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Interleukin-6 mediates low-threshold mechanical allodynia induced by intrathecal HIV-1 envelope glycoprotein gp120

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Abstract

Spinal cord glia (microglia and astrocytes) contribute to enhanced pain states. One model that has been used to study this phenomenon is intrathecal (i.t.) administration of gp120, an envelope glycoprotein of HIV-1 known to activate spinal cord glia and thereby induce low-threshold mechanical allodynia, a pain symptom where normally innocuous (non-painful) stimuli are perceived as painful. Previous studies have shown that i.t. gp120-induced allodynia is mediated via the release of the glial pro-inflammatory cytokines, tumor necrosis factor- α (TNF), and interleukin-1 β (IL-1). As we have recently reported that i.t. gp120 induces the release of interleukin-6 (IL-6), in addition to IL-1 and TNF, the present study tested whether this IL-6 release in spinal cord contributes to gp120-induced mechanical allodynia and/or to gp120-induced increases in TNF and IL-1. An i.t. anti-rat IL-6 neutralizing antibody was used to block IL-6 actions upon its release by i.t. gp120. This IL-6 blockade abolished gp120-induced mechanical allodynia. While the literature predominantly documents the cascade of pro-inflammatory cytokines as beginning with TNF, followed by the stimulation of IL-1, and finally TNF plus IL-1 stimulating the release of IL-6, the present findings indicate that a blockade of IL-6 inhibits the gp120-induced elevations of TNF, IL-1, and IL-6 mRNA in dorsal spinal cord, elevation of IL-1 protein in lumbar dorsal spinal cord, and TNF and IL-1 protein release into the surrounding lumbosacral cerebrospinal fluid. These results would suggest that IL-6 induces pain facilitation, and may do so in part by stimulating the production and release of other proinflammatory cytokines.

Keywords

interleukin-1; tumor necrosis factor; rat; HIV-1 gp120; allodynia; spinal cord

1. Introduction

Pain enhancement has classically been thought to result from the sensitization of dorsal horn spinal cord neurons by neuronally-derived substances such as substance P, glutamate, and nitric

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oxide (NO) (Haley et al., 1992). However, in recent years, it has become clear that spinal cord glia can be intimately involved in the creation and maintenance of diverse enhanced pain states (DeLeo and Yezierski, 2001; Watkins and Maier, 2003).

Although the involvement of glia in pain facilitation is now widely accepted, how exactly glia contribute to enhanced pain states is not fully understood. Upon activation, glia have been shown to release the pro-inflammatory cytokines, tumor necrosis factor- α (TNF), interleukin-1 β (IL-1), and interleukin-6 (IL-6). Currently the clearest evidence for proinflammatory cytokine involvement in pain enhancement comes from studies of TNF and IL-1 in spinal cord. Spinal TNF and IL-1 mRNA expression, protein production, and release are increased in response to various pain enhancing manipulations (DeLeo and Colburn, 1999; Milligan et al., 2001; Milligan et al., 2003). Both intrathecal (i.t.) TNF and i.t. IL-1 have been reported to elicit pain behaviors (Kwon et al., 2005) and enhance neuronal responsivity to nociceptive stimuli (Reeve et al., 2000). Moreover, disruption of TNF and IL-1 signaling in spinal cord, using TNF soluble receptors and/or IL-1 receptor antagonist (IL-1ra), has been reported to attenuate pain facilitation in diverse animal models (Chacur et al., 2004; DeLeo and Colburn, 1999; Laughlin et al., 2000; Milligan et al., 2001; Milligan et al., 2003; Sweitzer et al., 2001; Watkins et al., 1997).

In contrast, the pain modulatory effects of spinal IL-6 are far less clear. While IL-6 can act as a proinflammatory cytokine, under other circumstances it can act as an anti-inflammatory cytokine instead (Jordan et al., 1995; Tilg et al., 1997; Tilg et al., 1994). Given that IL-6 can exert diametrically opposing actions under different conditions, it is perhaps not surprising that both pain facilitatory and pain inhibitory effects of IL-6 have been reported (DeLeo et al., 1996; Flatters et al., 2003). We have recently documented that i.t. administration of human immunodeficiency virus-1 (HIV-1) envelope glycoprotein gp120 induces the production and release of IL-6, in addition to TNF and IL-1 (Holguin et al., 2004; Ledeboer et al., 2005). This raises the question of whether gp120-induced spinal IL-6, like TNF and IL-1 (Milligan et al., 2001), is important in the induction of gp120-induced mechanical allodynia or, in contrast, whether it acts to counter-regulate the actions of these latter two proinflammatory cytokines. Thus the purpose of the present study was to use i.t. gp120 to study the effect of blocking spinal IL-6 actions on both mechanical allodynia and the expression of TNF, IL-1, and IL-6.

