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Antinociceptive and immunosuppressive effects of opiate drugs: a structure-related activity study

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1 Although it is well known that morphine induces significant immunosuppression, the potential immunosuppressive activity of morphine derived drugs commonly used in the treatment of pain (codeine, hydromorphone, oxycodone) has never been evaluated.

2 We evaluated in the mouse the effect of the natural opiates (morphine and codeine) and synthetic derivatives (hydromorphone, oxycodone, nalorphine, naloxone and naltrexone) on antinociceptive thresholds and immune parameters (splenocyte proliferation, Natural Killer (NK) cell activity and interleukin-2 (IL-2) production).

3 Morphine displayed a potent immunosuppressive effect that was not dose-related to the antinociceptive effect, codeine possessed a weak antinociceptive effect and limited immunosuppressive activity; nalorphine, a μ-antagonist and κ-agonist, exerted a potent immunosuppressive effect, but had very weak antinociceptive activity. The pure κ-antagonist nor-BNI antagonized the antinociceptive, but not the immunosuppresive effect of nalorphine.

4 Hydromorphone and oxycodone, potent antinociceptive drugs, were devoid of immunosuppressive effects.

5 The pure antagonists naloxone and naltrexone potentiated immune responses.

6 Our data indicate that the C_6 carbonyl substitution, together with the presence of a C_{7-8} single bond potentiates the antinociceptive effect, but abolishes immunosuppression (hydromorphone and oxycodone).

7 The single substitution of an allyl on the piperidinic ring resulted in a molecule that antagonized the antinociceptive effect but maintained the immunosuppressive effect.

8 Molecules that carry modifications of C_6, the C_{7-8} bond and C_{14}, together with an allyl or caboxymethyl group on the piperidinic ring antagonized both the antinociceptive and the immunosuppressive effect of opiates and were themselves immunostimulants.

Keywords: Morphine; codeine; oxycodone; hydromorphone; naloxone; naltrexone; nalorphine; immunosuppression; analgesia

Introduction

For many years chemists and pharmacologists have tried to obtain a molecule that had the same or better antinociceptive activity of morphine accompanied by less side effects. The effort led to several new drugs, many of which were derived from slight modifications of the natural opiates morphine and codeine (Reisine & Pasternak, 1996). These drugs include the agonists hydromorphone and oxycodone, the oxidation products of morphine and codeine, respectively. In these drugs, the C_6 hydroxyl group of the native opiate has been substituted with a carbonyl group, and a single bond introduced at C_{7-8}, yielding an antinociceptive potency that exceeds that of original compounds. Naloxone and naltrexone, that carry an allyl or carboxymethyl substituent at the piperidinic ring of hydroxymorphone and an hydroxyl group at C_{14}, display a pure antagonistic effect. Finally, nalorphine, a morphine derivative with an allyl substituent at the piperidinic, but that retains the C_6 hydroxyl group, and the C_{7-8} bond unchanged, displays a mixture of agonist (on the κ receptor) and antagonist (on the μ receptor) properties.

The effects of these derivatives have been well documented as far as analgesia and respiratory depression are concerned (Rundlett Beyer & Elliot, 1976). However, great interest has recently been shown in the immunosuppressive effects induced by morphine (Bryant *et al*., 1988; Peterson *et al*., 1993; Carr *et al*., 1993; Carpenter *et al*., 1995; Fecho *et al*., 1996), since this could be relevant for the modulation of immune responses after both acute or chronic use of the drug, e.g. in the postoperative period, during treatment of chronic pain, or in the intake for abuse (Starec *et al*., 1993). The consequences of the immunosuppressive effect of the opiate could be an increased susceptibility to infections in the postoperative period, a possible lack of defences in cancer patients, and an increased susceptibility to HIV infection in drug abusers (Peterson *et al*., 1990). While these aspects of morphine pharmacology have been extensively studied in the last few years, very little or nothing is known of the effects of the other morphine congeners on immunity. Therefore, we thought it worthwhile to compare the effects of the acute administration of morphine, codeine, hydromorphone, oxycodone, nalorphine, naloxone and naltrexone on immune responses. To this end, after acute administration of the drugs to mice, we evaluated analgesia (hot-plate), concanavalin-A (Con-A)-induced proliferation of splenocytes, Natural Killer (NK) cell activity and interleukin-2 (IL-2) production by splenocytes.

