Abstract

To investigate the effects of morphine treatment on immune function in neuropathic pain state, we studied the effects of both acute and chronic treatment of morphine on T lymphocyte function, by evaluating Concanavalin-A (ConA) and phytohemagglutinin (PHA) induced splenocyte proliferation, and on natural killer (NK) cell activity and, at the same time, the effects of morphine on thermal hyperalgesia by measuring paw withdrawal latency (PWL) in rats receiving chronic constriction injury (CCI) of the sciatic nerve as described by Bennett and Xie. All CCI rats developed thermal hyperalgesia while sham-operated rats did not. Thermal hyperalgesia was reversed dose-dependently after acute (single injection) and chronic (daily injection for 7 days) administration of morphine but persisted in saline-treated CCI rats. There is no significant difference between sham and saline-treated CCI groups in splenocyte proliferation and NK cell activity. Acute morphine treatment induced a significant suppression of NK cell activity and splenocyte proliferation induced by ConA and PHA. The suppression is dose-dependent as well. The reversal of the
thermal hyperalgesia persisted over the period of chronic treatment. No tolerance developed to the suppression of NK cell activity and splenocyte proliferation after chronic morphine treatment. These data suggest that both acute and chronic treatment of morphine reverse thermal hyperalgesia and inhibit the NK cell activity and splenocyte proliferation dose-dependently in rats with sciatic CCI, without concomitant development of tolerance. It is recommended that opioid therapy for chronic neuropathic pain should be used cautiously especially in immune-compromised cases.

**Key Words**: opioid, morphine; natural killer cell activity; splenocyte proliferation; immunity; chronic sciatic constriction injury (CCI); neuropathic pain.

1. **Introduction**

Chronic neuropathic pain is one of the most difficult challenges to pain specialists. To date, there are still no absolute effective therapeutic modalities available. Although using opioids to control neuropathic pain has proved to be effective both in animal and human (Rowbotham et al., 1991; Hassenbusch et al., 1995; Backonja et al., 1995; Dellemijn and Vanneste, 1997; McQuay, 1997; Dellemijn et al., 1998), long-term follow-up study in non-cancer neuropathic pain patients showed that predisposition to side effects was one of the major reasons for discontinuation of opioid therapy. Among the unwarranted side effects of nausea, vomiting, sweating, fatigue, difficult micturation, constipation, psychomimetic disturbance, and dependence are the immunosuppressive qualities, particularly those which affect cell-mediated immunity. Considerable data suggest that opioids may negatively influence immune competency in pain-free subjects (Beilin et al., 1992; Garza et al., 1994; Yeager et al., 1995; Nelson et al., 1997). However, the evidence that neuropathic pain may suppress immune function emphasized the need for aggressive control of pain state (Herzberg et al., 1994). Use of the opioids on neuropathic pain led to serious concern regarding their effects on immune function. Thus, the opioid, immune and pain interactions are potentially complicated and important. To investigate these interactions, we studied the effects of both acute and chronic treatment of morphine on T lymphocyte function, by evaluating Concanavalin-A (ConA) and phytohemagglutinin (PHA) induced splenocyte proliferation, and on natural killer (NK) cell activity and, at the same time, we evaluated the effects of morphine on thermal hyperalgesia by measuring paw withdrawal latency in rats receiving chronic constriction injury (CCI) of the sciatic nerve as described by Bennett and Xie (1988).

2. **Methods**

2.1 **Animals**

This study adhered to the Ethical Guidelines of the International Association for the study of Pain (Zimmermann, 1983). Male Sprague-Dawley rats, weighing between 250 - 300 g, were obtained from Animal Center of the National Cheng Kung University (Tainan, Taiwan). Animals were housed at 21 ± 0.5°C in wire-mesh cages, with free access to food and water. The vivarium was maintained automatically on a 12/12 h light/dark cycle with lights on at 7:00 AM. In all experiments, each experimental group consisted of six animals.

2.2 **Surgery**

The animals were anesthetized with sodium pentobarbital 60 mg/kg, intraperitoneal. An incision was made from the left sciatic notch to the distal thigh. The subcutaneous tissue was bluntly dissected under the skin to expose the biceps femoris
muscle. The sciatic nerve was freed from its investing fascia. Four ligatures (4-0 chromic) were placed approximately 1 mm apart around the sciatic nerve and tightened until the suture barely indented the nerve. The muscle and skin were then closed. To observe the effect of CCI-induced pain on immune reaction, a group of rats received sham surgery consisting of exposure of the sciatic nerve in the same way but without ligation in the left limb.

