resulted in significant (P <0.05) reduction in spleen bacterial load (data not shown). Similarly, in the two dose regimen, 50 and 100 mg/kg morphine resulted in significant (P <0.01) reduction in bacterial load (Fig. 1), whereas low dose of morphine (5 mg/kg) had no effect. Further, no significant difference was observed in suppression of infection between the two regimens.

Spleen and lung pathology

On the day of sacrifice, spleen weights and gross lung pathology of *M. smegmatis*-infected mice were recorded. A direct correlation was observed between the weight and number of CFUs in the spleen of mice treated with single (data not shown) and two (Table 1) doses of morphine. There was no significant difference in spleen weights and CFU counts in either of the treatment regimens.

Effect of morphine on intramacrophage killing

The concentration-dependent effect of morphine was studied in *M. smegmatis*-infected macrophages. High concentration of morphine (100 μM) showed significant (P <0.05) reduction in intramacrophage bacterial load. However, lower concentrations of morphine were ineffective (Fig. 2). The effects of morphine were abrogated by naloxone (P <0.01) (Fig. 3) and AG (Fig.4).

Direct antimycobacterial activity of morphine

Different concentrations of morphine (1pM to 100 μM) lacked any direct antimycobacterial activity against *M. smegmatis* in the broth dilution assay (Table 2).

Discussion

Opioids can modulate the in vivo course of parasitic and bacterial infections in a dose-dependent manner [8, 9, 11, 15, 20]. In the present study, we report, apparently

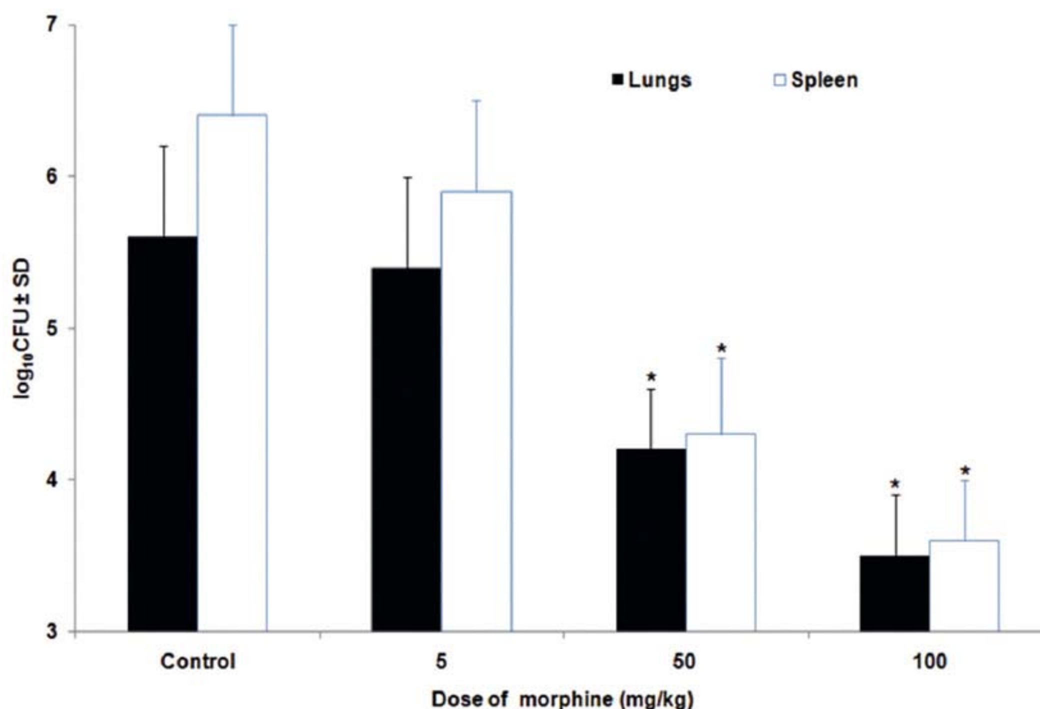

Fig. 1 Effect of morphine on lung and spleen CFU counts.
M. smegmatis-infected mice were treated with indicated doses of morphine on day 0 and day +3 and sacrificed to determine bacterial load in lungs and spleen as described in Materials and methods.
* P<0.01 as compared to control