Methods

Animals

Male Swiss mice (Charles River, Calco, Italy), 20–25 g body weight were used. Animals were housed at 22 ± 2°C, with a light:dark cycle of 14–10 h, food and water *ad libitum*. Each experimental group consisted of 8 animals. Different animals were used for the measurement of nociceptive thresholds and of immune parameters, in order to avoid the stressful effect of handling on immune parameters.

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Evaluation of the antinociceptive effect

Nociceptive thresholds were measured by the hot plate (54°C) method, as previously described (Bianchi *et al*., 1992) and the end point used was the licking of the hind paws. The results are expressed as percentage of the maximal possible effect (% MPE). MPE expresses the equation [(TL−BL)/ML−BL)]×100, where BL is the mean basal latency (8.0–10.0 s) measured before the first treatment was applied; TL is the test latency measured after treatments; ML is the maximal latency accepted (30 s), chosen in order to avoid tissue damage to the footpads.

Collection of cells

Spleen was removed by sterile procedures after cervical dislocation of animals, and cells were teased from the spleens by using 20-gauge sterile needles through an incision made in the spleen cuticle (Manfredi *et al*., 1993). Cells obtained from single spleens were checked for immune responses.

Splenocyte proliferation

Microcultures of splenocytes were set up (4×10^6 cells) in RPMI 1640, 10% FCS±Con-A (2.5 μg ml^{-1}, 5 μg ml^{-1}, 10 μg ml^{-1}). After 48 h incubation at 37°C, 1.0 μCi [^{3}H]-thymidine (specific activity 2 Ci mmol^{-1}, Amersham, U.K.) was added to all cultures. Eighteen hours later, cells were harvested by an automated cell harvester (Skatron) and radioactivity was measured in a liquid scintillation counter (Packard, Downers Grove, IL, U.S.A.). Background values, i.e. thymidine incorporation of resting cells, were subtracted from mitogen-induced proliferation (Manfredi *et al*., 1993).

The three concentrations of Con A were selected to provide sub-maximal as well as maximal stimulation of proliferation. The maximal proliferative response occurred at 5 μg ml^{-1} Con-A, and was selected for data analysis because it provided the most accurate measure of the overall proliferative responses of the lymphocytes. However, similar results were obtained at all mitogen concentrations, including sub-maximal concentrations.

Natural Killer cell activity

NK activity of splenocytes was evaluated by a 4 h ^{51}Cr release assay. Briefly, 5×10^6 YAC-1 cells (mouse lymphoblastoma cell line) were labelled by incubation with 100 μCi sodium chromate (Amersham, specific activity 250–500 mCi mg^{-1} chromium) in 0.2 ml of RPMI+10% FCS for 60 min at 37°C in 5% CO_2. After three washes, the YAC-1 cells were suspended in RPMI, 10% FCS at a concentration of 10^5 cells ml^{-1}. ^{51}Cr labelled YAC-1 cells were incubated with effector cells (splenocytes) in 96 well microtiter plates, at effector: target cell ratios (E:T) of 100:1 and 50:1. Each E:T ratio was tested in triplicate. Following an incubation of 4 h at 37°C in 5% CO_2, plates were centrifuged at $400\times g$ for 5 min, a 100 μl aliquot of supernatant was removed from each well, and counted in a Packard gamma counter (Sacerdote *et al*., 1994).

Maximum ^{51}Cr release and spontaneous release were determined in wells containing 3 M HCl or medium, respectively. Specific ^{51}Cr release was calculated according to the formula: 100×[(experimental release−spontaneous release)/(maximal release−spontaneous release)].

The E:T ratio of 100:1, at which the maximal killing occurred, was chosen for graphical presentation. However, the results obtained were identical also at an E:T of 50:1.

Measurement of interleukin-2

Spleen cells were adjusted to 4×10^6 cells ml^{-1} medium and incubated for 24 h with or without 10 μg ml^{-1} Con-A.

