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INCREASED SERUM IL-6 LEVEL TIME-DEPENDENTLY REGULATES HYPERALGESIA AND SPINAL MU OPIOID RECEPTOR EXPRESSION DURING CFA-INDUCED ARTHRITIS

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ABSTRACT

Interleukin (IL)-6 is known to cause pro- and anti-inflammatory effects during different stages of inflammation. Recent therapeutic investigations have focused on treatment of various inflammatory disorders with anti-cytokine substances. As a result, the aim of this study was to further elucidate the influence of IL-6 in hyperalgesia and edema during different stages of Complete Freund's Adjuvant (CFA)-induced arthritis (AA) in male Wistar rats. AA was induced by a single subcutaneous injection of CFA into the rats' hindpaw. Anti-IL-6 was administered either daily or weekly during the 21 days of study. Spinal mu opioid receptor (mOR) expression was detected by Western blotting. Daily and weekly treatment with an anti-IL-6 antibody significantly decreased paw edema in the AA group compared to the AA control group. Additionally, daily and weekly anti-IL-6 administration significantly reduced hyperalgesia on day 7 in the AA group compared to the AA control group; however, there were significant increases in hyperalgesia in the antibody-treated group on days 14 and 21 compared to the AA control group. IL-6 antibody-induced increases in hyperalgesia on the 14th and 21st days after CFA injection correlated with a time-dependent, significant reduction in spinal mOR expression during anti-IL-6 treatment. Our study confirmed the important time-dependent relationship between serum IL-6 levels and hyperalgesia during AA. These results suggest that the stages of inflammation in AA must be considered for anti-hyperalgesic and anti-inflammatory interventions via anti-IL-6 antibody treatment.

Keywords: Interleukin-6, hyperalgesia, inflammation, arthritis, CFA

INTRODUCTION

Inflammation results in rapid elevation of the secretion of inflammatory mediators, chemokines and cytokines, such as interleukins 1 and 6 (IL-1 and IL-6) and tumour necrosis factor α (TNFα). Inflammatory substances play a role in pain modulation by interfering with nociceptive transduction, conduction and transmission. Increasing evidence has demonstrated the importance of cytokines in acute and chronic pain (De Jongh et al., 2003a). IL-6 is a pleiotropic cytokine and was originally identified as a T-cell-derived B-cell differentiation factor, which showed different actions on various biological systems. Deregulation

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of IL-6 expression causes the synthesis and release of many inflammatory mediators, which may result in pain (Nishimoto and Kishimot, 2006). Due to its multiple stimulatory effects on cells of the immune system and vascular endothelial cells, it is believed that excess IL-6 plays a pathogenic role in the development of inflammation, resulting in hyperalgesia and edema (De Jongh et al., 2003b**)**. Modulation of immune responses to alleviate pain and inflammation has been of interest for many years. Due to the central role played by IL-6 in a number of manifestations of inflammatory diseases, therapeutic inhibition of IL-6 represents a novel approach to the treatment of chronic inflammation. Studies have demonstrated that some symptoms of inflammation with a significant cytokine component, such as rheumatoid arthritis, can be treated by inhibition of IL-6 (Van Snick, 1990). However, IL-6 has been shown to elicit both pro- and anti-inflammatory effects. IL-6 was shown to induce analgesia in an animal model of inflammation by contributing to the activation of the endogenous opioid system, which is induced in response to peripheral inflammation (Möller and Villiger, 2006). Complete Freund's Adjuvant (CFA)-induced arthritis (AA) in rats is an inflammatory model widely used in etiopathogenic investigational drug and molecular studies due to its similarity to human rheumatoid arthritis (RA). It has been shown that local injection of CFA in rats increases local and systemic secretion of cytokines, such as IL-1, IL-6 and TNFα (Zhang et al., 1998). Our previous study revealed that CFA-induced hyperalgesia on the 7th day following CFA injection was correlated with increases in inflammatory mediators, such as IL-6; however, hyperalgesia was found to be significantly reduced by the 3rd week. We hypothesised that activation of opioids is differentially regulated during different stages of AA, and these changes may be induced by variations in the secretion of certain mediators during inflammation. Mu-opioid receptors (mOR) are considered to be an important mediator of the analgesic effects of opioids. Mu-opioid receptors (mORs) are also up-regulated in inflammation, which may contribute to the anti-nociceptive effects of endogenous opioids (Zaringhalam et al., 2007). Moreover, Pol and Puig (2004) have demonstrated that mice with an IL-6 deficiency have reduced analgesic responses to morphine (mOR agonist) and a lower mOR receptor density in the grey matter of the midbrain (Pol and Puig, 2004). Further studies are necessary to evaluate the exact roles of IL-6 in pain after various forms of tissue or nerve injury. As a result of the two proposed roles of IL-6 during inflammation and recent investigations using anti-cytokine therapies to treat inflammatory disorders in several different animal models (Van Snick, 1990), we designed this study to further elucidate the influence of IL-6 in hyperalgesia and edema during different stages of CFA-induced arthritis (AA) by examining the effects of anti-IL-6 treatment on spinal mOR expression.