Table 1 Correlation between spleen weight and CFU counts

Treatment	Spleen weight (mg) ± SD	CFU counts (log_{10}CFU) ± SD
Control	150.0 ± 35.6	6.4 ± 0.6
Isoniazid	81.2 ± 9.1**	-*
Morphine (5 mg/kg)	165.1 ± 40.3	5.9 ± 0.6
Morphine (50 mg/kg)	145.3 ± 10.6	4.3 ± 0.5
Morphine (100 mg/kg)	120.6 ± 10.5**	3.6 ± 0.4**

M. smegmatis-infected mice were treated with indicated doses of morphine and sacrificed to determine bacterial load in spleen and spleen weight as described in Materials and methods.
* P<0.01 as compared to control
** P<0.05 as compared to control

Macrophages are the key cells responsible for in vivo killing of mycobacteria. Hence, we studied the intramacrophage killing of *M. smegmatis* at various concentrations of morphine. Morphine, at 100 µM concentration, showed a significant reduction in intramacrophage survival of *M. smegmatis,* whereas lower concentrations of morphine did not show a significant change (P>0.05) (Figure 2). Morphine lacked direct activity against *M. smegmatis* up to 100 µM concentration (Table 2) suggesting that the intramacrophage and in vivo killing of *M. smegmatis* is not due to the direct effect of morphine on the bacterium. Further, the intramacrophage killing of *M. smegmatis* was abrogated by naloxone (Fig. 3) indicating the role of naloxone-sensitive opioid receptors in the killing effects of morphine. These results support the proposition that morphine can modulate the intramacrophage survival of *M. smegmatis* by modulation of the immune system.