The levels of IL-2 in the supernatants were determined by enzyme linked immunosorbent assay (ELISA) protocol as standardized by Pharmingen (San Diego, California U.S.A.). Briefly, the anti IL-2 capture monoclonal antibody (mAb) was absorbed on a polystyrene 96 well plate and the IL-2 present in the sample was bound to the antibody coated wells. The biotinylated anti IL-2 detecting mAb was added to bind the IL-2 captured by the first antibody. After washing, avidin-peroxidase (Sigma) was added to the wells to detect the biotinylated detecting antibody and finally 2,2′-azino-bis (3-ethylbenthiazoline-6-sulphonic acid) (ABTS, Sigma) substrate was added and a coloured product was formed in proportion to the amount of IL-2 present in the sample, which was measured at OD 405 nm.

Drugs

The following opiate agonists and antagonists were used: morphine hydrochloride, codeine phosphate, nalorphine hydrochloride, oxycodone hydrochloride, naloxone hydrochloride, naltrexone hydrochloride (S.A.L.A.R.S., Como, Italy), hydromorphone hydrochloride (Dilaudid HP, Knoll Pharmaceuticals, Whippany, New Jersey), and the specific κ receptor antagonist non-binaltorphimine hydrochloride (nor-BNI; RBI, U.S.A.). Morphine.HCl (m. wt.321.8), oxycodone.HCl (m. wt. 351.8), nalorphine.HCl (m. wt. 347.8), hydromorphone.HCl (m. wt. 321.7), naloxone.HCl (m. wt. 363.8) and naltrexone.HCl (m. wt. 377.7) were administered at doses of 2.5, 5, 10, 20 mg kg^{-1}, s.c. Codeine.H_2PO_4 (m. wt. 397.4) at doses of 2.5, 5, 10, 20 and 100 mg kg^{-1} s.c., and nor-BNI (m. wt. 737.8) administered at a dose of 10 mg kg^{-1}, s.c., two hours before the administration of 10 mg kg^{-1} nalorphine for the evaluation of immunosuppression and 20 mg kg^{-1} of the opiate for antinociception. In the IL-2 experiments all drugs were used at the highest dose. All drugs were dissolved in sterile distilled water and administered subcutaneously, 60 min before the animals were killed, for the evaluation of immune parameters, or before the hot-plate test. The chemical structure of the drugs used in the study is presented in Table 1.

Statistical analysis

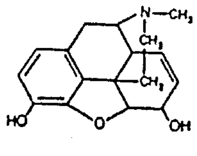
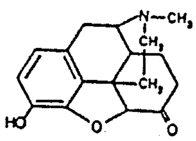
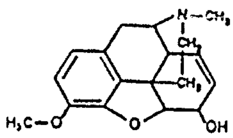
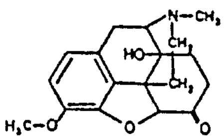
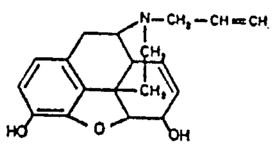
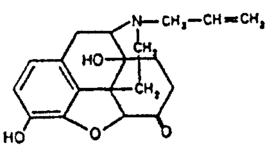
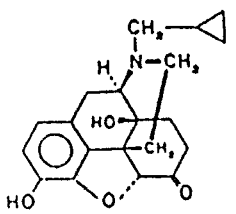
Statistical analysis for NK activity, cell proliferation and IL-2 production was performed by ANOVA, followed by Bonferroni test for multiple comparisons, while antinociceptive responses were evaluated by the Kruskall Wallis analysis of variance for non-parametric data.