2. Materials and Methods

2.1. Subjects

Pathogen-free adult male Sprague-Dawley rats (300-450 g; Harlan Labs, Madison, WI) were used in all experiments. Rats were housed in temperature ($23\pm 3^{\circ}\text{C}$) and light (12 h:12 h light:dark cycle; lights on at 0700 h) controlled rooms with standard rat chow and water freely available. Experiments were performed during the light cycle. Care and use of the rats were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2. Drugs

Lyophilized affinity purified polyclonal sheep anti-rat interleukin-6 (IL-6) neutralizing IgG (National Institute for Biological Standards and Control, Potters Bar, U.K.) was reconstituted at 1.3 $\mu\text{g}/\mu\text{l}$ in endotoxin-free sterile distilled water, aliquoted, and stored at -70°C . At the time of testing, a thawed aliquot of anti-rat IL-6 neutralizing antibody was diluted in sterile 0.9% saline to a final concentration of 0.065 $\mu\text{g}/5 \mu\text{l}$. Affinity purified normal sheep IgG (control; lot number 31K9105; Sigma, St. Louis, MO) was reconstituted to 1.3 $\mu\text{g}/\mu\text{l}$, aliquoted, stored, and diluted at the time of testing to 0.065 $\mu\text{g}/5 \mu\text{l}$ in an identical manner. Sterile aliquots of endotoxin-free recombinant HIV gp120 (1 $\mu\text{g}/\mu\text{l}$; product #1021; ImmunoDiagnostics,

Bedford, MA) were stored at -80°C. At the time of testing, gp120 was slowly thawed and diluted to a concentration of 0.5 µg/µl in a 0.1% rat serum albumin vehicle (RSA; Accurate Chemical & Scientific Corporation, Westbury NY), in endotoxin-free Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen Corp, Grand Island, NY).

2.3. Surgery and microinjections

Rats were anesthetized with isoflurane and chronic lumbosacral intrathecal (i.t.) indwelling catheters were implanted by lumbar approach as previously described (Milligan et al., 1999). Rats were allowed 4-10 days to recover prior to behavioral measures. Intrathecal injections were conducted as described previously (Milligan et al., 1999). Drugs were administered i.t. in a volume of 5 or 6 µl, followed by a flush of 8 µl saline to ensure complete drug delivery, and occurred over a 2 min period. I.t. catheter placements were verified upon completion of behavioral testing by visual inspection after sacrifice. Data were only analyzed from those rats with catheters verified as having the catheter tip at the lumbosacral spinal level.

2.4. Experimental design

To test whether IL-6 is involved in gp120-induced mechanical allodynia, glial activation, and cytokine production and/or release, IL-6 was prevented from binding to its receptor using a sheep anti-rat IL-6 neutralizing antibody. A 2 (anti-IL-6 antibody or control IgG) X 2 (gp120 or vehicle) design with 5-8 rats/group was used. After baseline behavioral assessments on the von Frey test, anti-IL-6 antibody (0.065 µg in 5 µl) or equivolume normal sheep IgG was intrathecally administered. This dose of sheep anti-rat IL-6 antibody was chosen based on prior studies (Milligan et al., 2003). Behavior was reassessed 1 h 55 min later, just prior to i.t. gp120 administration to confirm that the antibody had not affected baseline behavioral measures. Subsequently, i.t. gp120 (3 µg in 6 µl) or equivolume vehicle was administered. Behavioral testing was conducted every 20 min for 120 min after i.t. gp120. Immediately upon completion of testing, animals were anesthetized and CSF and lumbosacral spinal cord tissue were collected for ELISA and RT-PCR analyses.

2.5. von Frey test for mechanical allodynia

The von Frey test (Chaplan et al., 1994) measures paw withdrawal responses to a range of calibrated low-threshold mechanical stimuli and was performed as described previously (Milligan et al., 2000). Briefly, a logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) was applied randomly to the left and right hind paws to determine the threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by $\log_{10}$ (milligrams x 10) and ranged from 3.61 (407 mg) to 5.18 (15,136 mg). Assessments were made prior to (baseline) and at specific times after intrathecal drug administration, as detailed below. Behavioral testing was performed blind with respect to drug administration. Because there were no differences in thresholds between left and right hind paws throughout testing, data obtained from the left and right hind paws were averaged. The behavioral responses were used to calculate the 50% paw withdrawal threshold (absolute threshold), by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method (Harvey, 1986; Treutwein and Strasburger, 1999), as described in detail previously (Milligan et al., 2000). This fitting method allows parametric statistical analyses.

2.6. Spinal cord tissue and cerebrospinal fluid (CSF) collection

Immediately after behavioral testing, rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg; Abbott Labs, North Chicago, IL), supplemented with methoxyflurane (Pitman-Moore, Mundelein, IL) as required to maintain a surgical plane of anesthesia. Lumbosacral CSF was collected by aspiration and flash frozen in liquid nitrogen, as previously

described (Milligan et al., 2001). After verifying intrathecal catheter placement, the lumbosacral spinal cord was then dissected free and placed on an ice-chilled glass plate. The dorsal aspects of the tissue were subdivided into 2 halves and flash frozen in liquid nitrogen to allow for separate analyses of protein and mRNA. All samples were stored at -80°C until the time of assay. Left and right hemi-dorsal cords for each rat were randomly assigned to protein or mRNA analyses.

2.7. Enzyme linked immunosorbant assays (ELISA)

To detect rat IL-1 and rat TNF protein, samples were analyzed by ELISAs specific for these cytokines (R&D Systems, Minneapolis, MN). IL-6 protein was not assayed as the polyclonal sheep anti-rat IL-6 antibody used in the experiments binds to multiple epitopes on IL-6, thereby confounding the ability of the antibody-based ELISA to accurately measure it. TNF was not measured in spinal tissue as this assay has not been found to be reliable in our laboratory. Preparation of dorsal spinal cord tissue and CSF samples was as previously described (Milligan et al., 2001). ELISA of spinal tissue to measure IL-1 levels was performed according to the manufacturer's instructions. Total protein concentrations were determined by the Bradford assay (Bradford, 1976) and used to adjust results for sample size. CSF samples were assayed for TNF and IL-1 levels by serial ELISA for small samples, as described in detail previously (O'Connor et al., 2004).