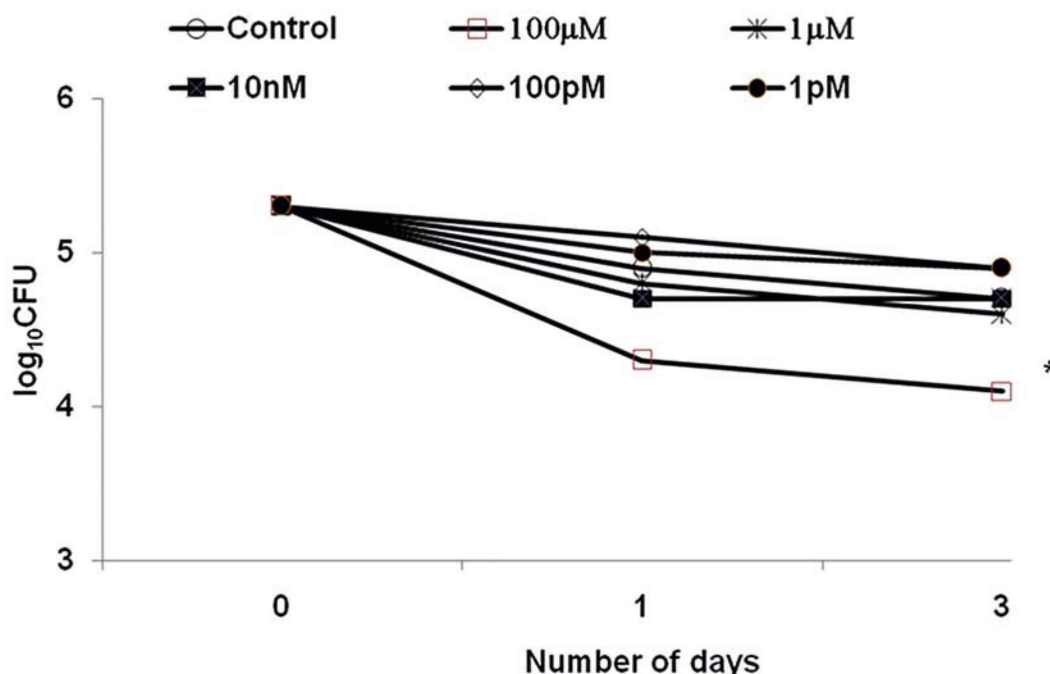

Fig. 2 Concentration-dependent effect of morphine on intracellular survival of *M. smegmatis*.
M. smegmatis-infected macrophages were incubated with indicated concentrations of morphine and lysed on day 0, +1 and +3 to determine intramacrophage killing as described in Materials and methods. Error bars are not shown for clarity.
* P<0.01 as compared to control
** P<0.05 as compared to control

for the first time, dose-dependent modulation of *M. smegmatis* infection in vitro and in vivo. The results of this study indicate that morphine can modulate the in vivo course of *M. smegmatis* infection in mice as evident from bacterial load in the target organs. Low dose of morphine (5 mg/kg) was found to be ineffective (P>0.05) while higher doses (50 and 100 mg/kg) showed a significant (P<0.01) suppression of infection. Further, bacterial load in control group was higher in spleen as compared to lungs which is a characteristic feature of non-virulent strains of *Mycobacterium* [21, 22].

The mechanisms involved in immunomodulatory effects of opioids are not well characterized. Opioids form a part of a complex network of common ligands and receptors, shared by the immune system and central nervous system. Opioids exert their effects by binding to their specific receptors expressed by the immune cells [2, 23] or in the central nervous system [4, 5] which account for their direct and

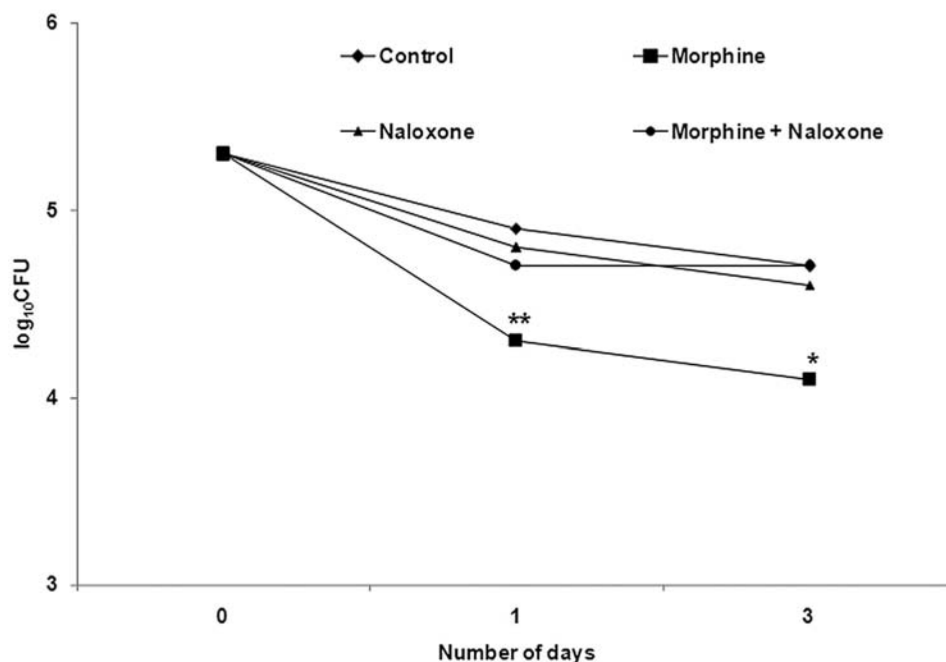

Fig. 3 Effect of naloxone on morphine-induced intramacrophage killing of *M. smegmatis.*
M. smegmatis-infected macrophages were incubated with 100 µM morphine in presence or absence of 10 µM naloxone and lysed on day 0, +1 and +3 to determine intramacrophage killing as described in Materials and methods. Error bars are not shown for clarity.
* $P<0.01$ as compared to control
** $P<0.05$ as compared to control