Results

The upper panel of Figure 1 shows that morphine exerts an inhibitory effect on Con-A-stimulated lymphocyte proliferation and IL-2 production at dose of 20 mg kg^{-1}, and on NK activity at doses of 10 and 20 mg kg^{-1}, while the antinociceptive effect was present at all the doses tested and reached a maximal effect at the dose of 10 mg kg^{-1}. The lower panel of the figure shows that the morphine derivative hydromorphone, does not affect either lymphocyte proliferation, IL-2 production or NK activity. However, the antinociceptive potency of hydromorphone is greater than that of morphine, since the antinociceptive effect is already maximal at the lowest dose tested (2.5 mg kg^{-1}). The natural compound codeine, that differs from morphine for the Methyl$_3$ substitution, as expected, exerts a much weaker antinociceptive effect than morphine (Figure 2, upper panel), since it induces a similar antinociceptive effect only at the dose of 100 mg kg^{-1}. At the same dose, but not at lower doses, codeine retained only a partial immunosuppressive activity, since it induced a significant decrease of NK activity and IL-2 production, while it did not affect splenocyte proliferation. Similar to that observed for the morphine derivative hydromorphone, the codeine derivative oxycodone (Figure 2, lower panel) showed a greater antinociceptive effect than the parent drug, and a complete loss of effect on the immune system. Figure 4 shows that nalorphine is equally potent with morphine in reducing Con-A induced proliferation and IL-2 production, and it is even more potent than the parent opiate on the inhibition of NK activity, while it induces a weak antinociceptive activity only at the highest dose. The immune effects were not blocked by the κ receptor antagonist nor-BNI, that by itself is not active, while it blocks the weak antinociceptive effect (Figure 3, lower panel).

The substitution of the piperidinic methyl of hydromorphone with an allyl group (naloxone) or a cyclopro-

Table 1 Drugs used in the study and summary of their effects

	Nociceptive thresholds	Proliferation	Natural Killer	IL-2
 Morphine	↑	↓	↓	↓
 Hydromorphone (Dilaudid)	↑	↔	↔	↔
 Codeine	↑	↔	↔↓	↔↓
 Oxycodone (Percodan)	↑	↔	↔	↔
 Nalorphine (Nalline)*	↔↑	↓	↓	↓
 Naloxone (Narcan)	↔	↑	↔	↑
 naltrexone	↔	↑	↔	↑

↑ = increase; ↓ = decrease; ↔ = no effect; ↔↑ = slight increase; ↔↓ = slight decrease