2.8. RNA extraction and cDNA synthesis

Total RNA was isolated after homogenization of the dorsal spinal cord tissue using TRIzol Reagent and RNase-free glycogen (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Samples were treated with DNase to remove contaminating DNA using the Ambion DNA-free kit (Ambion, Austin, TX). Concentration and purity of the total RNA were determined by measuring the absorbance at 260 and 280 nm by spectrophotometry.

Total RNA was reverse transcribed into cDNA using the Superscript II First-Strand Synthesis System from Invitrogen. First-strand cDNA was synthesized using 1.8 µg of total RNA, 50 ng of random DNA hexanucleotides, 0.5 mM dNTP mix, 5 mM MgCl₂, 10 mM dithiothreitol, 1 x RT buffer and 200 U of SuperScript II reverse transcriptase in a total volume of 20 µl. The reaction was carried out at 42°C for 50 min and terminated by deactivation of the enzyme at 70°C for 15 min. Control reactions lacking either reverse transcriptase or template were included to assess genomic DNA and non-specific contamination, respectively.

2.9. Real-time polymerase chain reaction (PCR)

Amplification of cDNA was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) on a MyiQ Single Color Real Time PCR Detection System (Bio-Rad, Hercules, CA), as described previously (Ledeboer et al., 2005). Primer specifications are listed in Table 1. The threshold cycle (C_T , the number of cycles to reach threshold of detection) was determined for each reaction, and the levels of the target mRNAs were quantified relative to the level of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) using the comparative C_T (ΔC_T) method (Livak and Schmittgen, 2001).

2.10. Data analysis

All statistical comparisons were computed using Statview 5.0.1 for Windows. Data from the von Frey test were analyzed as the interpolated 50% threshold (absolute threshold) in log₁₀ of stimulus intensity (monofilament stiffness in milligrams X 10). Pre-drug baseline measures were analyzed by one-way ANOVA. Post-drug time course measures were analyzed by repeated measures ANOVA with treatment (vehicle vs. gp120) and antibody (control IgG vs.

IL-6 antibody) as the between-subject factors, and time as the within-subject factor, followed by Fisher's protected least significant difference (PLSD) post hoc comparisons, where appropriate. ELISA and PCR data were analyzed by two-way ANOVA, followed by Fisher's PLSD post hoc comparisons, where appropriate.

3. Results

3.1. Effect of anti-IL-6 antibody on gp120-induced mechanical allodynia

All groups exhibited comparable baseline (BL) thresholds prior to drug treatments (Fig. 1; $F_{3,25} = 1.624$, $p > 0.05$). Baseline thresholds remained stable when reassessed 1 h 55 min after intrathecal (i.t.) anti-IL-6 antibody or control IgG injections ($F_{3,25} = 1.225$, $p > 0.05$). As in our previous studies (Holguin et al., 2004; Ledebøer et al., 2005; Milligan et al., 2000; Milligan et al., 2001), i.t. gp120 produced robust bilateral mechanical allodynia throughout the testing period (main effect of gp120, $F_{1,25} = 24.196$, $p < 0.0001$). Pretreatment with i.t. sheep anti-rat IL-6 antibody abolished the gp120-induced allodynia (main effect of antibody, $F_{1,25} = 23.438$, $p < 0.0001$; interaction gp120 x antibody, $F_{1,25} = 20.585$, $p < 0.0001$).

3.2. Effect of anti-IL-6 antibody on gp120-induced IL-1 and TNF protein levels

To examine the effect of anti-IL-6 antibody treatment on production and/or release of pro-inflammatory cytokines, protein levels of IL-1 and/or TNF were assessed in lumbar dorsal spinal cord and lumbosacral CSF samples that were collected 2 h after i.t. gp120. As previously reported (Holguin et al., 2004; Ledebøer et al., 2005; Milligan et al., 2001), i.t. gp120 elevated lumbar dorsal spinal cord content of IL-1 protein (Fig. 2A; $F_{1,16} = 13.667$, $p < 0.01$), and IL-1 protein levels in lumbosacral CSF (Fig. 2B; $F_{1,16} = 7.131$, $p < 0.05$). Pretreatment with i.t. anti-IL-6 antibody blocked the gp120-induced increases in IL-1 protein levels in lumbar dorsal spinal cord ($F_{1,16} = 10.569$, $p < 0.01$), and in CSF ($F_{1,16} = 7.682$, $p < 0.05$). Pretreatment with i.t. anti-IL-6 antibody did not significantly affect the i.t. gp120-induced increase in the lumbosacral CSF TNF- α protein levels.