MATERIALS AND METHODS

Laboratory animals

Adult male Wistar rats, weighing 200–220 g were used in all experiments. Rats were housed in polypropylene cages under hygienic and standard environmental conditions (22 ± 2 ºC, humidity 60-70 %, 12 h light/dark cycle). The animals were allowed for standard food and water. Each animal was used once only. All procedures were approved by the local ethics committee for the use of animals in research and we followed the guidelines of ethical standards for investigations of experimental pain in animals (Zimmermann, 1983).

Induction of adjuvant arthritis (AA)

AA was induced at day 0 as expressed in our previous studies. Briefly, a single subcutaneous injection (100 µl) of heat-killed mycobacterium tuberculosis suspended in sterile mineral oil (10 mg/ml), CFA (Sigma, St. Louis, MO/USA), into the left hind paw was performed. Left hind paws of control rats were injected with ster-

ile mineral oil only (100 µl). First day after CFA injection unilateral inflammation was established in injected hindpaw, then first week was considered as inflammatory phase. The third week after intervention was arthritic phase (Zaringhalam et al., 2007).

Assessment of CFA-induced arthritis (AA)

Arthritis due to CFA injection was assessed by measurements of paw volumes pre- and post-injection (on days 0, 7, 14 and 21). Measurements (paw volume) were conducted by displacement of an electrolyte solution in a plethysmometer (model 7141; Ugo Basile; Comerio VA, Italy), as described previously by our lab (Rezazadeh et al., 2009). Briefly, the rats' hindpaws were submerged up to the tibiotarsal joint in the electrolyte-filled Perspex cell of the plethysmometer. The volume of liquid displacement, which is correlated to the paw volume, was indicated on a digital display. Volume measurements were conducted twice for each paw, and the average volume displacement was calculated. The edema was quantified by measuring the difference in foot volume between day 0 and subsequent time points. The volumes measured on days post-injection were calculated as the percentage of the day 0 volume.

Behavioral test: Thermal hyperalgesia assessment

Paw withdrawal latency (PWL) from noxious heat by using the plantar test (Ugo Basile, Verse, Italy) was assessed in different experimental and control rats on days 0, 7, 14, and 21 as previously described (Zaringhalam et al., 2007) . Rats were placed in Plexiglas boxes for 10–15 min before testing in order to habituate to test environment. PWL automatically recorded by digital timer which connected to the heat source. If the rat did not withdraw its paw from stimulus by 20 s, the test was terminated and it was assigned as cut-off value. Withdrawal latency was measured three times for each hindpaw at an interval of 5–10 min and the mean latency was calculated. Then, the mean value for the affected paw (CFA-injected paw) was subtracted from that for the other paw and the result considered as the hyperalgesia sign in the injured paw.

Blood sampling and serum IL-6 measurements

The serum IL-6 levels of the rats were assayed by a rat standard ELISA kit (Bender Med System, UK) on day 0 (before CFA injection) and on days 7 (inflammatory phase), 14 and 21 (arthritic phase) following CFA injection (Zaringhalam et al., 2007). Rats were lightly anesthetised with methoxyflurane and retro-orbitally bled into heparinised tubes during the experimental procedure. The samples were centrifuged and stored at -70 °C. Blood samplings were done simultaneously for each group (8-8:15 AM). The collected serum showed 100 % cross-reactivity with the ELISA kit, and the limit of serum IL-6 detection was 31.2 pg/ml. ELISA assays were performed according to the manufacturer's protocol.