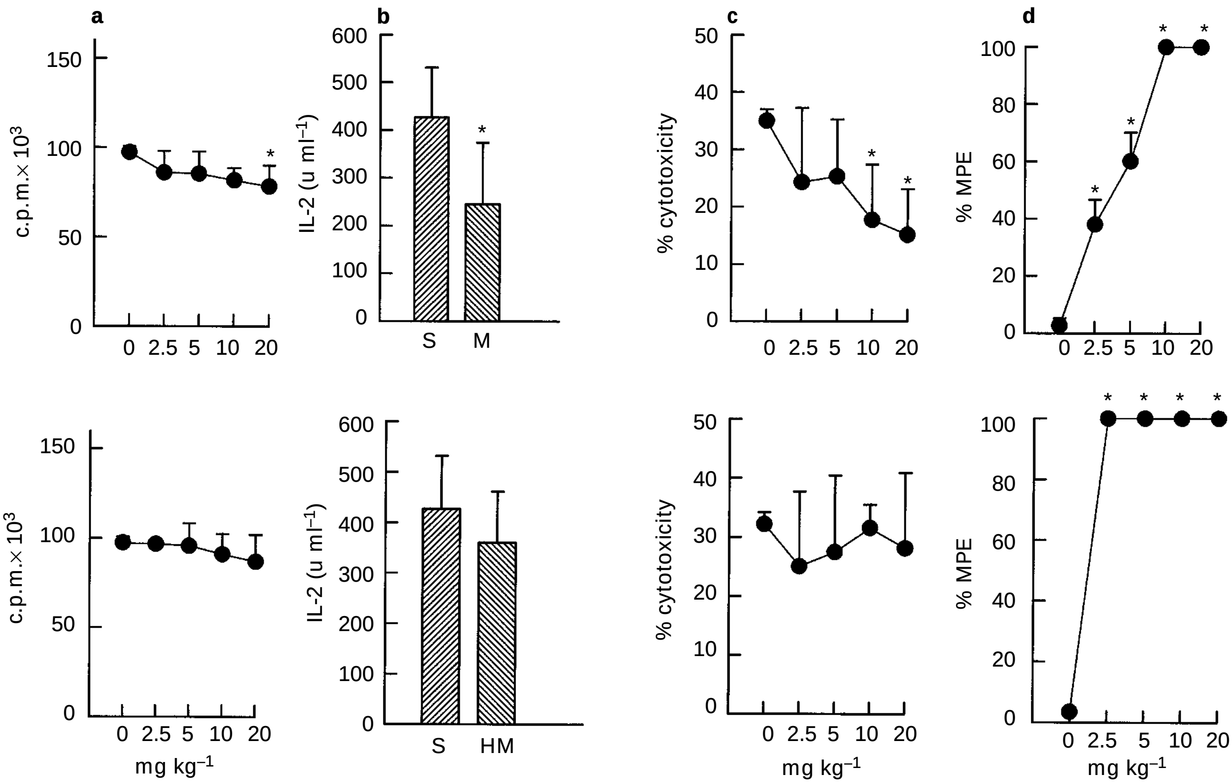


Figure 1 Effect of graded doses of morphine (upper panels) or hydromorphone (lower panels) on Con-A (5 μg ml^{-1})-induced splenocyte proliferation (a), stimulated IL-2 production (b), Natural Killer activity (effector:target cell ratio = 100:1) (c) and analgesic thresholds in the hot plate test (d). In (b) effect of morphine (M) or hydromorphone (HM) 20 mg kg^{-1} is shown compared to effect of saline (S). Values are mean and vertical lines show s.d.; *$P<0.01$ vs saline-control (0 mg kg^{-1}).