3.3. Effect of anti-IL-6 antibody on gp120-induced cytokine mRNA expression

To examine the effect of anti-IL-6 antibody treatment on pro-inflammatory and anti-inflammatory cytokine gene expression, mRNA levels were assessed in lumbar dorsal spinal cord collected 2 h after gp120. As previously reported (Holguin et al., 2004; Ledebøer et al., 2005), i.t. gp120 induced large increases in lumbar dorsal spinal cord mRNA levels of TNF, IL-1, and IL-6 (Fig. 3A-C; $F_{1,22} = 4.620$, $p = 0.05$; $F_{1,20} = 10.254$, $p < 0.005$; $F_{1,20} = 8.821$, $p < 0.01$, respectively). Pretreatment with i.t. anti-IL-6 antibody blocked the gp120-induced increases in lumbar dorsal spinal cord of TNF, IL-1, and IL-6 mRNA ($F_{1,22} = 4.822$, $p = 0.05$; $F_{1,20} = 3.761$, $p = 0.06$; $F_{1,20} = 5.454$, $p < 0.05$, respectively). Moreover, as shown previously (Ledebøer et al., 2005), i.t. gp120 induced a marked increase in both IL-1 α and IL-10 mRNA (Fig. 3D, E; $F_{1,20} = 10.878$, $p < 0.005$; $F_{1,20} = 5.934$, $p < 0.05$, respectively). These increases were largely blocked by anti-IL-6 antibody pretreatment ($F_{1,20} = 9.285$, $p < 0.01$; $F_{1,20} = 7.433$, $p < 0.05$, respectively).

3.4. Effect of anti-IL-6 antibody on gp120-induced glial activation, inferred by expression of mRNA for cell-type specific glial activation markers

To examine whether the decrease in allodynia induced by anti-IL-6 antibody pretreatment was associated with decreased glial activation as reflected by mRNA for glial activation markers, we assessed lumbar dorsal spinal cord mRNA levels of CD11b (complement receptor 3/CR3, Mac-1) and glial fibrillary acidic protein (GFAP), as a measure of microglial and astroglial activation, respectively. Both CD11b and GFAP mRNA were markedly induced by i.t. gp120 (Fig. 3F, G; $F_{1,20} = 7.512$, $p < 0.05$; $F_{1,20} = 4.738$, $p < 0.05$, respectively), consistent with

previous reports (Ledeboer et al., 2005). CD11b mRNA expression was attenuated by i.t. anti-IL-6 antibody pretreatment (interaction $F_{1,20} = 6.510$, $p < 0.05$), whereas GFAP mRNA levels were not significantly downregulated by the antibody treatment. While not reaching significance, it should be noted that GFAP mRNA levels in the anti-IL-6 + gp120 group were also not significantly different from those in either of the control groups (control IgG + vehicle and anti-IL-6 + vehicle).

3.5. Effect of anti-IL-6 antibody on cyclooxygenase (COX) mRNA expression

Finally, levels of COX-1 and COX-2 mRNA were assessed, since these enzymes have been thought to play a role in pain facilitation by generating prostaglandins within the spinal cord. No differences were observed in COX-1 mRNA expression levels between groups (Fig. 3H). In contrast, i.t. gp120 induced COX-2 mRNA levels (Fig. 3I; $F_{1,21} = 7.540$, $p < 0.05$), which were attenuated by anti-IL-6 antibody pretreatment ($F_{1,21} = 2.842$, $p = 0.10$).

4. Discussion

The present study indicates that blocking the effects of IL-6 via an anti-IL6 neutralizing antibody in lumbosacral spinal cord blocks i.t. gp120-induced: (a) mechanical allodynia, (b) increase in dorsal spinal cord IL-1, (c) release of IL-1 and TNF into surrounding cerebrospinal fluid, (d) increase in dorsal spinal cord mRNA for TNF, IL-1, and IL-6, (e) increase in dorsal spinal cord mRNA for complement type-3 receptor, a standard microglial activation marker, and (f) increase in dorsal spinal cord mRNA for COX-2.

While parallel elevations in TNF, IL-1, and IL-6 may at first glance appear to predict that IL-6, like TNF and IL-1, would exert pro-nociceptive effects, it is notable that IL-6 is not a classical proinflammatory cytokine. That is, IL-6 can exert anti-inflammatory as well as proinflammatory effects (Gosain and Gamelli, 2005). Anti-inflammatory cytokines, such as IL-4 and IL-10, have activities which oppose or down-regulate proinflammatory processes. These anti-inflammatory cytokines are frequently upregulated under the same conditions as are proinflammatory cytokines, and function as negative feedback signals to suppress proinflammatory processes (Gosain and Gamelli, 2005). It is noteworthy in this regard that IL-6 is not the only anti-inflammatory cytokine rapidly upregulated by i.t. gp120. It has recently been reported, and confirmed in the present study, that mRNA levels for the anti-inflammatory cytokines IL-1 receptor antagonist (IL1ra) and IL-10 are elevated by i.t. gp120 as well (Ledeboer et al., 2005). Unlike IL-6, IL-1ra and IL-10 are classic anti-inflammatory cytokines in that their presence does not predict proinflammatory effects (Gosain and Gamelli, 2005). Given that gp120 induces the expression of anti-inflammatory cytokines, in addition to proinflammatory ones, elevation of IL-6 in response to i.t. gp120 did not necessarily predict a pro-nociceptive action.

The finding that gp120 exerts its pain enhancing effects via IL-6 is consistent with other studies showing that IL-6 protein levels rise significantly following administration of gp120 both *in vivo* (Holguin et al., 2004; Ledeboer et al., 2005) and *in vitro* when added to mixed glial cultures (Kong et al., 1996). Furthermore, spinal cord IL-6 has been implicated in other exaggerated pain states, such as allodynia induced by peripheral nerve injury (Arruda et al., 2000; DeLeo et al., 1996), sciatic inflammatory neuropathy (Chacur et al., 2004; Milligan et al., 2003), and intrathecal fractalkine (Milligan et al., 2005). IL-6 has been suggested to be involved in nociception at the level of the skin, nerve, dorsal root ganglia (DRG), and spinal cord, and its expression is induced in response to nerve injury in spinal cord, DRG sensory neurons (Arruda et al., 1998; Lee et al., 2004; Murphy et al., 1995), and in peripheral nerves (Ma and Quirion, 2005; Okamoto et al., 2001).