Anti-IL-6 antibody administration

To further evaluate the role of IL-6 in AA symptoms, rats were treated with polyclonal anti-IL-6 antibodies to deplete serum IL-6 levels. The anti-rat IL-6 antibody was obtained from R&D Systems (Abcam/UK). This antibody was produced in goats immunised with purified, Escherichia coli-derived, recombinant rat IL-6. In addition, some investigators have demonstrated reactivity of this antibody preparation in vivo in rat models (Agarwal et al., 2010). According to the manufacturer, the neutralisation dose 50 (ND_{50}) for this anti-rat IL-6 antibody (catalogue number ab9770) was approximately 0.003-0.005 µg/ml in the presence of 0.1 ng/ml rat IL-6. The IL-6 neutralising antibody was dissolved in sterile phosphate-buffered saline (PBS) for intraperitoneal (i.p.) injection, and control animals received PBS. This solution was prepared fresh at a maximum of 30 min prior to injection.

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Spinal mOR expression detection by Western blotting

After behavioral tests, Western blot was used to examine the variations of mOR expression in spinal cord as previously was described (Back et al., 2006). Rats were sacrificed under isoflorane anesthesia, individual spinal cords were rapidly removed on ice and homogenized (Brinkman Polytron Homogenizer, 20.000 rpm 30 s) in extraction buffer containing a cocktail of proteinase inhibitors (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 % NP40, 0.5 % sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 2.5 µg/ml aprotinin, 2 µg/ml leupetin, 2 µg/ml pepstatin A). Then, boiled for 5 min and centrifuged at 10.000 rpm (15 °C) for 10 min. The supernatant was removed for analysis and protein concentration was determined (Bradford, 1976). (Equal amounts of protein (60 µg) were diluted with loading buffer (4 % SDS, 25 mM Tris–HCl pH 6.8, 5 % glycerol, 0.5 % 2-mercaptoethanol, 0.005 % bromophenol blue). After boiling for 10 min, an aliquot of the diluted sample (12 µl) was loaded on polyacrylamide gels (Pager Gels 10 % Tris-Glycine, Bio-Rad) and samples were separated by electrophoresis (120 V for 60 min). A sample from an individual spinal cord was loaded on each lane. Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using the miniprotean II (Bio-Rad) at 100 V for 85 min. Nonspecific binding sites on the membrane were blocked by incubation (90 min at 24 °C or overnight at 4 °C) in blocking buffer (0.2% Aurora Blocking Reagent; 1X Phosphate Buffered Saline: 0.058 M Na_2HPO_4, 0.017 M NaH_2PO_4, 0.068 M NaCl; 0.05 % Tween-20 from ICN Biomedicals, Costa Mesa, CA) followed by incubation (1 h, 24 °C) with primary antibody in blocking buffer (Rabbit polyclonal IgG for mOR (1:1000); Abcam/CA). Membranes were washed twice with blocking buffer and then incubated (1 h, 24 °C) with secondary antibody in blocking buffer (Anti-rabbit IgG (1:10000), Abcam/CA). Membranes were then washed thrice with blocking buffer, followed by two quick rinses with assay buffer (20 mM Tris-HCl, pH 9.8, 1 mM $MgCl_2$). The immunoreactivity of the proteins on the membrane was visualized using the chemiluminescence detection system (ECL, Amersham). The membranes were then incubated in stripping buffer (100 µM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris [pH 6.7]) at 50 °C for 30 min and reprobed with beta-actin primary antibody (1:5000; Cell Signaling) as a loading control. Band intensity was measured densitometrically using NIH Image (1.60), and expressed as the ratio of the intensity of the mOR band to that of β-actin to account for any differences in starting mOR proteins. Each experiment was replicated three times with new groups of rats.

Experimental procedures

Rats were randomly divided into different experimental groups (n=6-7 per group). AA was induced by subcutaneous injection of CFA in the left hindpaw on day 0 (under light anaesthesia) in experimental groups. To determine the effects of serum IL-6 levels on pain and edema and whether a time-dependent relationship exists, different experiments were performed. A neutralising dose of anti-IL-6 diluted in PBS (0.1 µg/ml) was injected (i.p.) from the first day after CFA injection until the 21st day of the study, either daily or weekly (Liang et al., 2009; Serada et al., 2008). As different phases were described for CFA-induced AA (Cicala et al., 2000; Taniguchi et al., 2004), the molecular, behavioural, paw edema and serum IL-6 levels were assessed on day 0 (immediately before CFA injection), and on days 7 (inflammatory phase), 14 and 21 (arthritic phase), 30 min before anti-IL-6 administration. Spinal mOR expression was assessed by Western blotting on days 0, 7, 14 and 21 in AA, AA plus anti-IL-6 (either daily or weekly injections) and AA plus PBS (vehicle) groups. This procedure was also applied in control groups. Animals were killed by decapitation at the end of each experiment (under

