Immunosuppression by δ-Opioid Antagonist Naltrindole: δ- and Triple μ/δ/κ-Opioid Receptor Knockout Mice Reveal a Nonopioid Activity

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ABSTRACT
The δ-opioid antagonist naltrindole has been shown to inhibit graft rejection in vivo and suppress allogeneic mixed lymphocyte reaction (MLR) in vitro, similarly to cyclosporin A. We investigated whether this action is mediated by δ-opioid receptors using both genetic and pharmacological tools. Naltrindole and two related compounds, 7-benzylidene-7-dehydronaltrexone and naltriben, inhibited MLR performed with lymphocytes from wild-type and δ-opioid receptor knockout mice, with comparable potency. Furthermore, these compounds suppressed the proliferation of spleen cells from triple δ/μ/κ-opioid receptor-deficient animals as well. Finally, the highly δ-selective, but structurally distinct, antagonist N,N-dimethyl-Dmt-Tic-OH and the general opioid antagonist naltrexone were inactive in the MLR assay. In conclusion, we demonstrate for the first time that the immunosuppressive activity of naltrindole and close derivatives is not mediated by any of the three cloned opioid receptors. Therefore, the postulated inhibitory activity of naltrindole in the graft rejection process is mediated by a target, which remains to be discovered.

Morphine and related opiate agonists are first line medication for moderate-to-severe pain (Mather and Smith, 1999). Opiate compounds also exhibit many other pharmacological activities (Vaccarino and Kastin, 2000), including the alteration of immune responses and there is evidence that the endogenous opioid system plays an important role in regulating immunity (Eisenstein and Hilburger, 1998). Opiate drugs and endogenous opioid peptides elicit their biological actions through three highly homologous G protein-coupled receptors, μ-, δ-, and κ (for review, see Kieffer, 1997), which are differentially implicated in opioid function.

Highly interesting was the observation that the prototypic δ-opioid antagonist naltrindole (Portoghese et al., 1988) reduces graft rejection in vivo and inhibits allogeneic mixed lymphocyte reaction (MLR) in vitro (Arakawa et al., 1993). In the latter assay, naltrindole showed a potency comparable to that of cyclosporin A, considered to be the most efficient immunosuppressant in the graft rejection process and used clinically for more than two decades (Bush, 1999). This striking finding brought naltrindole as a potential immunosuppressive agent in organ transplantation and prompted further characterization of this activity. Because the existence of two δ-receptor subtypes (δ1 and δ2) has been suggested by the pharmacology (Zaki et al., 1996), another study used naltrindole (δ1 and δ2), 7-benzylidene-7-dehydronaltrexone HCl (BNTX, δ1; Portoghese et al., 1992), and naltriben methanesulfonate (naltriben, δ2; Soffuolo et al., 1991). The results indicated that naltrindole and BNTX, but not naltriben, suppress several immune responses in vitro (House et al., 1995). In similar experiments, peptidic δ-antagonists proved little active (House et al., 1997). Another BNTX-related δ1-opioid receptor antagonist, 7-benzyspiroidanylnaltrexone, also prolonged renal allograft survival in the rat (Linner et al., 1998). Together, the data suggest that selective blockade of δ-receptors, mainly of the δ1 subtype, by nonpeptidic opioid compounds could inhibit the graft rejection process.

The purpose of this study was to clarify the molecular basis for naltrindole immunosuppressive activity in the MLR reaction, considered to be a well accepted in vitro model of T-lymphocyte response to allogeneic transplantation. In this assay, we have used novel genetic and pharmacological tools. We first have determined whether mice lacking the δ-opioid receptor (DOR) gene (Filliol et al., 2000), as well as mice lacking all three opioid receptor genes that we have generated (Kieffer, 1999; Simonin et al., 2001), respond to naltrindole, BNTX, and naltriben in the allogeneic MLR reaction.

ABBREVIATIONS: MLR, mixed lymphocyte reaction; BNTX, 7-benzylidene-7-dehydronaltrexone; DOR, δ-opioid receptor; MOR, μ-opioid receptor; KOR, κ-opioid receptor; IL-2, interleukin-2.
We also have examined the activity of a novel δ-selective antagonist, \( N,N \)-dimethyl-Dmt-Tic-OH, which displays a δ-selectivity far greater than naltrindole (Bryant et al., 1998; Lazarus et al., 1998). Finally, we have investigated the effect of naltrexone, a general opioid antagonist. Together, our results demonstrate that the immunosuppressive activity of naltrindole in the MLR reaction is not mediated by opioid receptors.