Previous studies have shown that the proinflammatory cytokines IL-1 and TNF are key mediators in the mechanical allodynia produced by i.t. gp120 (Milligan et al., 2001). Furthermore, nitric oxide (NO) has been shown to be necessary for i.t. gp120-induced elevations in mRNA, protein and release of IL-1, TNF, and IL-6, as well as the resultant mechanical allodynia (Holguin et al., 2004). Thus, in prior investigations, increases and decreases in IL-6 dorsal spinal cord content and release were directly correlated with concomitant changes in TNF and IL-1. The present experiment adds further understanding of the gp120-induced proinflammatory cytokine profile by including IL-6 as another key mediator in gp120-induced mechanical allodynia and as a regulator of the production and release of both IL-1 and TNF.

TNF, IL-1, and IL-6 are known to be intimately related, and form a highly regulated cytokine network. Generally, the proinflammatory cascade begins with TNF, which then stimulates the release of IL-1, then both cytokines are thought to stimulate the release of IL-6 (Gershewald et al., 1990). However, the data in the present study would implicate IL-6 as having a direct effect on the release of TNF and IL-1 as well, suggesting that it is possible that there may be alternative pathways involved. There is evidence that IL-6 induces production of IL-1 in the brain (Miller et al., 1997; Rothwell et al., 1991). However, the present results appear to be the first evidence that IL-6 may induce the release of TNF as well. While gp120 is known to upregulate mRNA for IL-6, IL-1, and TNF, no previous report suggests that IL-6 modulates rises in IL-1 and TNF mRNA.

As noted previously, conflicting data exist on the effects of IL-6 on pain states. In terms of its pro-nociceptive effects beyond the present study, intracerebroventricular injection of IL-6 induced thermal hyperalgesia in naïve rats (Oka et al., 1995), intrathecal injection of IL-6 induced touch-evoked allodynia in naïve rats (DeLeo et al., 1996), thermal hyperalgesia in rats with a sciatic nerve lesion (DeLeo et al., 1996), and increased cold allodynia in neuropathic rats (Vissers et al., 2005). In addition, an anti-IL-6 antibody blocked or attenuated the mechanical allodynia induced by peripheral nerve trauma (Arruda et al., 2000) or peripheral nerve inflammation (Chacur et al., 2004; Milligan et al., 2003), and CCI-induced cold allodynia (Vissers et al., 2005). Also, IL-6 in combination with its soluble receptor can sensitize skin nociceptors to heat (Obreja et al., 2002; Oprea and Kress, 2000). Thus, both endogenous IL-6 (Arruda et al., 2000; Chacur et al., 2004; Milligan et al., 2003), as in the present study, and exogenous IL-6 (DeLeo et al., 1996; Vissers et al., 2005) have been reported to have pro-nociceptive effects. Moreover, in IL-6 knockout mice, mechanical allodynia that is induced in wild-type mice following chronic constriction injury or spinal nerve injury is absent or delayed compared to wild-type mice, further indicating that endogenous IL-6 may mediate hypersensitivity responses associated with neuropathic pain (Murphy et al., 1999; Ramer et al., 1998). In terms of its anti-nociceptive effects, intrathecal IL-6 elicited anti-nociceptive effects in neuropathic rats (Flatters et al., 2003), and intracisternally injected IL-6 had anti-nociceptive effects in an acute orofacial pain model (Choi et al., 2003). Interestingly, Choi et al. showed in their study that intracisternal IL-6 could also have a hyperalgesic response depending on the orofacial pain model used, and this was blocked by pretreatment with IL-1ra, suggesting that this response is mediated by the IL-1 receptor.

There are many potential explanations for these differences. One may be endogenous versus exogenous IL-6. Generally there has been a trend of endogenous IL-6 having pro-nociceptive effects on exaggerated pain states as revealed by use of anti-IL-6 neutralizing antibodies (Arruda et al., 2000; Chacur et al., 2004; DeLeo et al., 1996; Milligan et al., 2003); present study) and exogenous IL-6 having anti-nociceptive effects as revealed by administration of IL-6 protein (Choi et al., 2003; Flatters et al., 2003). However, three studies using exogenous IL-6 did report pro-nociceptive effects (DeLeo et al., 1996; Oka et al., 1995; Vissers et al., 2005). Comparison of the dosages between these studies does not indicate that these differences in

effects are seen because of low vs. high drug doses, as these studies used several different drug doses.

Another possible explanation could be the specific site of IL-6 action. Exogenous IL-6 has been shown to have pro-nociceptive effects particularly in the spinal cord (DeLeo et al., 1996). Pro-nociceptive effects mediated by endogenous spinal IL-6 have also been observed in peripheral nerve injury- and inflammation-induced mechanical allodynia (Arruda et al., 2000; Chacur et al., 2004; Milligan et al., 2003). However, spinal application of exogenous IL-6 resulted in anti-nociceptive effects in electrophysiology studies (Flatters et al., 2003). On the other hand, using similar endpoints, nociceptive responses to heat were also inhibited by *peripheral* IL-6 (Flatters et al., 2004), suggesting that the site of action of IL-6 in these studies may not necessarily be at the level of the spinal cord or DRG. It has also been suggested in this study that the anti-nociceptive effects of IL-6 following nerve injury are modality-specific, i.e. only neuronal responses to heat in neuropathic rats were inhibited. Whether the results observed by Flatters et al. also relate to the inflammatory conditions inherent in acutely traumatic preparations required for spinal cord electrophysiology, the effect of anesthetics on glial function, suppressive effects previously observed for IL-6 on dorsal root function (Ozaktay et al., 2002), or another unknown factor, remains to be defined. Clearly, the electrophysiological studies of Flatters et al. differ from the other studies of spinal IL-6 effects in significant ways.