methoxyflurane anaesthesia), and whole spines were dissected, immediately snap-frozen in liquid nitrogen and kept at -80 °C until mOR expression assessment was conducted by Western blot.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Western blot experiments were replicated two or three times, and the results were analysed by performing an unpaired *t*-test. Thermal hyperalgesia, paw edema and serum IL-6 levels were analysed by one-way analysis of variance (ANOVA) (Statistica 6). Post hoc analysis was performed with Tukey's multiple comparison test where appropriate. Statistical significance was accepted at $P<0.05$.

RESULTS

Paw edema variations during different stages of AA

CFA injection increased ipsilateral paw (left paw) volume, which was continuous until the 21st day of the study. Paw volume significantly increased on the 7th, 14th and 21st days after CFA injection compared to day 0. Paw volume was significantly increased on the 21st day compared to the 7th and 14th days after CFA injection ($P<0.01$ and $P<0.05$, respectively). Daily and weekly anti-IL-6 antibody administration (i.p.) in the AA group significantly reduced paw edema compared to the AA control group throughout this study ($P<0.001$). However, even with anti-IL-6 antibody treatment, the volume of the injected paw was significantly greater on days 7, 14 and 21 than on day 0 ($P<0.001$). There were no significant differences in reduction in paw volume between daily and weekly anti-IL-6 treatment. Additionally, there were no significant differences in paw volume between CFA injected rats and rats injected with CFA and treated with vehicle, PBS (Fig. 1).

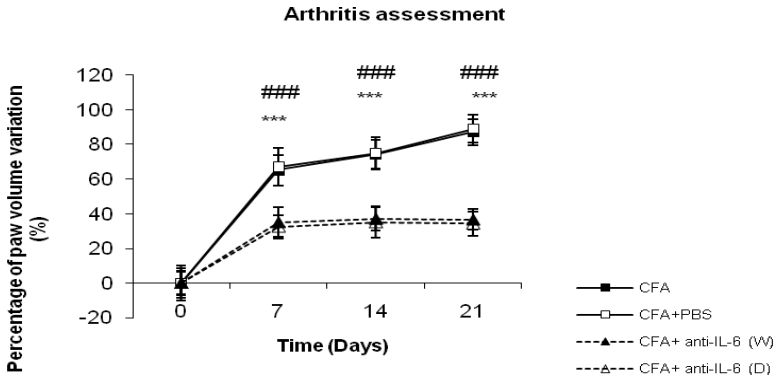

Fig. 1: CFA injection caused significant edema in affected paw which continued till 21 days of AA. Anti-IL-6 antibody treatment significantly reduced inflamed paw volume. Data are presented as mean ± SEM (n=6/group). ***P<0.001 indicate significant increase in CFA injected paw during different days of study compared to day 0. ### P<0.001 comparison of paw volume variation between CFA and CFA+ anti-IL-6 antibody treated rats.

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Variations in thermal hyperalgesia during different stages of AA

Thermal hyperalgesia varied in the left (injected) paw during different stages of AA. Hyperalgesia significantly increased on the 7th day after CFA injection compared to day 0 (P<0.001). PWL measurements in the AA group indicated that hyperalgesia significantly decreased on the 14th and 21st days after CFA injection compared to day 7 (P<0.001). There was a significant decrease in hyperalgesia on the 21st day compared to day 14 after CFA injection (P<0.001). Daily or weekly anti-IL-6 antibody administration in AA rats significantly reduced hyperalgesia on the 7th day after CFA injection compared to the AA group (P<0.001), but these two antibody-treated experimental groups showed significant increases in hyperalgesia on days 14 and 21 compared to the AA control group (P<0.01 and P<0.001, respectively). There were no significant differences in hyperalgesia between daily treatment with anti-IL-6 antibody (i.p.) and weekly administration. Also, there were no significant differences in hyperalgesia during different phases of AA between the CFA injected group and the CFA injected group given PBS vehicle (Fig. 2).