Materials and Methods

Knockout Mice. DOR-deficient mice (129 × C57BL/6 genetic background) were constructed by homologous recombination as described in Filiol et al. (2000). Tripotent \( \mu \) (MOR\( ^{\delta} \)) (KOR\( ^{\delta} \)) deficient mice were generated by interbreeding of single MOR (Matthes et al., 1996), DOR (Filiol et al., 2000), and KOR (Simonin et al., 1998) knockout mice (Simonin et al., 2001). Animals were maintained under standard housing conditions in a 12-h dark/light cycle with free access to food and water. Animal care was in accordance with ethical guidelines (National Institutes of Health, 1995; Council of Europe, 1996) and approved by a local ethical committee. Seven- to 13-week-old sex-matched animals were used in the experiments.

Concanavalin A-Induced Lymphocyte Proliferation. Spleocytes from five mice of each genotype were distributed into flat-bottom microtiter plates (5 × 10^5 cells/well) and stimulated with Concanavalin A (2 μg/ml, Sigma, St. Quentin Fallavier, France) for T-lymphocyte stimulation (Gavériaux-Ruff et al., 1998). Mitogen concentration was adjusted to provide maximal T-cell proliferation. Triplicate cultures in supplemented synthetic culture medium (Ultraculture; Biowhittaker, Verviers, Belgium) were pulsed with 0.5 μCi of [\(^{3}H\)]methyl-thymidine (PerkinElmer Life Science, Paris, France) after 48-h incubation at 37°C and harvested 16 h later. Incorporation of [\(^{3}H\)]methyl-thymidine was determined by filtration on a cell harvester (Inotech, Dottikon, Switzerland) and counting using a Packard beta counter (Rungis, France).

MLR. We used a one-way MLR assay. Responders, i.e., splenocytes (10^5 cells) from wild-type, DOR knockout, and triple knockout mice were cultured with allogeneic stimulators (4 × 10^3 mitomycin-C-treated BALB/c total spleen cells) for 5 days in flat-bottom 96-well plates in 200 μl of RPMI 1640 culture medium (supplemented with 10% heat-inactivated fetal calf serum, glutamine, and antibiotics, all from Life Technologies, Cergy Pontoise, France), in the absence or presence of a range naltrindole, BNTX, naltrexone (Sigma/RBI, Natick MA) or N,N-dimethyl-Dmt-Tic-OH concentrations. N,N-Dimethyl-Dmt-Tic-OH was a kind gift from Lawrence H. Lazarus (Peptide Neurochemistry, National Institute of Environmental Health Sciences, Research Triangle Park, NC). Cyclosporin A was from Sigma. IL-2 was obtained as a culture supernatant of a stable X63 transfectant (IG-BMC, Illkirch, France). Proliferation was assessed by [\(^{3}H\)]methyl-thymidine incorporation in the last 18 h of culture as described above.

Measurement of IL-2 Production in MLR. A range of naltrindole and cyclosporin A concentrations (0.03–50 μM and 0.3–300 nM, respectively) was added in the MLR at day 0 as described above. Cultures were harvested 24 h later, supernatants collected, and stored at −80°C until quantification of IL-2 by a standard enzyme-linked immunosorbent assay technique. Anti-mouse IL-2 (clone JES6-5H4) and biotinylated anti-mouse IL-2 (clone JES6-1A12) antibodies used for coating and detection were purchased from Immunotech (Coulter-Beckman, Roissy, France). Standard recombinant mouse IL-2 was from Calbiochem (Merck Eurolab, Strasbourg, France). Extravidin-alkaline phosphatase conjugate was from Sigma. Independent experiments on individual mice were performed in triplicate.

Statistical Analysis. Statistical analysis of results was performed with the unpaired Student's t test (Statview, Berkeley, CA).

Results

δ-Receptor Is Not Necessary for Naltrindole, BNTX, and Naltriben Immunosuppression in MLR. Because the expression of δ-receptors has been shown regulated on T lymphocytes following activation in vitro (Miller, 1996; Sharp et al., 1997), we first verified whether T-lymphocyte proliferation in the MLR assay could be affected by the absence of δ-receptors in the DOR mutant mice. We prepared splenocytes from wild-type and DOR-deficient mice and obtained similar numbers of cells from both genotypes (8 × 10^5 ± 0.8 × 10^5 cells/spleen in wild-type mice, 10 × 10^5 ± 0.8 × 10^5 cells/spleen in DOR knockout mice, \( P = 0.1 \)). We first compared proliferative responses of splenocytes in response to the T-cell mitogen concanavalin A. Cells from both mouse strains exhibited the same proliferation, as measured by [\(^{3}H\)]thymidine incorporation (142,374 ± 10,109 cpm for wild-type mice, 133,792 ± 25,964 cpm for DOR knockout mice, \( P = 0.77 \); data not shown). Furthermore, we exposed splenocytes from wild-type and mutant mice to allogeneic stimulator BALB/c cells (see Materials and Methods) in the MLR assay. Again, splenocytes from both genotypes displayed similar [\(^{3}H\)]thymidine uptake (Fig. 1). Together, these data indicate that the absence of δ-opioid receptors does not alter T-lymphocyte response in the MLR.