Other factors that may influence the discrepancy in actions of IL-6 are the timing, modality and method of assessment of nociceptive responses, and/or the type and site of nerve injury, which may in turn influence the extent of inflammation. Also, IL-6 is known to have pleiotropic actions on both glial cells and neurons, and may induce the synthesis or release of both pain-enhancing or pain-inhibitory, neuroprotective or neuromodulatory factors. For example, IL-6 knockout mice exhibit decreased allodynia following CCI, but they also show decreased expression of substance P, involved in pain transmission, in sensory neurons (Murphy et al., 1999). In addition, differences in IL-6 receptors and/or signaling may account for differential effects of IL-6.

In summary, this current study suggests that in addition to the other proinflammatory cytokines (TNF & IL-1), endogenous IL-6 is also a key mediator in the exaggerated pain states induced by i.t. gp120. The data suggest that IL-6 may act on spinal cord glial cells and/or neurons presumably via autocrine or paracrine pathways to facilitate gp120-induced enhanced pain. The present data also suggest that alternative proinflammatory cytokine pathways may exist other than those previously thought with IL-6 mediating the release of both TNF and IL-1. The data from this study would suggest that the actions of IL-6 in gp120-enhanced pain states are pro-nociceptive in nature.

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References

- Arruda JL, Colburn RW, Rickman AJ, Rutkowski MD, DeLeo JA. Increase of interleukin-6 mRNA in the spinal cord following peripheral nerve injury in the rat: potential role of IL-6 in neuropathic pain. *Brain Res Mol Brain Res* 1998;62:228–235. [PubMed: 9813345]
- Arruda JL, Sweitzer S, Rutkowski MD, DeLeo JA. Intrathecal anti-IL-6 antibody and IgG attenuates peripheral nerve injury-induced mechanical allodynia in the rat: possible immune modulation in neuropathic pain. *Brain Res* 2000;879:216–225. [PubMed: 11011025]

- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
- Chacur M, Milligan ED, Sloan EM, Wieseler-Frank J, Barrientos RM, Martin D, Poole S, Lomonte B, Gutierrez JM, Maier SF, Cury Y, Watkins LR. Snake venom phospholipase A2s (Asp49 and Lys49) induce mechanical allodynia upon peri-sciatic administration: involvement of spinal cord glia, proinflammatory cytokines and nitric oxide. *Pain* 2004;108:180–191. [PubMed: 15109522]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994;53:55–63. [PubMed: 7990513]
- Choi HS, Ju JS, Lee HJ, Kim BC, Park JS, Ahn DK. Effects of intracisternal injection of interleukin-6 on nociceptive jaw opening reflex and orofacial formalin test in freely moving rats. *Brain Res Bull* 2003;59:365–370. [PubMed: 12507687]
- DeLeo, JA.; Colburn, RW. Proinflammatory cytokines and glial cells: Their role in neuropathic pain. In: Watkins, L., editor. *Cytokines and Pain*. Birkhauser: Basel; 1999. p. 159-182.
- DeLeo JA, Colburn RW, Nichols M, Malhotra A. Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res* 1996;16:695–700. [PubMed: 8887053]
- DeLeo JA, Yezierski RP. The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain* 2001;90:1–6. [PubMed: 11166964]
- Flatters SJ, Fox AJ, Dickenson AH. Spinal interleukin-6 (IL-6) inhibits nociceptive transmission following neuropathy. *Brain Res* 2003;984:54–62. [PubMed: 12932839]
- Flatters SJ, Fox AJ, Dickenson AH. Nerve injury alters the effects of interleukin-6 on nociceptive transmission in peripheral afferents. *Eur J Pharmacol* 2004;484:183–191. [PubMed: 14744602]
- Gershenswald JE, Fong YM, Fahey TJ 3rd, Calvano SE, Chizzonite R, Kilian PL, Lowry SF, Moldawer LL. Interleukin 1 receptor blockade attenuates the host inflammatory response. *Proc Natl Acad Sci U S A* 1990;87:4966–4970. [PubMed: 1695006]
- Gosain A, Gamelli RL. A primer in cytokines. *J Burn Care Rehabil* 2005;26:7–12. [PubMed: 15640726]
- Haley JE, Wilcox GL, Chapman PF. The role of nitric oxide in hippocampal long-term potentiation. *Neuron* 1992;8:211–216. [PubMed: 1371216]
- Harvey LO. Efficient estimation of sensory thresholds. *Behav Res Meth Instrum Comput* 1986;18:623–632.
- Holguin A, O'Connor KA, Biedenkapp J, Campisi J, Wieseler-Frank J, Milligan ED, Hansen MK, Spataro L, Maksimova E, Bravmann C, Martin D, Fleshner M, Maier SF, Watkins LR. HIV-1 gp120 Stimulates proinflammatory cytokine-mediated pain facilitation via activation of nitric oxide synthase-I (nNOS). *Pain* 2004;110:517–530. [PubMed: 15288392]
- Jordan M, Otterness IG, Ng R, Gessner A, Rollinghoff M, Beuscher HU. Neutralization of endogenous IL-6 suppresses induction of IL-1 receptor antagonist. *J Immunol* 1995;154:4081–4090. [PubMed: 7706746]
- Kong LY, Wilson BC, McMillian MK, Bing G, Hudson PM, Hong JS. The effects of the HIV-1 envelope protein gp120 on the production of nitric oxide and proinflammatory cytokines in mixed glial cell cultures. *Cell Immunol* 1996;172:77–83. [PubMed: 8806809]
- Kwon MS, Shim EJ, Seo YJ, Choi SS, Lee JY, Lee HK, Suh HW. Differential modulatory effects of cholera toxin and pertussis toxin on pain behavior induced by TNF-alpha, interleukin-1beta and interferon-gamma injected intrathecally. *Arch Pharm Res* 2005;28:582–586. [PubMed: 15974446]
- Laughlin TM, Bethea JR, Yezierski RP, Wilcox GL. Cytokine involvement in dynorphin-induced allodynia. *Pain* 2000;84:159–167. [PubMed: 10666520]
- Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR. Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain* 2005;115:71–83. [PubMed: 15836971]
- Lee HL, Lee KM, Son SJ, Hwang SH, Cho HJ. Temporal expression of cytokines and their receptors mRNAs in a neuropathic pain model. *Neuroreport* 2004;15:2807–2811. [PubMed: 15597059]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408. [PubMed: 11846609]