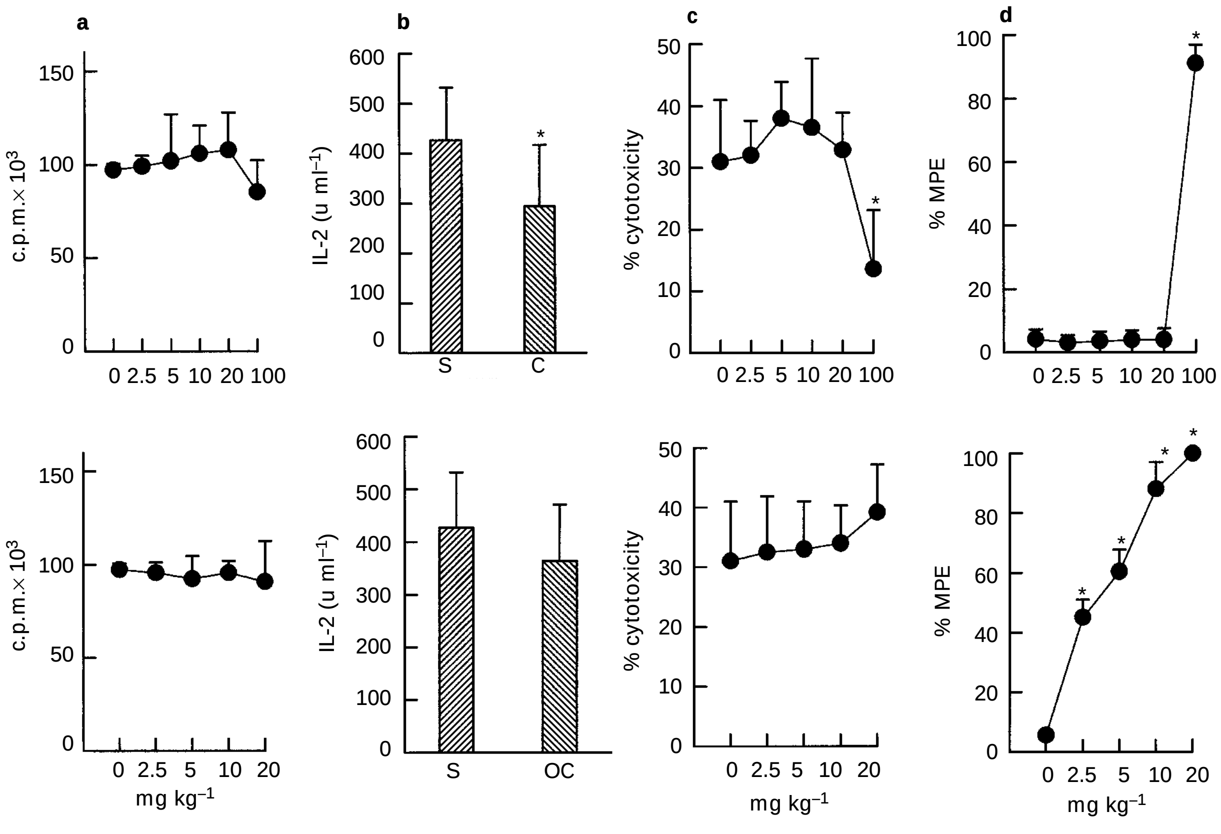


Figure 2 Effect of graded doses of codeine (upper panels) or oxycodone (lower panels) on Con-A (5 μg ml^{-1})-induced splenocyte proliferation (a), stimulated IL-2 production (b), Natural Killer activity (effector:target cell ratio = 100:1) (c) and analgesic thresholds in the hot plate test (d). In (b) effect of codeine (C) 100 mg kg^{-1} and oxycodone (OC) 20 mg kg^{-1} are shown compared to effect of saline (S). Values are mean and vertical lines show s.d.; *$P<0.01$ vs saline-control (0 mg kg^{-1}).

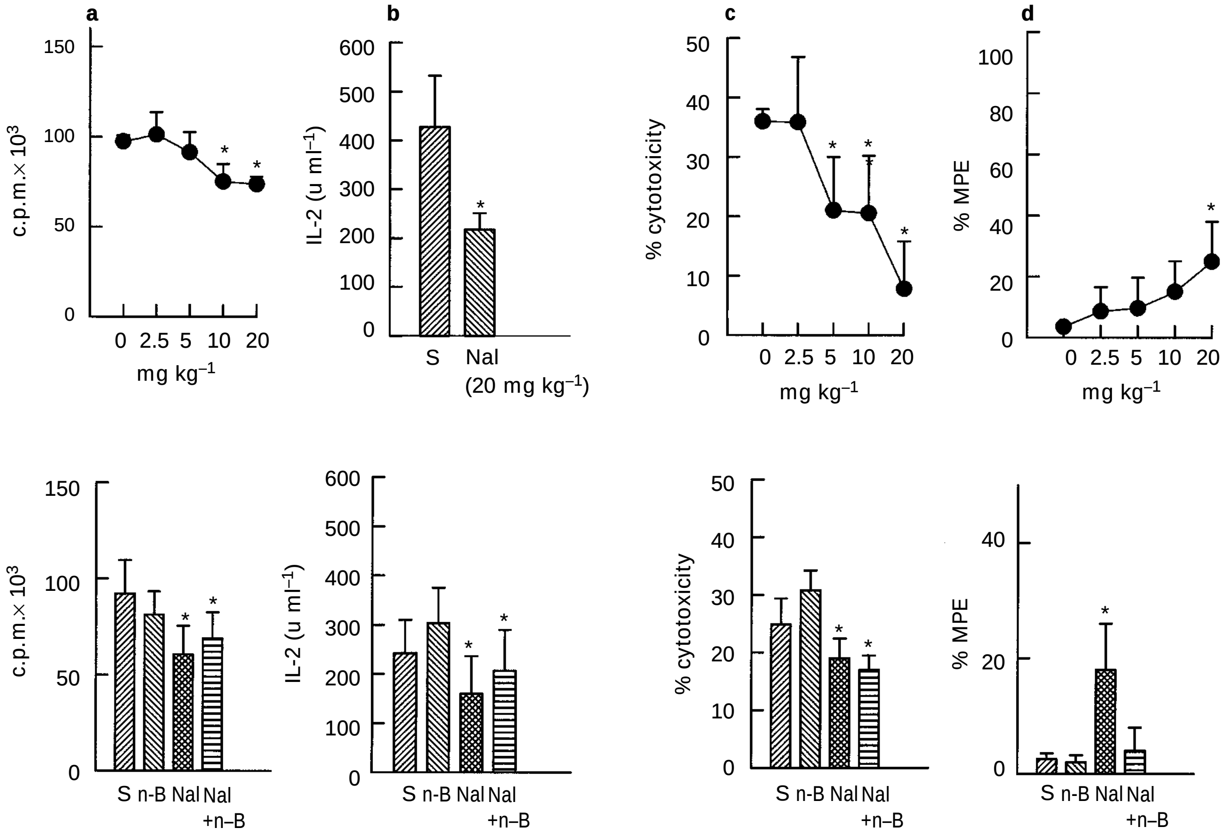


Figure 3 Effect of graded doses of nalorphine (Nal, upper panels) on Con-A (5 μg ml^{-1})-induced splenocyte proliferation (a), stimulated IL-2 production (b), Natural Killer activity (effector:target cell ratio = 100:1) (c) and analgesic thresholds in the hot plate test (d). The lower panels show the modulation by the κ-receptor antagonist nor-BNI (n-B, 10 mg kg^{-1}) of the effects of nalorphine (10 mg kg^{-1}). In the hot plate test, nalorphine was administered at a dose of 20 mg kg^{-1}. Values are mean and vertical lines show s.d.; $*P<0.01$ vs saline-control (S, 0 mg kg^{-1}).