We then tested the activity of naltrindole, which prolongs rat survival following kidney transplantation and suppresses lymphocyte proliferation in a MLR assay using rat splenocytes (Arakawa et al., 1993). We also tested the closely re-
lated compounds BNTX and naltriben (Fig. 2). BNTX has been described as an immunosuppressive drug using in vitro B-cell and T-cell proliferation, as well as Natural Killer cell activity assays in mice (House et al., 1995). When splenocytes from wild-type mice were incubated with allogeneic cells, the three compounds inhibited T-cell proliferation in a dose-dependent manner (Fig. 3). IC_{50} values were comparable across compounds and were in the micromolar range (naltrindole, 5 µM; BNTX, 7 µM; naltriben, 10 µM), consistent with previous observations (Arakawa et al., 1993; House et al., 1995). This demonstrates that the immunosuppressive activity of naltrindole, reported earlier in a rat MLR assay, is also observed in mouse and shows that the mouse model is appropriate in our study. It also shows that the two other nonpeptidic δ-antagonists naltriben and BNTX exhibit a similar activity in this model of allogeneic stimulation in vitro, extending evidence for an immunosuppressive activity of these two compounds.

Most interestingly, naltrindole, BNTX, and naltriben also exhibited inhibitory activity on spleen cells from DOR-deficient mice, which was comparable to that of wild-type splenocytes (Fig. 3). This demonstrates that δ-receptors are not required and that naltrindole, BNTX, and naltriben compounds inhibit lymphocyte proliferation through another mechanism.

**Inhibitory Activity of Naltrindole, Naltriben, and BNTX Is Not Mediated by μ-, δ-, or κ-Opioid Receptors.** Although the three antagonists are considered among the best δ-selective compounds, the high concentrations that were needed to achieve half-maximal inhibition of T-cell proliferation suggest that the compounds may act via μ- or κ-opioid receptors, which could be present on lymphocytes (Sharp et al., 1998). Selective opioid compounds indeed have been shown to cross-react over several opioid receptors under some experimental conditions, and the issue of pharmacological selectivity is often a matter of debate (Kieffer, 1999). As an example, naltrindole has been suggested to exhibit κ-receptor-mediated antinociceptive activity (Takemori et al., 1992). We therefore tested whether any opioid receptor is involved in the inhibition of MLR. To this aim, we examined the activity of naltrindole on splenocytes from wild-type and triple MOR/DOR/KOR knockout mice. As in the previous experiment, naltrindole inhibited the MLR in a dose-depen-

**Fig. 2.** Structure of opioid antagonists used in the study. Naltrindole, BNTX, and naltriben are structurally related selective δ-opioids. Naltrexone is a nonselective opioid and N,N-dimethyl-Dmt-Tic-OH is the most selective δ-compound.

**Fig. 3.** Effects of several δ-selective opioid antagonists on MLR using splenocytes from wild-type and DOR knockout mice. Naltrindole, BNTX, naltriben, and N,N-dimethyl-Dmt-Tic-OH were added in the MLR coculture at day 0 and proliferation measured after 5 days. Proliferation of cells from wild-type (●) and DOR knockout (▲) mice are shown and values are mean ± S.E.M. of four independent experiments in triplicate (four mice for each genotype). There was no significant difference between responses in wild-type and mutant mouse splenocytes.