- Ma W, Quirion R. Up-regulation of interleukin-6 induced by prostaglandin E from invading macrophages following nerve injury: an in vivo and in vitro study. *J Neurochem* 2005;93:664–673. [PubMed: 15836625]
- Miller AJ, Hopkins SJ, Luheshi GN. Sites of action of IL-1 in the development of fever and cytokine responses to tissue inflammation in the rat. *Br J Pharmacol* 1997;120:1274–1279. [PubMed: 9105702]
- Milligan E, Zapata V, Schoeniger D, Chacur M, Green P, Poole S, Martin D, Maier SF, Watkins LR. An initial investigation of spinal mechanisms underlying pain enhancement induced by fractalkine, a neuronally released chemokine. *Eur J Neurosci* 2005;22:2775–2782. [PubMed: 16324111]
- Milligan ED, Hinde JL, Mehmert KK, Maier SF, Watkins LR. A method for increasing the viability of the external portion of lumbar catheters placed in the spinal subarachnoid space of rats. *J Neurosci Methods* 1999;90:81–86. [PubMed: 10517276]
- Milligan ED, Mehmert KK, Hinde JL, Harvey LO, Martin D, Tracey KJ, Maier SF, Watkins LR. Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. *Brain Res* 2000;861:105–116. [PubMed: 10751570]
- Milligan ED, O'Connor KA, Nguyen KT, Armstrong CB, Twining C, Gaykema RP, Holguin A, Martin D, Maier SF, Watkins LR. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *J Neurosci* 2001;21:2808–2819. [PubMed: 11306633]
- Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, Tracey K, Martin D, Maier SF, Watkins LR. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J Neurosci* 2003;23:1026–1040. [PubMed: 12574433]
- Murphy PG, Grondin J, Altares M, Richardson PM. Induction of interleukin-6 in axotomized sensory neurons. *J Neurosci* 1995;15:5130–5138. [PubMed: 7623140]
- Murphy PG, Ramer MS, Borthwick L, Gauldie J, Richardson PM, Bisby MA. Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. *Eur J Neurosci* 1999;11:2243–2253. [PubMed: 10383613]
- Obreja O, Schmelz M, Poole S, Kress M. Interleukin-6 in combination with its soluble IL-6 receptor sensitises rat skin nociceptors to heat, in vivo. *Pain* 2002;96:57–62. [PubMed: 11932061]
- O'Connor KA, Holguin A, Hansen MK, Maier SF, Watkins LR. A method for measuring multiple cytokines from small samples. *Brain Behav Immun* 2004;18:274–280. [PubMed: 15050654]
- Oka T, Oka K, Hosoi M, Hori T. Intracerebroventricular injection of interleukin-6 induces thermal hyperalgesia in rats. *Brain Res* 1995;692:123–128. [PubMed: 8548295]
- Okamoto K, Martin DP, Schmelzer JD, Mitsui Y, Low PA. Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain. *Exp Neurol* 2001;169:386–391. [PubMed: 11358451]
- Opree A, Kress M. Involvement of the proinflammatory cytokines tumor necrosis factor- α , IL-1 β , and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. *J Neurosci* 2000;20:6289–6293. [PubMed: 10934280]
- Ozaktay AC, Cavanaugh JM, Asik I, DeLeo JA, Weinstein JN. Dorsal root sensitivity to interleukin-1 β , interleukin-6 and tumor necrosis factor in rats. *Eur Spine J* 2002;11:467–475. [PubMed: 12384756]
- Ramer MS, Murphy PG, Richardson PM, Bisby MA. Spinal nerve lesion-induced mechanoallodynia and adrenergic sprouting in sensory ganglia are attenuated in interleukin-6 knockout mice. *Pain* 1998;78:115–121. [PubMed: 9839821]
- Reeve AJ, Patel S, Fox A, Walker K, Urban L. Intrathecally administered endotoxin or cytokines produce allodynia, hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli in the rat. *Eur J Pain* 2000;4:247–257. [PubMed: 10985868]
- Rothwell NJ, Busbridge NJ, Lefevre RA, Hardwick AJ, Gauldie J, Hopkins SJ. Interleukin-6 is a centrally acting endogenous pyrogen in the rat. *Can J Physiol Pharmacol* 1991;69:1465–1469. [PubMed: 1777846]