2.3 Paw withdrawal latency (PWL) test

The development of thermal hyperalgesia was assessed by measurement of PWLs to radiant heat according to the method of Hargreaves and colleagues (1988). A planter test apparatus was used (Ugo Basile, Comerio, Italy). Briefly, the rats were placed in a clear plastic chamber and left to acclimatize for at least 10 min before testing. The radiant heat from a high intensity lamp bulb, projecting through a round aperture and the base of the plastic box, was delivered to the plantar surface of the rat’s hindpaw. The time taken for the animal to withdraw its hindpaw was measured.

2.4 Pharmacological experiments

Morphine chloride was purchased from Narcotic Control Bureau of Taiwan. Six animals were used for each treatment group. PWLs (the mean of 3 consecutive stable values which did not differ more than 15%) were obtained one day before surgery (day 0) and the 4th day after surgery (day 5) to confirm the development of thermal hyperalgesia. On day 6, animal was administered subcutaneously (s.c.) with normal saline or morphine at doses of 5, 10, and 15 mg/kg respectively in the acute treatment groups, and PWLs were measured 4 hours after treatment. The animals were killed by cervical dislocation 60 min after treatment for immunological studies. In the chronic treatment groups, morphine was administered s.c. at the single daily dose 5, 10, or 15 mg/kg respectively, for 7 days. PWLs were evaluated on the 1st, 3rd, 5th and 7th day of the treatment (day 6, 8, 10 and 12 respectively). The animals were sacrificed 4 h after the last injection of the drug for immunological studies. In both acute and chronic treatment groups, control animals received the same volume of saline (1 ml) s.c. Saline was also injected in the sham-operated group.

2.5. Preparation of spleen cells

All spleens were aseptically removed and teased on a steel mesh immersed in chilled RPMI-1640 (GIBCO BRL, Grand Island, NY) in a plastic dish. The cells that passed through the mesh were washed twice with RPMI-1640. The erythrocytes were lysed with double distilled water, while the remaining cells were suspended in Dulbecco’s Modified Eagle medium (DMEM, GIBCO BRL) with 10% fetal bovine serum (FBS, GIBCO BRL) containing 2 mM glutamine (Sigma Chemical, St. Louis, MO), 100 IU/ml penicillin G, and 100 µg/ml streptomycin.

2.6. Cytotoxicity assay

A modified lactic acid dehydrogenase (LDH) release assay as described previously (Korzeniewski & Callewaert, 1985) was used in 4 h standard cytotoxicity assays. All cells were transferred into RPMI 1640 (GIBCO BRL, Grand Island, NY) medium without phenol red. Effector cells at various concentrations were incubated with 1 x 10^4 target YAC-1 cells (NK-sensitive cell line) in 96-well round-bottom microplates (Corning Glass Works, Corning, NY). An effector-to-target cell ratios of 5:1, 2.5:1, 1.25:1 or 0.625:1 were made by different dilution of 50 µl effector cells, 50 µl target
cells, or 100 µl culture medium in each well. After incubation, the plates were centrifuged at 800 g for 10 min. Aliquots of cell-free supernatant (100 µl) were transferred into corresponding wells of flat-bottom microplates (Nunc, RosRilde, Denmark). LDH release was determined as described previously (Mueller et al., 1989).

LDH release assay kit was purchased from Boehringer Mannheim (GmbH, FRG). The microplates were read at 490 nm on a Mutyskan photometer (MR 5000, Dynatech, McLean, Va.) To determine the LDH activity in these supernatants, add 100 µl reaction mixtures to each well and incubated for 30 min at room temperature. The percentage of specific lysis was calculated by the following formula:

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\%\text{ specific cytolysis} = \frac{(\text{test} - \text{spontaneous})}{(\text{maximal} - \text{spontaneous})} \times 100
\]

where test = OD released in the presence of effector cells, spontaneous = OD released from target cells added to culture medium alone, and maximal = OD released from target cells obtained by adding 150 µl 2% Triton X – 100 to 50 µl of target cell suspension. (Merck, Darmstadt, FRG).

All groups were tested in triplicate.

2.7. Mitogenic stimulation assay

The responses of spleen cells to mitogenic stimulation were performed as previously described (Korzeniewski and Callewaert, 1985). Spleen cells were suspended in DMEM containing 10% FBS, 2 mM glutamine and antibiotics and the final concentration of the spleen cells was 4 x 10^6 cells/ml for Concanavalin A (Con A, Sigma) and 6 x 10^6 cells/ml for phytohemagglutinin (PHA, Murex Biotech, Dartford, England). Each assay flat-bottom microtiter plates (Nunc) contained 100 µl diluted cells and 100 µl mitogen or 100 µl of culture medium. After 72 h of incubation, the cells were pulsed with 0.5 µCi of [³H] thymidine (6.7 Ci/mmol) (DuPont NEN, Boston, Mass.) per well for 5 h. The cells were harvested on glass fiber filters (Skatron, VA) with an automatic cell harvester (Cambridge, Watertown, Mass.). The radioactivity incorporated was assayed in a liquid scintillation counter (LS 5000 TA; Beckman, Fullerton, CA.).