**Fig. 4.** Effects of naltrindole and naltrexone on MLR using splenocytes from wild-type and MOR/DOR/KOR triple knockout mice. Naltrindole and naltrexone were added in the MLR assay at day 0 and proliferation measured after 5 days. Proliferation of cells from wild-type (●) and triple MOR/DOR/KOR knockout (▲) mice are shown and values are mean ± S.E.M. of four independent experiments in triplicate (four mice for each genotype). There was no significant difference between responses in wild-type and mutant mouse splenocytes.
view, see Bryant et al., 1998). These peptides exhibit antagonist activity, with an unusual high selectivity toward the δ-receptor. Among the numerous derivatives that have been synthesized and tested, the N,N-dimethyl-Dmt-Tic-OH peptide (Fig. 2) shows a 20,000-fold selectivity for δ versus μ-opioid receptor (Salvadori et al., 1997), while naltrindole, BNTX, and naltriben are in the 100- to 1000-fold range only (Raynor et al., 1994). This compound exhibits structural features that differ from naltrindole, BNTX, and naltriben. N,N-Dimethyl-Dmt-Tic-OH was assayed in MLR at concentrations ranging from 3 nM to 30 nM in both wild-type and DOR-deficient mice. It showed no inhibitory activity in any of the two splenocyte preparations (Fig. 3). This result shows that selective blockade of δ-receptors with a compound structurally distinct from the previously used antagonists has no influence on allogeneic T-cell proliferation.

In correlation to our findings on triple opioid receptor mutant mice, we also tested whether pharmacological blockade of all three opioid receptor subtypes using the nonselective antagonist naltrexone would impair the MLR. We tested the compound on splenocytes from wild-type and the triple knockout mutant mice and found no change in T-cell proliferation in response to allogeneic cells exposure, at concentrations up to 100 μM (Fig. 4). The other commonly used general opioid antagonist naloxone was also inactive in this assay (data not shown). Together, these data show that both the highly δ-selective antagonist N,N-dimethyl-Dmt-Tic-OH and the general opioid antagonists have no measurable immunosuppressive activity in the MLR assay, under conditions where naltrindole, BNTX, and naltriben do. This suggests that the three related compounds naltrindole, BNTX, and naltrindole act via a specific and nonopioid mechanism.

Naltrindole Inhibits MLR by an IL-2-Dependent Pathway. To further probe the mechanism of naltrindole immunosuppression in the MLR, we tested whether naltrindole activity is due to a specific immune-mediated mechanism rather than a general cytotoxic effect. Cyclosporin A is the prototypic graft rejection suppressor and potently inhibits the MLR in vitro. Cyclosporin A is known to suppress T-cell proliferation in the MLR by inhibiting IL-2 synthesis and release, which follows exposure to allogeneic cells (Bunjes et al., 1981). Therefore, cyclosporin A-induced suppression of MLR can be reversed by the addition of IL-2 in the assay. We tested whether IL-2 could also reverse naltrindole-induced inhibition of T-cell proliferation in the MLR assay. Results are shown in Fig. 5. In the absence of IL-2, both naltrindole and cyclosporin A decreased lymphocyte proliferation. Cyclosporin A abolished cell proliferation at 0.2 μM concentration, as previously reported (Arakawa et al., 1993). Naltrindole dose-dependently inhibited lymphocyte response at 10, 30, and 100 μM concentrations, as in our previous experiments (Figs. 3 and 4). Cyclosporin A-induced inhibition was reversed by IL-2 at a concentration of 2 ng/ml, as expected. Under those conditions, IL-2 also partially reversed naltrindole-induced inhibition of MLR.

Finally, we tested whether naltrindole inhibits IL-2 production. We measured IL-2 in the supernatant of MLR cultures performed in the absence or presence of naltrindole or cyclosporin A as a positive control. As expected, cyclosporin A suppressed IL-2 production with an IC50 of 13 ± 2.8 nM (Table 1). Naltrindole also decreased IL-2 production at concentrations similar to those inhibiting lymphocyte proliferation (IC50 of 18 ± 0.88 μM; Table 1). Together, these data indicate that naltrindole inhibits lymphocyte proliferation in the MLR, partially at least, via an IL-2-dependent mechanism.

Discussion

Opiate antagonists have been developed in the past as pharmacological tools, to selectively block the action of opioid agonists at μ-, δ-, and κ-opioid receptors and clarify the role of each receptor in opioid physiology. They also are used clinically to reverse heroin overdose. Interestingly, opioid antagonists have been shown to exhibit pharmacological properties per se, indicating that blockade of endogenous opioid activity could influence the physiology and be of medical interest. As an example, the general opioid antagonists

![Fig. 5](https://via.placeholder.com/150.png)

**Fig. 5.** Reversal of naltrindole- and cyclosporin A-induced inhibition of MLR by IL-2. A representative experiment using spleen cells from a wild-type mouse is shown. Values represent incorporated [3H]thymidine. Each column shows the mean ± S.E.M. of triplicate wells. IL-2 added alone in the MLR assay at 2 and 8 ng/ml produced a high [3H]thymidine uptake of 102,867 and 138,300 cpm, respectively (NTI, naltrindole; CsA, cyclosporin A; UnS, unstimulated; S, stimulated).

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Effect of naltrindole and cyclosporin A on IL-2 production in mixed lymphocyte reaction.

Mixed lymphocyte reaction was performed in the absence or presence of naltrindole or cyclosporin A and IL-2 was measured in cell supernatant after 24 h of culture using an enzyme-linked immunosorbent assay technique as described under Materials and Methods. Data are means ± S.E.M. of three independent experiments performed in triplicate.
naloxone and naltrexone have proved efficient in reducing cocaine euphoria, as well as alcohol dependence and relapse in humans (for review, see van Ree et al., 1999). In this context, selective δ-antagonists may also be promising compounds in the treatment of addiction to opiates, as well as nonopioid drugs of abuse (Dondio et al., 1997). Another aspect of the activity of δ-antagonist compounds, which has been investigated for many years, is immunosuppression. The finding of a potent inhibitory activity of naltrexone in allograft rejection is particularly intriguing, and this activity is also found in the in vitro MLR assay, which reflects allogeneic T-cell response in vivo. The purpose of our study was to clarify the mechanism of naltrexone action in this assay, and our data demonstrate that the suppressive activity of naltrexone is nonopioid in nature. Our conclusion is based on two lines of evidence. We show that 1) the immunosuppressive activity of naltrexone, BNTX, and naltiben is maintained in mice lacking either µ-, δ-, and κ-receptors; and that 2) a highly δ-selective antagonist (N,N-dimethyl-Dmt-Tic-OH), as well as a general opioid antagonist (naltrexone) show no biological activity in this assay. Our study demonstrates for the first time a nonopioid activity for the prototypic δ-antagonist naltrexone and its close derivatives BNTX and naltiben.

The suppression of MLR by δ-opioid antagonists clearly appears dependent on the chemical nature of the compounds. This activity has been described previously for naltrexone (Arakawa et al., 1993) and confirmed here. In this study we have also observed similar activity for the two closely related compounds BNTX and naltiben. Earlier reports have indicated immunosuppressive activity for BNTX using several other in vitro assays of immune function (House et al., 1995). In these assays, naltiben was little active and peptidic-type opioid antagonists, such as TIPP (Tyr-Tie-Phe-Phe-OH) and ICI174864, were almost ineffective (House et al., 1997). In our MLR assay, the pseudodipeptide N,N-dimethyl-Dmt-Tic-OH showed no inhibitory activity. Therefore, together with previous reports, our data suggest that a common determinant in the chemical structures of naltrexone, BNTX, and naltiben does confer an immunosuppressive activity that 1) is specific to those compounds, 2) is absent in peptidic-type antagonists, and 3) is not mediated by opioid receptors.

The mechanism for the nonopioid immunosuppressive activity of naltrexone in the MLR remains to be determined. We suggest that structural determinants of the indole moiety of naltrexone, of the corresponding benzofuran group in naltiben, and of the benzylidine substituent in BNTX (Fig. 2), which are absent in naltrexone, could be critical in allogeneic MLR suppression. The N,N-dimethyl-Dmt-Tic-OH compound would not contain this nonopioid pharmacological determinant and would be inefficient in the MLR. Our finding that naltrexone inhibits IL-2 production suggests that a specific interaction of naltrexone, and its close derivatives, with an unknown target impairs allogeneic-evoked IL-2 production in T lymphocytes. Naltrexone may act by other mechanisms as well, such as the modulation of other cytokine production, apoptosis, or cell cycling. Further investigations should clarify the molecular basis for this nonopioid activity of the prototypic δ-antagonist naltrexone.

Finally, this nonopioid immunosuppressive activity is not detected for the general opioid antagonists naloxone and naltrexone in our assay. These compounds have been studied previously for their action on graft rejection in vivo and results have appeared variable. Naltrexone has been reported to increase grafted cardiac tissue survival and inhibit MLR in vitro (Li et al., 1998), whereas naloxone decreased skin graft survival (Sacerdote et al., 1998). Together with our data, this suggests that general opioid antagonists may influence allogeneic reactions in vivo by opioid mechanisms, but that this is not necessarily detectable in the MLR assay in vitro. In fact, one cannot exclude that opioid and nonopioid mechanisms do contribute to naltrexone-suppressive activity after organ transplantation in vivo. The activation of δ-receptors has been reported to enhance or inhibit immune cell functions (Shahabi and Sharp, 1995; House et al., 1996; Kowalski, 1998) and it is likely that, in vivo, naltrexone could inhibit graft rejection both by blocking an endogenous δ-receptor tone and via the nonopioid mechanism reported here.

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References


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