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Contribution of interleukin-1β to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia

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1 Peripheral inflammation is associated with the local production of neuroactive inflammatory cytokines and growth factors. These may contribute to inflammatory pain and hyperalgesia by directly or indirectly altering the function or chemical phenotype of responsive primary sensory neurones.

2 To investigate this, inflammation was produced by the intraplantar injection of complete Freund's adjuvant (CFA) in adult rats. This resulted in a significant elevation in interleukin-1β (IL-1β) and nerve growth factor (NGF) levels in the inflamed tissue and of the peptides, substance P and calcitonin gene-related peptide (CGRP) in the L4 dorsal root ganglion 48 h post CFA injection.

3 The effects of a steroidal (dexamethasone) and a non-steroidal (indomethacin) anti-inflammatory drug on the levels of NGF and IL-1β in inflamed tissue were investigated and compared with alterations in behavioural hyperalgesia and neuropeptide expression in sensory neurones.

4 Systemic dexamethasone (120 μg kg^{-1} per day starting the day before the CFA injection) had no effect on the inflammatory hyperalgesia. When the dose was administered 3 times daily, a reduction in mechanical and to a lesser extent thermal sensitivity occurred. Indomethacin at 2 mg kg^{-1} daily (i.p.) had no effect on the hyperalgesia and a dose of 4 mg kg^{-1} daily was required to reduce significantly mechanical and thermal hypersensitivity.

5 The increase in NGF produced by the CFA inflammation was prevented by both dexamethasone and indomethacin, but only at the higher dose levels. Dexamethasone at the lower and higher dose regimes diminished the upregulation of IL-1β whereas indomethacin had an effect only at the higher dose.

6 The increase in SP and CGRP levels produced by the CFA inflammation was prevented by dexamethasone and indomethacin at the lower and higher dose regimes.

7 Intraplantar injections of IL-1β (0.01, 0.1 and 1 ng) produced a brief (6 h) thermal hyperalgesia and an elevation in cutaneous NGF levels which was prevented by pretreatment with human recombinant IL-1 receptor antagonist (IL-1 ra) (0.625 μg, i.v.). The thermal hyperalgesia but not the NGF elevation produced by intraplantar IL-1β (1 ng) was prevented by administration of a polyclonal neutralizing anti-NGF serum.

8 IL-1 ra significantly reduced the mechanical hyperalgesia produced by CFA for 6 h after administration as well as the CFA-induced elevation in NGF levels. Anti-NGF pretreatment substantially reduced CFA-induced mechanical and thermal hyperalgesia without reducing the elevation in IL-1β.

9 Intraplantar NGF (0.02, 0.2 and 2 μg) injections produced a short lasting thermal and mechanical hyperalgesia but did not change IL-1β levels in the hindpaw skin.

10 Our results demonstrate that IL-1β contributes to the upregulation of NGF during inflammation and that NGF has a major role in the production of inflammatory pain hypersensitivity.

Keywords: Pain; inflammation; neurotrophins; cytokines; sensory neurone; neuropeptides; interleukin-1β; nerve growth factor; NSAIDs; steroids

Introduction

Inflammation is associated with a complex pattern of local and systemic changes including inflammatory cell migration, cytokine release, oedema, erythema, release of acute phase proteins, fever, pain and hyperalgesia. The precise molecular events responsible for the sensory changes at the site of the inflammation and surrounding tissue are not yet fully understood, but changes both in the transduction sensitivity of the high threshold nociceptors and in excitability in the central nervous system secondary to the activation of chemosensitive nociceptors by inflammatory mediators are involved (Treede *et al.*, 1992; Reeh, 1994). The mediators responsible include K^+, H^+, ATP, arachidonic acid derivatives, cytokines, bradykinin, tachykinins, 5-hydroxytryptamine and histamine, operating together in a synergistic way (Treede *et al.*, 1992; Reeh, 1994). Recently it has become apparent that the neurotrophin nerve growth factor (NGF) also plays a major role in the production of inflammatory hyperalgesia (Woolf *et al.*, 1994; Lewin *et al.*, 1994).