2.8. Statistical analysis

Statistical analysis for NK cell activity and mitogen-induced cell proliferation was performed by two-way analysis of variance (ANOVA), followed by Bonferroni’s test. PWLs percent of day 0 value were analyzed using a one-way ANOVA for repeated measures followed by a post hoc Tukey’s test for multiple comparisons. Data are presented as mean ± SEM. A P value of less than 0.05 was considered significant.

3. Results

There were no significant differences in weights among the animals in this study. All rats with loose ligation of the sciatic nerve developed a neuropathic pain syndrome as described by Bennett and Xie (1988) and thermal hyperalgesia as of the 4th day following the surgery (day 5), which was indicated by a significant reduction in PWLs of CCI limb (P<0.001) when compared to preoperative (day 0) value, while sham-operated rats did not. Thermal hyperalgesia to radiant heat was still present postoperatively up to day 12 in the control (saline) group. (Fig. 1).

In both acute and chronic treatment groups, there were no intergroup differences in PWL at preoperative baseline (day 0)
Fig. 1. Dose-related reversal of paw withdrawal latency (PWL), expressed as % of day 0 value was shown. Preoperative values were obtained on day 0. Chronic constriction injury (CCI) of sciatic nerve was induced on day 1. Development of thermal hyperalgesia was confirmed on day 5 by the significant reduction of PWL \((p < 0.001)\). Values are means ± SEM. Drug was administered on day 6 daily for 7 days. PWL were evaluated on day 6, 8, 10, and 12. **\(p < 0.01\) vs. saline group.

Fig. 2: Effect of acute and chronic treatment of morphine at doses of 0, 5, 10, and 15 mg/kg on ConA-induced (vehicle, 1 and 2 µg/ml) splenocyte proliferation. Values are means ± SEM. **\(p < 0.01\) vs. saline group. #\(p < 0.001\) vs. saline group.

Fig. 3: Effect of acute and chronic treatment of morphine at doses of 0, 5, 10, and 15 mg/kg on PHA-induced (vehicle, 2 and 4 µg/ml) splenocyte proliferation. Values are means ± SEM. **\(p < 0.01\) vs. saline group. HP < 0.001 vs. saline group.

Fig. 4: Effect of acute (A) and chronic (B) treatment of morphine at doses of 0, 5, 10, and 15 mg/kg on natural killer cell activity (effector: target = 0.625:1, 1, 2.5, 5, and 10:1) incorporation (CPM). Values are means ± SEM. *\(p < 0.05\) vs. saline group. **\(p < 0.01\) vs. saline group. ***\(p < 0.001\) vs. saline group.
1.25: 1, 2.5: 1 and 5: 1) Values are means ± SEM. *P < 0.05 vs. saline group. **P < 0.01 vs. saline group. #P < 0.001 vs. saline group.

to the inhibition of ConA and PHA induced proliferation. Moreover, it inhibits the activity of NK cells, a subset of lymphocytes capable of mediating spontaneous cytotoxicity against tumor cells (Whiteside, 1989) and appear to have an important role in immune defense against viral infections (Bron, 1989). The immune effects of morphine are observed at lower dose; in fact, the effects on lymphocyte proliferation and NK activity are already evident at the dose of 5 mg/kg. It is shown that the reverse of thermal hyperalgesia of morphine in CCI neuropathic pain model is dose-dependent, so are immunosuppressive effects. This study suggests that morphine’s immunomodulatory effects are concordant with it’s reversal of thermal hyperalgesia both in acute and chronic treatment.

Considerable evidence suggests that opioids interact with the immune system. Opioid anesthesia suppresses natural killer cell activity (Beilin et al., 1992). Immunological dysfunction developed in heroin addicts (Brown SM et al., 1974). Chronic opioid administration results in depressed lymphocyte proliferative responses and reduced expression of cell surface antigens specific to T-lymphocytes and T-helper/inducer (CD4+) and T-suppressor/cytotoxic (CD8+) lymphocyte subsets in mice (Kimes et al., 1992). Morphine-exposed rats demonstrate suppression of NK cell cytotoxicity (Shavit et al., 1984). Morphine inhibits spontaneous and cytokine-enhanced natural killer cell cytotoxicity at doses within the range of analgesic use in volunteers (Yeager et al., 1995). However, these studies were all in pain-free subjects.