Variations in serum IL-6 levels during different stages of AA

CFA injection caused significant increases in serum IL-6 levels on the 7th, 14th and 21st days after CFA injection compared to day 0 (P<0.001). Anti-IL-6 antibody treatment reduced serum IL-6 levels to near basal levels (day 0). Daily or weekly treatment of AA groups with an anti-IL-6 antibody significantly attenuated serum IL-6 levels on the 7th, 14th and 21st days compared to the AA group (P<0.001). There were no significant differences in serum IL-6 levels between daily and weekly anti-IL-6 antibody treated groups. Groups treated with CFA or CFA plus PBS showed no significant differences in serum IL-6 levels (Fig. 3).

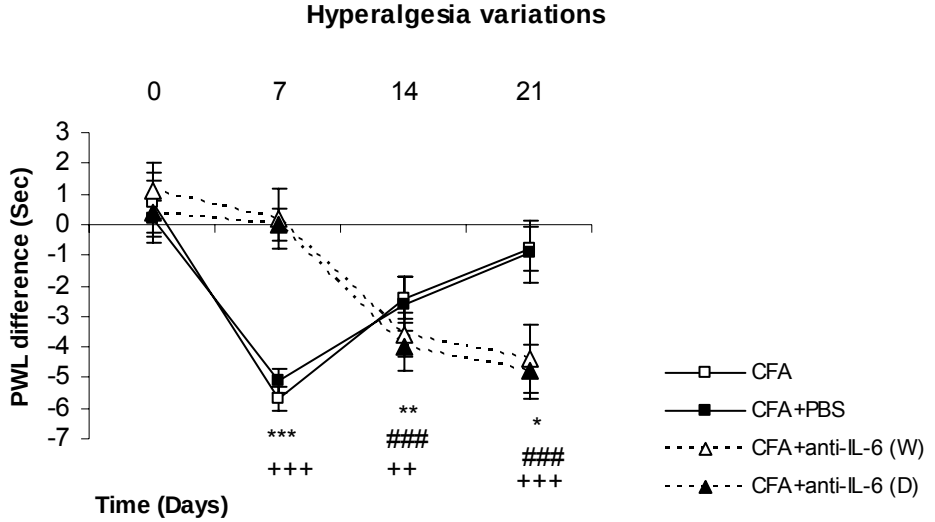

Fig. 2: CFA injection into the hind paw of rats cause significant hyperalgesia at 7th day but, hyperalgesia reduced at 14th and 21st days. Daily/weekly administration of anti-IL-6 changed hyperalgesic procedure during AA. Data are presented as mean ± SEM (n=6/group). *** P<0.001, **P<0.01, *P<0.05 for comparing the hyperalgesia between day 0 and other days of AA. ###P<0.001 for comparing hyperalgesia variation between day 7 and other days in AA group. +++P<0.001, ++P<0.01 Indicate hyperalgesia difference between CFA and CFA+ anti-IL-6 antibody treated groups.

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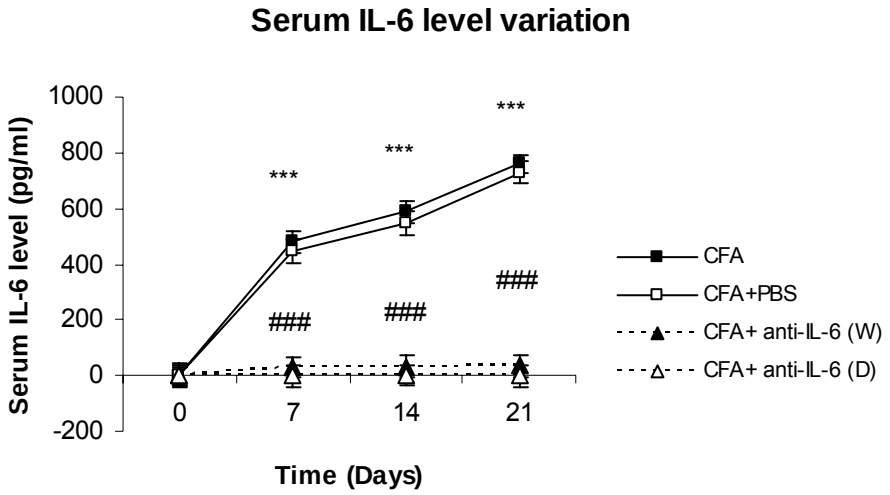

Fig. 3: Serum IL-6 level significantly increased during different stages of AA. Anti-IL-6 antibody treatment during AA caused significant reduction in serum IL-6 level. Data are presented as mean ± SEM (n=6/group). ***P<0.001 indicate serum IL-6 increase during different stages of AA compared to day 0. ### P<0.001 comparison of serum IL-6 level variation between CFA and CFA+ anti-IL-6 antibody treated rats.