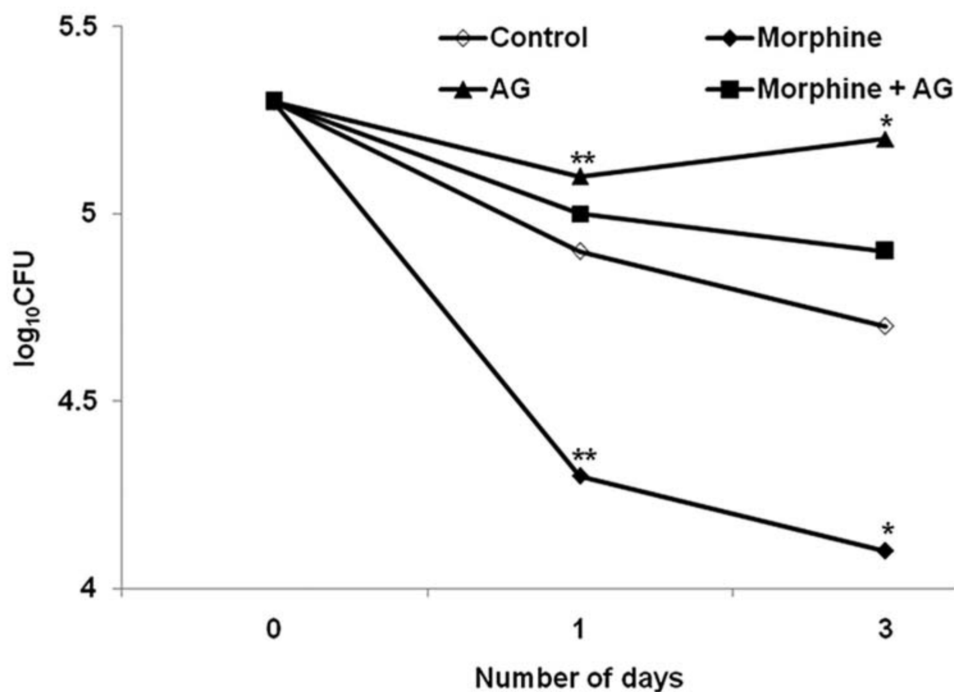

Fig. 4 Effect of AG on morphine-induced intramacrophage killing of *M. smegmatis*.
M. smegmatis-infected macrophages were incubated with 100 µM morphine in presence or absence of 100 µM AG and lysed on day 0, +1 and +3 to determine intramacrophage killing as described in Materials and methods. Error bars are not shown for clarity.
* $P<0.01$ as compared to control
** $P<0.05$ as compared to control

Table 2 Direct antimycobacterial activity of morphine

Treatment	% Growth inhibition ± SD
Control	0 ± 0.5
Isoniazid (100μM)	100.0 ± 5.4*
Morphine (1pM)	2.3 ± 0.4
Morphine (100pM)	3.7 ± 0.9
Morphine (10nM)	4.1 ± 0.5
Morphine (1μM)	1.6 ± 0.6
Morphine (100μM)	3.3 ± 0.9

The direct antimycobacterial activity of morphine was determined by broth dilution method. Log-phase cultures of *M. smegmatis* were incubated with indicated concentrations of morphine for 72 hours and growth was monitored spectrophotometrically at 600 nm and confirmed by plating on Middlebrook 7H10 medium.
* $P<0.01$ as compared to control

indirect immunomodulatory effects, respectively. Apart from the classical opioid receptors, atypical opioid receptors are also expressed by immune cells [24, 25].