pylmethyl group (naltrexone) and the introduction of a C_{14} hydroxyl completely antagonizes the antinociceptive effect of the parent compound (data not shown), while, as shown in Figure 4, it enhanced the Con-A stimulated proliferation and IL-2 production, without modifying NK activity.

Discussion

Our data show that the immunosuppressive effect of opiates is independent of the antinociceptive effect and that specific structural modifications of the morphine molecule can account for the difference.

Substitution of the C_3 hydroxyl group decreased the antinociceptive potency of the molecule, but only partially affected the immunosuppressive effect of the opiate, as it is exemplified by codeine, which, at doses equianalgesic to morphine, is able to decrease NK activity.

In contrast, the substitution with a carbonyl group at C_6, together with a single bond in C_{7-8} and an hydroxyl group at C_{14} increased the antinociceptive effect in comparison to the parent natural opiate, i.e. hydromorphone vs. morphine and oxycodone vs. codeine, but completely blocked the immunosuppressive effect. In fact, both hydromorphone and oxycodone do not affect lymphocyte proliferation, NK activity and also IL-2 production, at doses at which the antinociceptive responses are higher than those of morphine. It is well known that the substitution of the methyl group on the piperidinic ring with a larger alkyl chain, like in nalorphine, naloxone and naltrexone, leads to compounds that lack any anticociceptive effect and behave as antagonists. Nalorphine, that compared to morphine carries unchanged C_3 and C_6 groups, maintained a potent immunosuppressive effect on all the immune parameters that we studied, while it antagonized the antinociceptive effect of morphine due to the presence of the allyl substitution. The weak antinociceptive effect of nalorphine is known to be due to its agonistic effect on the κ-opiate receptor and in our hands this effect was blocked by nor-BNI. However, the immunosuppressive effect cannot be ascribed to such an interaction, since it is not affected by the specific κ receptor antagonist. Moreover, both naloxone and naltrexone, that, compared to morphine, carry the same modifications at C_6 of hydromorphone, plus the substitution on the piperidinic ring, do not induce immunosuppression, but on the contrary potentiate Con-A-induced proliferation and IL-2 production, but not NK activity. We previously demonstrated that this pattern of effects of the two antagonists on immune responses is due to their blockade of the tonic suppression exerted by the endogenous opioid, β-endorphin, on mitogen induced proliferation, but not on NK activity (Manfredi *et al.*, 1993; Panerai *et al.*, 1995).

From these data, it emerges that those molecules that carry the hydroxyl group at both C_3 and C_6 possessed the strongest immunosuppressive activity (morphine and nalorphine); modification at C_3 lead to the partial loss of immunosuppressive activity (codeine), while substitution with a carbonylic group at C_6 completely abolished the immunosuppression (hydromorphone and oxycodone). Finally, modification at the piperidinic ring in the absence of modifications at C_3 or C_6, such as in nalorphine, does not seem to be relevant for immunosuppression.

The reason for the dissociation of the antinociceptive and immunosuppressive effects of opiates remains elusive. All the compounds we tested, in fact, are thought to bind to the μ opiate receptor (Makman, 1994) and their antinociceptive