- Sweitzer S, Martin D, DeLeo JA. Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience* 2001;103:529–539. [PubMed: 11246166]
- Tilg H, Dinarello CA, Mier JW. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol Today* 1997;18:428–432. [PubMed: 9293158]
- Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 1994;83:113–118. [PubMed: 8274730]
- Treutwein B, Strasburger H. Fitting the psychometric function. *Percept Psychophys* 1999;61:87–106. [PubMed: 10070202]
- Vissers KC, De Jongh RF, Hoffmann VL, Meert TF. Exogenous interleukin-6 increases cold allodynia in rats with a mononeuropathy. *Cytokine* 2005;30:154–159. [PubMed: 15863388]
- Watkins LR, Maier SF. Glia: a novel drug discovery target for clinical pain. *Nat Rev Drug Discov* 2003;2:973–985. [PubMed: 14654796]
- Watkins LR, Martin D, Ulrich P, Tracey KJ, Maier SF. Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. *Pain* 1997;71:225–235. [PubMed: 9231865]

Fig 1.

Intrathecal anti-IL-6 antibody inhibits gp120-induced mechanical allodynia. Low-threshold mechanical sensitivity was assessed by the von Frey test, before (baseline, BL) and 20-120 min after intrathecal administration of gp120 (3 ug) or vehicle. Anti-rat IL-6 neutralizing IgG (0.065 ug) or control IgG was given 2 h prior to gp120 injection. Values represent average thresholds of left and right paws (mean $\pm$ SEM). * $p < 0.0001$ vs. all other groups.

Fig 2.

Effects of intrathecal anti-IL-6 antibody on gp120-induced IL-1 and TNF protein levels in lumbar dorsal spinal cord and/or lumbosacral CSF. Intrathecal gp120 induced an increase in IL-1 protein levels in spinal cord (**A**) and IL-1 and TNF protein levels in CSF (**B, C**), compared to controls. Pretreatment with anti-IL-6 IgG inhibited gp120-induced IL-1 levels in spinal cord and CSF, but not TNF levels in CSF. In all cases, levels in rats treated with anti-IL-6 IgG + vehicle were not significantly different than those in control IgG + vehicle-treated rats. Data represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. control IgG + vehicle; # $p < 0.05$, ## $p < 0.01$ vs. control IgG + gp120.

Fig 3.

Effects of intrathecal anti-IL-6 antibody on TNF, IL-1, IL-6, CD11b, GFAP, and COX mRNA expression in lumbar dorsal spinal cord. Intrathecal gp120 induced an increase in spinal cord TNF (A), IL-1 (B), IL-6 (C), IL-1ra (D), IL-10 (E), CD11b (F), GFAP (G), and COX-2 (I) mRNA expression, but did not significantly induce spinal cord COX-1 (H) mRNA levels, compared to controls. Pretreatment with anti-IL-6 IgG inhibited the gp120-induced mRNA expression of TNF, IL-1, IL-6, IL-1ra, IL-10, CD11b, and COX-2, but not of GFAP. Anti-IL-6 IgG did not significantly affect COX-1 mRNA levels. In all cases, levels in rats treated with anti-IL-6 IgG + vehicle were not different than those in control IgG + vehicle-treated rats. Data represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. control IgG + vehicle; # $p < 0.05$, ## $p < 0.01$ vs. control IgG + gp120.

Table 1
Oligonucleotide primers used for the amplification of rat cDNAs^a

Gene	GenBank Accession no.	Sequence (5'-3')
GAPDH	M17701	GTTTGTGATGGGTGTGAACC (forward) TCTTCTGAGTGGCAGTGATG (reverse)
CD11b	NM_012711	CTGGGAGATGTGAATGGAG (forward) ACTGATGCTGGCTACTGATG (reverse)
GFAP	NM_017009	AGGGACAATCTCACACAGG (forward) GACTCAACCTTCCTCTCCA (reverse)
IL-1 β	M98820	CATTGTGGCTGTGGAGAAG (forward) ATCATCCACGAGTCACGA (reverse)
IL-6	NM_012589	ACTTCACAGAGGATACCAC (forward) GCATCATCGCTGTTTCATAC (reverse)
TNF- α	D00475	CTTCAAGGGACAAGGCTG (forward) GAGGCTGACTTTCTCCTG (reverse)
IL-1RA	M63101	GTCTGGAGATGACACCAAG (forward) TCGGAGCGGATGAAGGTAA (reverse)
IL-10	NM_012854	TAAGGGTTACTTGGGTTGCC (forward) CTGTATCCAGAGGGTCTTCA (reverse)
COX-1	S67721	AACTGGTCTGCCTCAACAC (forward) AACCCACATCAAGGACTGTC (reverse)
COX-2	S67722	CTTCGCCTCTTTCAATGTGC (forward) GGTCAGTAGACTCTTACAGC (reverse)

^aPrimers were purchased from Prologo (Boulder, CO).