The role of NGF in the development and maintenance of peripheral sympathetic and nociceptive sensory neurones is well established (Johnson, Jr. *et al.*, 1986; Barde, 1989; Korsching, 1993). NGF is produced in the peripheral target, binds to a high affinity receptor tyrosine kinase, *trkA*, on the neurone and after internalization is retrogradely transported to the cell body where, by activation of second messenger signals

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and changes in transcription factor expression, it controls the survival, growth and phenotype of immature neurones (Rich *et al.*, 1987; Matsuda *et al.*, 1988; Barde, 1989; Chao, 1992; Glass & Yancopoulos, 1993). In addition to this specific neurotrophic action during development, a constant supply of NGF from the periphery may be important for the maintenance of normal phenotype in *trkA* receptor expressing nociceptive adult primary sensory neurones (Lindsay & Harmar, 1989). Removal of NGF results in a down regulation of several transmitters and proteins (Rich *et al.*, 1984; Lindsay *et al.*, 1989; Gold *et al.*, 1991) while an excess results in abnormal sensitivity (Lewin & Mendell, 1993). Systemic NGF administration, for example, induces thermal and mechanical hyperalgesia in the neonatal and adult rat (Lewin *et al.*, 1993) while intraplantar NGF produces localized thermal hyperalgesia in the adult rat (Woolf *et al.*, 1994).

NGF levels have been found to be elevated during inflammation in inflammatory cells (mast cells) inflammatory exudates (ascites/synovial fluid), inflamed skin and in the nerves innervating inflamed tissue (Weskamp & Otten, 1987; Varilek *et al.*, 1992; Donnerer *et al.*, 1992; Leon *et al.*, 1994; Woolf *et al.*, 1994). We have found that the hyperalgesia generated by the inflammation resulting from complete Freund's adjuvant (CFA) injection, is significantly reduced by systemic administration of a specific anti-NGF antiserum (Woolf *et al.*, 1994), implying that NGF contributes directly or indirectly to inflammatory sensitivity changes. The indirect effects of NGF may result from its cytokine-like actions, including stimulation of growth and differentiation of human B lymphocytes (Otten *et al.*, 1987; Thorpe *et al.*, 1988; Treede *et al.*, 1992), the release of inflammatory mediators from lymphocytes and basophils (Bischoff & Dahinden, 1992; Horigome *et al.*, 1993) and a degranulation of mast cells (Aloe & Levi-Montalcini, 1977; Bohm *et al.*, 1986; Mazurek *et al.*, 1986; Pearce & Thompson, 1986). Direct effects could either be due to a *trkA* receptor-tyrosine kinase-mediated phosphorylation at the nociceptor terminal, increasing transduction sensitivity, or a consequence of a change in the expression of transmitter/neuromodulators such as substance P and calcitonin gene-related peptide (CGRP) in the cell body (Lindsay & Harmar, 1989; Woolf *et al.*, 1994), amplifying the central actions of the nociceptor in the spinal cord (Lewin *et al.*, 1994). Substance P and CGRP are present in C-fibres and coexist in up to 20% of dorsal root ganglion (DRG) neurones (Lee *et al.*, 1985). Expression of both peptides is modulated by NGF in adult sensory neurone cultures (Lindsay & Harmar, 1989; Lindsay *et al.*, 1989), and both are upregulated during inflammation (Noguchi *et al.*, 1988; Donaldson *et al.*, 1992; Donnerer *et al.*, 1992; Smith *et al.*, 1992; Woolf *et al.*, 1994). Anti-NGF prevents the inflammatory increase in the peptides (Donnerer *et al.*, 1992; Woolf *et al.*, 1994). These neuropeptides are released from the central terminals of C-fibres and have excitatory effects on dorsal horn neurones controlling the gain of nociceptive transmission (Woolf, 1991). Whether the role of NGF in inflammatory hyperalgesia is related to alteration in neuropeptide levels in sensory neurones is not known.

A key issue is what controls the production of NGF during inflammation. One candidate is interleukin-1β (IL-1β), a pleiotropic cytokine, involved in a variety of inflammatory responses (Movat, 1987; Dinarello, 1991; Bianchi *et al.*, 1992). IL-1β is produced by activated macrophages and a large variety of other cell types including B-lymphocytes and endothelial cells (Libby *et al.*, 1986; Dinarello, 1991) and its administration causes hyperalgesia (Ferreira *et al.*, 1988; Schweizer *et al.*, 1988; