The opioid antagonist naloxone induces a shift from Type 2 to Type 1 cytokine pattern in BALB/cJ mice

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Opioid peptides affect different immune functions. We present evidence that these effects could be mediated by the modulation of Th1/Th2 cytokine production. BALB/cJ mice were immunized with 50 or 100 µg of the protein antigen keyhole-limpet hemocyanin (KLH), and treated acutely or chronically with the opioid antagonist naloxone. One and 2 weeks after immunization, the production of cytokines by splenocytes was evaluated by in vitro restimulation with KLH. The acute and chronic treatment with the opioid receptor antagonist naloxone decreased the production of interleukin (IL)-4 by splenocytes of BALB/cJ mice. In contrast, IL-2 and interferon-γ levels increased after naloxone treatment. Finally, the opioid antagonist diminished the serum immunoglobulin G anti–KLH antibody titers. These results suggest that naloxone increases Th1 and decreases Th2 cytokine production. The effect of naloxone could be ascribed to the removal of the regulatory effects exerted by endogenous opioid peptides, which could therefore activate Th2 and suppress Th1 cytokines. (Blood. 2000;95:2031-2036)

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with the use of 20-gauge sterile needles through an incision made in the
spleen capsule, centrifuged, and washed twice in Hanks balanced salt
solution. Cells were suspended in RPMI supplemented with 10% fetal calf
serum (FCS), 1% glutamine, 2% antibiotics, 10 mmol/L Hepes, and 50
mmol/L 2-ME (all from Sigma) and were plated at 7 \times 10^6 cells in 24-well
plates containing a final concentration of 80 µg/mL KLH in a total volume
of 1 mL. Plates were incubated at 37°C in 5% CO₂ and 95% air.
Supernatants were collected after 48 and 72 hours in culture and
stored frozen at −80°C for cytokine analysis. The concentration of 80 µg/mL
used in vitro was chosen on the basis of previous pilot experiments. This
concentration, in fact, induced an easily measurable, but not maximal,
stimulation of cytokine production, in order to be able to detect a possible
stimulation as well as any inhibition induced by naloxone treatment.

Measurement of Interleukin-2, interleukin-4, and interferon-γ

The levels of IL-2 in 48- and 72-hour supernatants were determined by
enzyme-linked immunosorbent assay (ELISA) protocol as standardized by
Pharmingen (San Diego, CA). Briefly, the anti–IL-2 capture monoclonal
antibody (mAb) 1(µg/mL) 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 (0.5 µg/mL) 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 (3ethylbenzthiazoline-6-sulfonic acid) (ABTS,
Sigma) substrate was added, and a colored product was formed in
proportion to the amount of IL-2 present in the sample, which was
measured at optical density 405 nm.

IL-4 production was measured in 48- and 72-hour supernatants with the
use of the ELISA protocol outlined above with mAb anti–IL-4 at the same
concentrations used for IL-2. IFN-γ was also evaluated with the same
ELISA protocol except for the use of anti–IFN-γ capture and detecting
antibody at 2 µg/mL and 1 µg/mL, respectively (all mAbs were from
Pharmingen).

Anti–keyhole-limpet hemocyanin antibody enzyme-linked
immunosorbent assay

Blood was collected at the time of sacrifice, and sera were stored at −20°C.
Plates were coated overnight with 10 µg/mL KLH in a carbonate coating
buffer, pH 9.6. Mice sera were diluted 1:25, 1:50, and 1:100 in phosphate-
buffered saline (PBS)/Tween containing 1 mol/L NaCl and incubated for 3
hours at 37°C. Alkaline-phosphate–conjugated goat anti-mouse IgG (chain
specific, Sigma), diluted 1:6000 in PBS/Tween, or anti-mouse IgG (chain
specific, Sigma), diluted 1:6000 in PBS/Tween, was then added, and plates
were incubated overnight at 4°C. After washing, p-nitrophenyl-phosphate
substrate at 1 mg/mL in carbonate buffer was added, and the colored
product formed was measured at OD 405 nm. Serum from nonimmunized
mice served as control.

Statistical analysis

Cytokine data were analyzed by means of 2-way analysis of variance
(ANOVA), with treatments and hours of culture, doses of KLH, and time of
immunization as factors, followed by a Tukey test for multiple comparis-
ons. Acute and chronic saline groups were treated as a single group. Serum
antibody titers were analyzed by 2-way ANOVA, with treatments and
serum dilution as factors.

Results

Keyhole-limpet hemocyanin–stimulated cytokine production

The stress linked to simple manipulation and handling of animals
did not modify cytokine secretion, since levels of all cytokines did
not differ in acutely or chronically saline-injected mice, in compari-
son with mice that were not further injected after KLH immuniza-
tion (data not shown). Moreover, since no difference was observed
in acute or chronically saline-treated animals, in the statistical
analysis they were considered as a single group. Naloxone treat-
ment induces significant changes. Figure 1 reports the effect of the
acute and chronic treatment with naloxone on IL-2 production by
splenocytes.

In the acute experiments, animals were treated with 1 injection
of naloxone or saline at the moment of in vivo immunization with
50 or 100 µg KLH, and 7 or 14 days later, splenocytes were
cultured in vitro with or without KLH for 48 and 72 hours. In the
chronic experiments, animals were treated daily with naloxone or
saline from the day of immunization to the day of sacrifice. With
respect to the levels of IL-2 in all of the in vitro KLH-stimulated

cultures, the 2-way ANOVA on data revealed a main effect of both acute and chronic treatment (F2,300 ≈ 37.2, P < .001), with no significant difference between 48- and 72-hour cultures. Also, no significant difference was observed between 50 and 100 µg KLH (F1,300 = 0.6, P = .4) or between 1 and 2 weeks of immunization (F1,300 = 0.83, P = .36). The effect of naloxone treatments on spontaneous IL-2 production was still significant, although less evident (F2,300 = 4.54, P = .011). Moreover, in the saline group, the IL-2 levels were lower in 72-hour culture than in 48-hour culture (Tukey, q = 2.83, P < .05). In terms of the results in detail, when animals were immunized in vivo with 50 µg KLH, 1 week after immunization no effect of naloxone treatment was observed in the 48-hour cultures (Figure 1A); however, in the 72-hour supernatant, a significant increase in KLH-stimulated production was present after acute treatment with naloxone (Figure 1B). After 14 days of immunization with 50 µg KLH, a significant increase of IL-2 in KLH-stimulated cells was observed in 48-hour (Figure 1C) and 72-hours cultures (Figure 1D) after chronic naloxone treatment. The production of this cytokine showed an increase after naloxone treatment in the animals immunized with 100 µg KLH: 1 week after immunization, the enhancement of IL-2 production was evident in the 48-hour cultures after acute naloxone treatment in the unstimulated cultures and after both treatments in the stimulated ones (Figure 1E), while an increase was present after acute naloxone treatment in the 72-hour stimulated supernatant (Figure 1F). At 14 days after immunization, the increase was present after chronic naloxone treatment in KLH-stimulated cells in the 48-hour cultures, while in the 72-hour cultures the effect of chronic naloxone was also present in the unstimulated cell cultures (Figure 1G, 1H).

Independently of treatment, IFN-γ levels were higher in the 72-hour than in the 48-hour cultures (spontaneous secretion: F1,317 = 175.3, P < .001; KLH stimulated: F1,317 = 10.123, P < .001). Naloxone treatments induce a significant increase of IFN-γ levels in the in vitro KLH-stimulated cultures (F2,317 = 10.1, P < .001). Moreover, an interaction between the dose of KLH used for immunization and naloxone effect was evident, as the treatments were effective only in the animals immunized with 100 µg KLH (F2,317 = 11.26, P = .001). In fact, in the animals immunized with 50 µg KLH, the treatment with naloxone did not affect the production of IFN-γ (Figure 2A-2D). On the contrary, in the 100 µg KLH–immunized animals, a significant increase of the cytokine was present. When IFN-γ was measured 7 days after immunization, a significant enhancement was evident in both the 48- and the 72-hour supernatant after acute treatment with naloxone for unstimulated as well as in vitro KLH-stimulated cultures (Figure 2E, 2F). At 2 weeks after immunization with 100 µg KLH, in 48-hour stimulated cultures a significant increase of IFN-γ was observed after chronic naloxone treatment in unstimulated cells, and after both acute and chronic naloxone treatment in KLH-stimulated cells (Figure 2G). In the 72-hour culture, an increase after chronic naloxone treatment in KLH-stimulated cells was evident (Figure 2H).

Independently of treatment, the production of IL-4 was barely detectable in the 48-hour cultures (data not shown). Figure 3 reports the levels of IL-4 in the 72-hour cultures. The levels of IL-4 in the unstimulated cultures were also very low. The effect of naloxone treatment on KLH-stimulated IL-4 production is indeed opposite to the one observed on IL-2 and IFN-γ. A significant main effect of naloxone is in fact present (F2,151 = 38.29, P < .001), since both acute and chronic naloxone decrease IL-4 production.

Figure 2. Effect of acute and chronic naloxone treatment on KLH-stimulated IFN-γ production by splenocytes. Animals were immunized with either KLH (panels A,B,C,D) or 100 µg KLH (panels E,F,G,H) and killed after 1 week (panels A,B,E,F) or 2 weeks (panels C,D,G,H). Splenocytes were cultured with 0 or 80 µg/ml KLH for 48 h (panels A,C,E,G) and 72 h (panels B,D,F,H). Acute saline control is represented by white bars; chronic saline control is represented by right-slanted striped bars; acute naloxone treatment is represented by left-slanted striped bars; chronic naloxone treatment is represented by hatched bars. Results are expressed as mean ± SE. * = P < .01 versus corresponding saline controls.

The effect is present with both doses of in vivo KLH and is present 1 week as well as 2 weeks after immunization.

Keyhole-limpet hemocyanin antibody response

KLH IgM serum titers are reported in Figure 4. The IgM titers were higher (F1,71 = 8.21, P = .006) in the groups of animals immunized with 100 µg KLH (panels A,B,D) or 100 µg KLH (panels E,F,G,H) and killed after 1 week (panels A,B,E,F) or 2 weeks (panels C,D,G,H). KLH IgM serum titers. * = P < .01 versus corresponding saline controls.
immunized with 50 µg KLH (Figure 5A), the titers of anti-KLH IgG were lower than in the animals treated with 100 µg (Figure 5B) \( (F_{1,83} = 5.908, \ P = .003) \); moreover, higher titers were present 2 weeks after immunization (Figure 5C, D) in comparison with 1 week after (Figure 5A, B) \( (F_{1,83} = 30.17, \ P < .001) \).

A significant overall effect of naloxone treatment on IgG was present \( (F_{2,251} = 10.29, \ P < .001) \) (Figure 5). The post hoc comparison indicated that the antibody titers were in fact reduced in the animals chronically treated with naloxone 1 week after immunization with 50 µg KLH \( (P < .05) \) (Figure 5A, B), while in animals immunized with 100 µg KLH the IgG titers appeared to be reduced after both acute and chronic naloxone treatment \( (P < .05) \) (Figure 5D). No significant interactions (week × KLH dose × treatment) were present \( (F_{2,251} = .765, \ P = .466) \).

Discussion

The administration of the opioid antagonist naloxone appears to affect the pattern of cytokine production by splenocytes of BALB/cJ mice immunized with the protein antigen KLH. Acute as well as chronic naloxone treatments decrease IL-4 production in both unstimulated and in vitro KLH-stimulated splenocyte cultures. On the contrary, the production of the TH1 cytokines IL-2 and IFN-γ is increased by naloxone. Although slight differences are present depending on the dose of KLH used to immunize animals, the duration of immunization, and the duration of in vitro culture, no qualitative difference is observed: TH1 cytokines show a trend to increase and TH2 cytokines to decrease after naloxone.

Independently of treatment, although higher levels of IFN-γ and IL-4 are present after 72 hours of in vitro stimulation, the opposite is true for IL-2, since a decline of the levels of the cytokine is observed. IL-2 acts as an autocrine growth factor for T cells: IL-2, secreted by TH cells following stimulation by antigen, upregulates the expression of its own receptors, and the subsequent binding of IL-2 to this high-affinity receptor results in proliferation of the antigen-activated T cells. However, the half-life of cell-bound IL-2 is 25 to 30 minutes, with the IL-2/IL-2R complex being removed from the cell surface by internalization.17 The fact that lower amounts of free IL-2 are found at cells cultured for longer times could therefore be explained by massive utilization of the cytokine by the activated cells.

When TH1 cytokines are evaluated 7 days after immunization with the highest dose of KLH, acute treatment with naloxone is sufficient to increase TH1 cytokines, while chronic treatment is more efficacious in increasing TH1 cytokines 2 weeks after immunization.

We do not at the moment have a satisfactory explanation for this time course.

IL-4 production is greatly affected by naloxone treatment. A significant decrease of this cytokine is in fact present at both doses of antigen, at both 1 and 2 weeks after immunization, and after treatment with both acute and chronic naloxone. In BALB/cJ mice, one type of TH helper population dominates over the other. BALB/c mice are susceptible to infection by intracellular pathogens, have a weak cell-mediated immune response, and consistently present a TH2 dominance.18,19 We cannot say, at the moment, whether naloxone could primarily act on the dominant TH2 population, affecting IL-4 secretion. Since IL-4 is inhibitory for the differentiation and effector function of the TH1 subset,11,12 the IL-4 decrease induced by naloxone could thereafter permit the production of TH1 cytokines. Alternatively, the effect on TH1 cytokines can precede and thereafter affect IL-4 production.

![Figure 3. Effect of acute and chronic naloxone treatment on KLH-stimulated IL-4 production in vitro by splenocytes. Animals were immunized with either 50 µg KLH (panels A,B) or 100 µg KLH (panels C,D) and killed after 1 week (panels A,C) or 2 weeks (panels B,D). Spleen cells were cultured with 0 or 80 µg/ml KLH for 72 h. Acute saline control is represented by white bars; chronic saline control is represented by right-slanted striped bars; acute naloxone treatment is represented by left-slanted striped bars; chronic naloxone treatment is represented by hatched bars. Results are expressed as mean ± SE. * = P < .01 versus saline controls.](https://www.bloodjournal.org/content/95/6/2034/F3)

![Figure 4. Effect of acute and chronic naloxone treatment on serum IgM anti-KLH antibody response. Either animals were immunized with 50 µg KLH and killed after 1 week (A) or 2 weeks (C), or they were immunized with 100 µg KLH and killed after 1 week (B) or 2 weeks (D). Results are expressed as mean ± SE of 10 animals per group. * = P < .01 versus saline control.](https://www.bloodjournal.org/content/95/6/2034/F4)
Further studies are in progress in order to find a potential temporal dependence.

Consistent with the decrease of IL-4 production, naloxone also affects the primary antibody response, as shown by the low serum IgG levels that are present in the mice after naloxone treatment, confirming the link between IL-4 and antibody production. On the whole, the data obtained suggest that treatment with naloxone tends to skew the T-cell balance toward a TH1 pattern.

Given that naloxone is an almost pure antagonist at the μ-opioid receptor and is devoid of any intrinsic activity at the μ receptor, the effects of the drug are likely to be due to the removal of a regulatory tone exerted by endogenous opioid peptides. We previously showed that, in rat and human, naloxone increased T-lymphocyte proliferation, increased NK activity, and worsened the development of inflammatory responses. Similar effects were also shown by our laboratory with the use of a neutralizing antibody against the opioid peptide BE. It can rather be hypothesized that the opioid might exert an inhibitory control of TH1 cell populations, probably through the modulation of TH1/TH2 cytokine balance and released by the cells of the immune system and that it can bind specific opioid receptors present on immunocytes. Evidence therefore exists for an autocrine/paracrine activity of the opioid.

The data reported in this paper contribute a new insight on the role of opioid peptides in the modulation of the immune system. It becomes in fact questionable to claim a unique immunosuppressive or immunostimulatory role for opioid peptides and BE. It can rather be suggested that the opioid might exert an inhibitory control of TH1 cell populations, probably through the stimulation of TH2 cell types. In line with this hypothesis, the literature is often contradictory on the effects of opioid peptides on the immune system. Depending on the immune function evaluated (eg, cellular versus humoral) and on the preexisting TH1/TH2 balance (eg, after previous exposure of animals to different pathogens or by genetic predisposition), inhibition or stimulation of classical laboratory immune parameters has been reported.

The effect of chronic naloxone treatment on TH1 cytokines seems to be in contrast with what we previously reported in a different experimental model. In the earlier experiments, in fact, naloxone was given chronically to naive rats with a resting immune system, and lymphoproliferation, tested in vitro upon mitogen stimulation, was decreased. In the present experiments, the opioid antagonist is administered to immunized animals that present an in vivo stimulated immune response. Similarly, chronic naloxone treatment was able to increase TH1 cytokines in skin graft experiments. In this case, the drug was also given to an already activated immune system. These observations indicate that the status of the immune system of the host can be relevant in order to direct the opioid control of the immune responses.

The disruption of a correct TH1/TH2 balance is involved in the development of several immune diseases. As a consequence, the effects that opioid peptides and naloxone exert on TH1 cell types can be relevant in immunopathology. Consistently, we demonstrated that the administration of naloxone, which, as shown in the present paper, increases the production of TH1 cytokines, worsened the development of experimental autoimmune encephalomyelitis where the TH1-type effector cell response is dominant. Moreover, in a murine model of skin allograft rejection, which is driven by the development of a TH1 response, we showed that naloxone significantly anticipated, while BE delayed, the onset of the rejection. We can hypothesize that the use of opioid peptides or their modulation and/or the use of opioid antagonists might be interesting new tools to achieve immune deviation.

In conclusion, our data indicate a role for opioid peptides in the modulation of TH1/TH2 balance in the complex network of immunoregulatory signals and offer a clue to the interpretation of conflicting results present in the literature.

References

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