As a stressful stimulus, pain leads to a very complex response of the immune
In the study of Herzberg et al. (1994), CCI rats were used as an animal model of chronic mononeuropathic pain to investigate the immune function. Delayed-type hypersensitivity response was increased after sensitization to keyhole limpet hemocyanin, whereas the specific immunoglobulin production to the antigen was decreased. In the present study, ConA and PHA induced splenocyte proliferation was not significantly different from those of sham groups (no CCI). These findings were consistent with those of Dhabhar and McEwen's study (1996) in which splenocyte proliferation to the T-cell mitogens Con-A and PHA and the B-cell mitogen lipopolysaccharide showed no alteration between control and stressed rats. Stress-induced enhancement of antigen-specific cell-mediated immunity, but no effect on the immune reaction that does not involve an Ag-specific memory response. Experimental models of stress have been shown to affect different immune cell populations depending on stress (Wood et al., 1993). Moreover, experimental models of stress have been shown to affect different immune cell populations depending on stress intensity, duration, frequency etc. (Labeur et al., 1995).

In the present study, reverse of thermal hyperalgesia by morphine in CCI rats was dose-dependent, significantly greater than the saline group at dose of 10 mg/kg and reaching the nearly complete reverse at the dose of 15 mg/kg. The sensitivity to the drug of the immune parameters measured is different. The immunosuppressive effects of morphine are observed at lower dose of morphine compared to reverse of thermal hyperalgesia. In fact, the effects on PHA-induced splenocyte proliferation and NK cell activity are already evident (p < 0.05) at the dose of 5 mg/kg, whereas ConA-induced proliferation is suppressed significantly at dose of 10 and 15 mg/kg. This observation is not surprising, since a different sensitivity of NK cell and T lymphocytes to pharmacological treatments has been reported for opiate induced immunosuppression. This study suggest that immunomodulatory effects of morphine are dose dependency and not uniform across the compartments of the immune system (Lysle et al., 1993).

After long-term administration or continuous infusion of morphine in animals, tolerance to morphine antinociception develops rapidly in pain-free subjects. Adaptation of the immune system has been reported to the effects of pharmacological as well as stress stimuli (Bryant et al., 1988; Sacerdote et al., 1994). Tolerance also develops to the immunosuppressive effects of morphine. However, pain significantly attenuated the development of morphine tolerance to antinociception after morphine infusion (Ho et al., 1999). Morphine tolerance may not occur in rats treated with formalin injections into a paw model. Vaccarino and colleagues (1993) found that tolerance to morphine antinociception did not occur after repeated injection s.c. of high dose morphine in the presence of formalin-induced pain. Clinically, patients who receive morphine for persistent pain do not develop marked tolerance (Collin, 1993). In our study, morphine tolerance neither to thermal hyperalgesia nor to immunosuppressive reaction developed after morphine injection for 7 days, which is considered long-term for animal model.

The mechanism of morphine effects on immunomodulation is still not completely understood. Studies have suggested that morphine's immunomodulatory effects may not be mediated via mechanisms similar to
morphine’s antinociceptive effects. Central opioid pathways have proved to be involved in the immunosuppressive effects of morphine and these pathways may be distinct from those participating in opioid-induced analgesia and adrenal activation (Hernandez et al, 1993). Moreover, direct effects of morphine on the receptors of immune cells may play a role as well. Morphine might also act via non-opiate mechanisms on immune function (Peterson et al., 1993).

Opioid-immune interactions are potentially important. A better understanding of interactions between opioids and the immune system has important clinical implications. In treatment of neuropathic pain with opioids, it might need higher dosage relatively to non-neuropathic pain (Cherny et al., 1994). In our study, the data showed that the analgesic effects of morphine in neuropathic pain model are dose-dependent, and so is immune modulation but seem to be more sensitive to the immunosuppressive function. Differential sensitivity between analgesic effects and immunosuppressive effects may lead to adverse immunosuppression for reaching the maximal analgesic effects of opioids with no ceiling effect. Further clarification of the mechanisms responsible for the interaction between morphine’s immunologic and analgesic effects in neuropathic pain state would provide beneficial information in clinical setting. It is recommended that opioid therapy for neuropathic pain should be decided cautiously especially to immunocompromised patients. Whether suppressive immunological effects of morphine in neuropathic pain also apply in situations of acute, perioperative or chronic pain remains to be determined.

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References