Variations in spinal mOR expression during different stages of AA

Western blot analysis of mOR protein expression showed similar bands with molecular masses of approximately 48 kDa in tissue from the spinal cords of all experimental groups, which is consistent with earlier reports. Immunospecificity was confirmed by the absence of immunoreactive bands when the membrane was pre-incubated with an antigenic peptide prior to antibody incubation (data not shown). Following quantification of mOR-immunoreactive bands, membranes were stripped and re-probed for β-actin as a loading control (43 kDa) (Fig. 4). To normalise differences in protein-loading, all data were expressed as mOR/β-actin ratios. Analysis by densitometry demonstrated that AA time-dependently led to a significant increase in spinal mOR protein expression when compared to the control group. There was no significant increase in spinal mOR on the 7th day after AA (3 ± 1.15 %) compared to day 0 (2 ± 1.2 %). Results indicated that spinal mOR expression was significantly increased on days 14 (28.4± 1.3 %) and 21 (54± 1.15 %) following CFA injection compared to day 0 (P<0.001 for both). Spinal cord mOR protein expression in AA groups significantly increased on the 21st day compared to the 14th day after CFA injection (P<0.001) (Fig. 5).

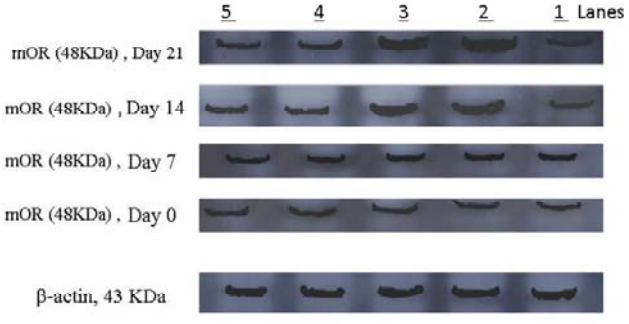

Fig. 4: Immunoblots of mOR receptor protein extracted from the spinal cord during different stages of AA (days 0, 7, 14, 21).
Lanes:
1: Control group
2: AA group
3: AA+PBS group
4: Daily anti-IL-6 antibody received group
5: Weekly anti-IL-6 antibody received group.
β-actin was considered as a loading control.

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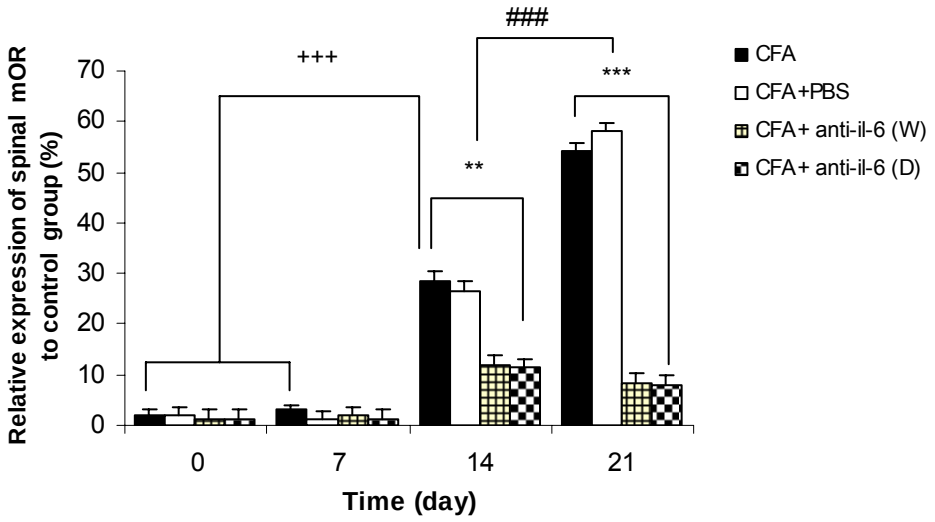