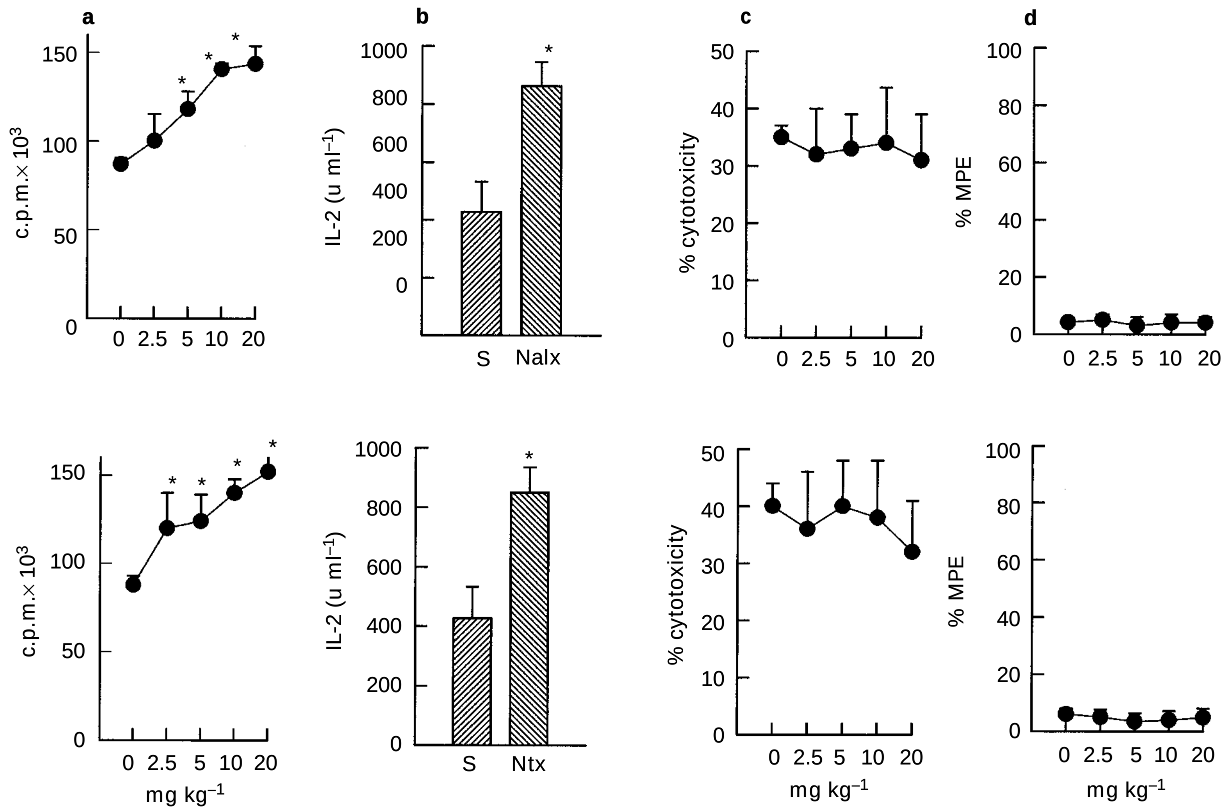


Figure 4 Effect of graded doses of naloxone (Nalx, upper panels) or naltrexone (Ntx, lower panels) on Con-A (5 μg ml^{-1})-induced splenocyte proliferation (a), stimulated IL-2 production (b), Natural Killer activity (effector:target cell ratio = 100:1) (c) and analgesic thresholds in the hot plate test (d). In the IL-2 experiment, both opiate receptor antagonists were administered at the dose of 10 mg kg^{-1}. Values are mean and vertical lines show s.d.; *$P<0.01$ vs saline-control (S, 0 mg kg^{-1}).

effect was reversed by classical μ opiate receptor antagonists. Consistently, the immunosuppressive effect of morphine can be reversed by μ opiate receptor antagonists (Carpenter *et al*., 1995). However, why the substitutions at C_6, C_{7-8}, and C_{14} potentiate the antinociceptive effect, while abolishing the immunosuppressive effect, remains to be determined.

In conclusion, our data open interesting aspects of opiate pharmacology. In fact, the use of potent antinociceptive drugs, like hydromorphone, that lack immunosuppressive effects, could be beneficial in many situations where a predisposition for opportunistic infections, e.g. in AIDS, or a diminution of tumour surveillance mechanisms, e.g. in cancer patients, may be contraindicated. Moreover, the existence of compounds like nalorphine or the development of novel opiate drugs, with no antinociceptive activity, but with immunosuppressive properties, may lead to new therapies in areas such as transplantation and autoimmune diseases.

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