Macrophages are important cellular target for opioids. Morphine can alter normal macrophage functions such as phagocytic activity and burst phenomena and may account for the immunosuppressive effects of morphine; however, morphine can also exhibit immunostimulant effects [10]. Macrophages are the key cells involved in resistance to *M. tuberculosis* and other related species. However, these bacteria reside and divide in the very cells that are responsible for their killing, i.e. macrophages. The intracellular killing of bacteria is achieved by production of NO by the enzyme nitric oxide synthase (NOS). NOS activation is, in turn, under the control of cytokines like interferon-γ and tumor necrosis factor-α. Morphine can alter the generation of NO through modulation of NOS activity [3, 8, 26] or cytokine production by immune cells [8, 10], leading to alteration in host susceptibility to infections. In the present study, the effect of dexamethasone (an immunosuppresant) and AG (NOS inhibitor; Fig. 4) on intramacrophage killing of *M. smegmatis* was studied. The intramacrophage killing of *M. smegmatis* by morphine was abrogated in presence of dexamethasone (data not shown) and AG (Fig. 4). Further, the intracellular killing was found to be dependent on the nitrite concentration in culture supernatants of morphine-treated, *M. smegmatis*-infected mouse peritoneal macrophages (data not shown). These results suggest that morphine can stimulate the macrophages, possibly involving induction of NOS, and result in reduced intramacrophage survival of *M. smegmatis*.

In conclusion, morphine can modulate the in vitro and in vivo survival of *M. smegmatis*. Previous reports on immunomodulatory activity of morphine have demonstrated the protective effects of low doses of morphine in experimental models of malaria [11], leishmania [8, 9] and TB [15]. However, relatively higher doses of morphine were found to be protective in *M. smegmatis* infection in vivo. Further, higher concentrations of morphine were required to activate *M. smegmatis*-infected macrophages. Hence, there can not be a fixed dose of morphine showing protective effect against infections. The protective dose may depend on the nature of infection and the immune status of the host. Further studies are required to elucidate the mechanisms responsible for differential effects of morphine in different infections.

Acknowledgments We are grateful to Prof. P. Ramarao, Director, National Institute of Pharmaceutical Education and Research (NIPER), for his help and encouragement. We thank Mr. Vijay Kumar Misra for excellent technical assistance. This is NIPER communication No. 436.

References

1. Risdahl JM, Khanna KV, Peterson PK and Molitor TW (1998) Opiates and infection. J Neuroimmunol 83:4–18
2. Sharp BM, Roy S and Bidlack JM (1998) Evidence for opioid receptors on cells involved in host defense and the immune system. J Neuroimmunol 83:45–56
3. Stefano GB, Cadet P, Fimiani C and Magazine HI (2001) Morphine stimulates iNOS expression via a rebound from inhibition in human macrophages: nitric oxide involvement. Int J Immunopathol Pharmacol 14:129–138
4. Vallejo R, de Leon-Casasola O and Benyamin R (2004) Opioid therapy and immunosuppression: a review. Am J Ther 11: 354–365
5. Mellon RD and Bayer BM (1998) Evidence for central opioid receptors in the immunomodulatory effects of morphine: review of potential mechanism(s) of action. J Neuroimmunol 83:19–28
6. Fuggetta MP, Di Francesco P, Falchetti R, Cottarelli A, Rossi L, Tricarico M and Lanzilli G (2005) Effect of morphine on cell-mediated immune responses of human lymphocytes against allogeneic malignant cells. J Exp Clin Cancer Res 24:255–263
7. Sheridan PA and Moynihan JA (2005) Modulation of the innate immune response to HSV-1 following acute administration of morphine: role of hypothalamo-pituitary-adrenal axis. J Neuroimmunol 158:145–152
8. Singh PP and Singal P (2007) Morphine-induced neuroimmunomodulation in murine visceral leishmaniasis: the role(s) of cytokines and nitric oxide. J Neuroimmune Pharmacol 2: 338–351
9. Singal P, Kinhikar AG, Singh S and Singh PP (2002) Neuroimmunomodulatory effects of morphine in *Leishmania donovani*-infected hamsters. Neuroimmunomodulation 10: 261–269
10. Singal P and Singh PP (2005) *Leishmania donovani* amastigote component-induced colony-stimulating factor produc-