Fig. 5: CFA injection caused significant increase in spinal mOR expression time dependently. Anti-IL-6 antibody administration significantly reduced spinal mOR expression during different stages of AA. Ratio of spinal mOR protein band intensity in AA group significantly decreased at 14th and 21st days after anti-IL-6 antibody treatment compared to AA control group. Data are presented as mean ± SEM (n=6/group). +++ P<0.001 comparison of ratio of spinal mOR protein band intensity between different days of AA. ** P<0.01, ***P<0.001 comparison of spinal mOR protein band intensity between AA and AA+ anti-IL-6 antibody treated rats. ### P<0.001 indicated spinal mOR protein band intensity difference in AA and AA + anti-IL-6 antibody treated rats between 14th and 21st days.

The effect of anti-IL-6 antibody treatment on spinal mOR expression during different stages of AA

Daily or weekly treatment with an anti-IL-6 antibody significantly reduced spinal mOR protein expression in a time-dependent manner. Anti-IL-6 antibody administration caused a significant reduction in spinal mOR protein expression on the 14th (11.9 ± 2.1 %) and 21st (8.5 ± 1.8 %) days following CFA injection, compared to AA control groups (P<0.01 and P<0.001, respectively); however, spinal mOR expression on days 14 and 21 was significantly higher than on day 0 (P<0.01). There were no significant differences in spinal mOR protein expression between daily and weekly anti-IL-6 antibody treatment groups on different days of study. Statistical analysis showed that mean spinal mOR protein expression decreased due to anti-IL-6 antibody treatment on the 21st day after AA compared to the AA control group and was significantly higher than on day 14 (P<0.001). PBS administration in AA rats, the anti-IL-6 antibody vehicle, did not change spinal mOR protein expression compared to the AA group (Fig. 5).

DISCUSSION

The immune and nervous systems express similar molecules, such as cytokines and neurotransmitter receptors, which facilitate the bidirectional coordination between these two systems. This coordination is essential for managing and modulating the immunoneurobiological responses to stressors such as inflammation. The analgesic effects of peripherally applied opioids are augmented under conditions of tissue injury and inflammation (Zöllner et al., 2003). Our study, in addition to previous studies, demonstrated that one of the underlying mechanisms of these effects is the up-regulation of spinal mOR under persistent inflammatory conditions. Cytokines may play an important role in this mechanism by up-regulating mOR expression in the central nervous system and peripheral tissues. IL-6 is a multifunctional cytokine with pro-

and anti-inflammatory properties. IL-6 mediates both acute and chronic phases of inflammatory responses and is therefore involved in both early and late stages of inflammation (Möller and Villiger, 2006). Our investigation showed that AA-associated inflammation increased serum IL-6 levels, which remained elevated until the 21st day after initiating AA by CFA injection. Circulating serum IL-6 has been shown to be increased in multiple inflammatory diseases, including RA, systemic juvenile idiopathic arthritis and Crohn's disease (Van Snick, 1990). A clear correlation between IL-6 levels and different markers of inflammation was established in patients with RA; however, IL-6 induces a variety of opposing effects in the nervous system, which are not well understood (Leon, 2002). Our study demonstrated that serum IL-6 time-dependently affects hyperalgesia during different stages of AA. During first week of study, daily or weekly systemic administration of anti-IL-6 reduced hyperalgesia. We predicted this effect because previous studies have demonstrated that the hyperalgesic responses of AA in the acute phase are partially related to the release of cytokines, such as IL-1, IL-6 and TNFα, which can directly or indirectly stimulate nociceptors **(**De Jongh et al., 2003a)**.** IL-6 is one of the chief stimulators of the production of most acute phase proteins and is critical in controlling the extent of local and systemic acute inflammatory responses, such as hyperalgesia (Van Snick, 1990). As a result, these data and the findings of this study suggest that IL-6 may play an important role in the induction of hyperalgesia during the inflammatory (acute) phase of CFA-induced AA. Anti-IL-6 antibody (i.p.) administration in AA rats significantly alleviated paw edema on all days of investigation. This might be due to IL-6 stimulatory roles on endothelial cell production, release of IL-8 and monocyte-derived chemoattractant proteins, expression of adhesion molecules and recruitment of leukocytes to inflammatory sites **(**Lipsky, 2006)**,** which can be blocked by anti-IL-6 antibody treatment. Other animal studies have demonstrated that IL-6 is critical in the development of experimental arthritis (edema). The development of carrageenan-induced arthritis in anti-mouse IL-6 antibody-treated mice was significantly inhibited (Takagi et al., 1998). Anti-IL-6 antibody treatment during this study reduced paw edema in AA rats; however, affected paw volumes of anti-IL-6 antibody treated rats was higher than on day zero (before CFA injection). Several mediators, such as histamine and bradykinins, and cytokines, such as, IL-1, TNF α and IL-6, are thought to influence inflammation (Möller and Villiger, 2006). Therefore, continuation of paw edema despite treatment with a neutralising dose of anti-IL-6 antibody during AA was expected.

Furthermore, our results indicated that spinal mOR expression on the 14th and 21st days of AA increased with concurrent decreases in hyperalgesia. These findings, in addition to our previous study, which revealed that naloxone (as mOR antagonist) has hyperalgesic effects on the 21st day of AA, confirm the important role of spinal mOR in decreasing hyperalgesia during the chronic phase (arthritic phase) of AA. It has been shown that the transition from acute to chronic inflammation is likely to involve several systems, including changes in cytokine signalling both centrally and peripherally; however, the mechanisms involved in these changes are poorly understood (Vadivelu and Sinatra, 2005). The pattern of cytokine production and secretion in the acute and chronic phase responses varies in different inflammatory conditions. IL-6 is important cytokine for transitioning between acute and chronic inflammation, and it is involved in different stages of RA (Nishimoto and Kishimot, 2006). Some studies have indicated that IL-6 exhibits pro-inflammatory features in carrageenan-induced chronic inflammation and induces hyperalgesia (Takagi et al., 1998), but our results demonstrated anti-inflammatory effects of serum IL-6 during the chronic phase of AA. Our study emphasised that systemic anti-IL-6 antibody administration

(either daily or weekly) reduced spinal mOR expression during the chronic phase of AA and subsequently increased hyperalgesia. These different findings concerning the hyperalgesic roles of IL-6 during chronic inflammation may be due to different models of inflammation, the time of inflammatory symptom assessment and different methods of assessing IL-6 levels (both local and systemic). In this in vivo study, we assessed the role of systemic and chronic increases of IL-6 levels on hyperalgesia induced by CFA injection. Moreover, our results and results from previous studies demonstrated that IL-6 administered one week after injection of CFA into the rats' hindpaw resulted in immediate analgesia (<5 min), which was blocked by naloxone. This analgesia for sustained inflammatory pain was attributed to a local release of endogenous opioid peptides by immune cells after IL-6 challenge. Some studies have also shown that IL-6 can induce mOR mRNA up-regulation in a human neuroblastoma cell line (SH SY5Y cells) (De Jongh et al., 2003b). It has been suggested that IL-6 may serve as a regulator of inflammatory pain and may be essential in the immune-opioid pathway (De Jongh et al., 2003a**)**. Therefore, it is expected that at least part of the anti-hyperalgesic effect of serum IL-6 during the arthritic (chronic) phase of AA can be mediated via increases in spinal mOR expression. Our results also demonstrated that daily or weekly administration of a neutralising dose of anti-IL-6 antibody (0.1 µg/ml) was effective at reducing elevated serum IL-6 levels during AA, and there were no significant differences between daily and weekly injections. This suggests that a maximum threshold of anti-IL-6 activity may be achieved at or even below the 0.1 µg/ml/week dose level; thus, the current study cannot provide pharmacokinetic data on the efficacy of anti-IL-6 antibody therapy beyond the observation of a marked reduction in disease parameters. In sum, this study confirmed the important time-dependent relationship between serum IL-6 levels and CFA-induced inflammation. An important finding of this study was that increases in serum IL-6 levels reduced hyperalgesia during the chronic (arthritic) phase of AA, which may be mediated by increased spinal mOR expression. On the other hand, due to the central role played by IL-6 in some manifestations of inflammatory diseases, inhibition of IL-6 may provide a novel approach for the treatment of RA. However, our study suggests that the stages of arthritis must be considered for anti-hyperalgesic and anti-inflammatory intervention via anti-IL-6 antibody treatment. Additional studies are necessary to establish the procedure of anti-IL-6 antibody treatment in the clinic.

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