tion by macrophages: modulation by morphine. Microbes Infect 7: 148–156

11. Singh PP, Singh S, Dutta GP and Srimal RC (1994) Immunomodulation by morphine in *Plasmodium berghei*-infected mice. Life Sci 54:331–339
12. Durante AJ, Selwyn PA and O'Connor PG (1998) Risk factors for and knowledge of *Mycobacterium tuberculosis* infection among drug users in substance abuse treatment. Addiction 93:1393–1401
13. MacGregor RR, Dunbar D and Graziani AL (1994) Tuberculin reactions among attendees at a methadone clinic: relation to infection with the human immunodeficiency virus. Clin Infect Dis 19:1100–1104
14. Peterson PK, Gekker G, Hu S, Sheng WS, Molitor TW and Chao CC (1995) Morphine stimulates phagocytosis of *Mycobacterium tuberculosis* by human microglial cells: involvement of a G protein-coupled opiate receptor. Adv Neuroimmunol 5:299–309
15. Singh RP, Jhamb SS and Singh PP (2008) Effects of morphine during *Mycobacterium tuberculosis* H37Rv infection in mice. Life Sci 82:308–314
16. Jhamb SS, Singh RP and Singh PP (2008) A comparison of conventional and radiometric methods for the assessment of antitubercular activity of drugs against *Mycobacterium tuberculosis* in mouse and macrophage models. Indian J Tuber 55:70–76
17. Lenaerts AJM, Gruppo V, Brooks JV and Orme IM (2003) Rapid in vivo screening of experimental drugs for tuberculosis using gamma interferon gene-disrupted mice. Antimicrob Agents Chemother 47:783–785
18. Kaufmann SH (2002) Protection against tuberculosis: cytokines, T cells, and macrophages. Ann Rheum Dis 61: ii54–ii58
19. Rawat M, Uppal M, Newton G, Steffek M, Fahey RC and Av-Gay Y (2004) Targeted mutagenesis of the *Mycobacterium smegmatis* mca gene, encoding a mycothiol-dependent detoxification protein. J Bacteriol 186:6050–6058
20. Kaur A, Kinhikar AG and Singh PP (2004) Bioimmunotherapy of rodent malaria: co-treatment with recombinant mouse granulocyte-macrophage colony-stimulating factor and an enkephalin fragment peptide. Tyr-Gly-Gly Acta Trop 91: 27–41
21. North RJ and Izzo AA (1993) Mycobacterial virulence. Virulent strains of Mycobacteria tuberculosis have faster in vivo doubling times and are better equipped to resist growth-inhibiting functions of macrophages in the presence and absence of specific immunity. J Exp Med 177:1723–1733
22. Pierce CH, Dubos RJ and Schaefer WB (1953) Multiplication and survival of tubercle bacilli in the organs of mice. J Exp Med 97:189–206
23. Sharp BM (2004) Opioid receptor expression and function. J Neuroimmunol 147:3–5
24. Navolotskaya EV, Kolobov AA, Kampe-Nemm EA, Zargarova TA, Malkova NV, Krasnova SB, Kovalitskaya YA, Zav'yalov VP and Lipkin VM (2003) Synthetic peptide VKGFY and its cyclic analog stimulate macrophage bactericidal activity through non-opioid beta-endorphin receptors. Biochemistry (Mosc) 68:34–41
25. Navolotskaya EV, Zargarova TA, Malkova NV, Zharmukhamedova TY, Kolobov AA, Kampe-Nemm EA, Yurovsky VV and Lipkin VM (2003) Macrophage-stimulating peptides VKGFY and cyclo (VKGFY) act through nonopioid beta-endorphin receptors. Biochem Biophys Res Comm 303: 1065–1072
26. Pacifici R, Minetti M, Zuccaro P and Pietraforte D (1995) Morphine affects cytostatic activity of macrophages by the modulation of nitric oxide release. Int J Immunopharmacol 17:771–777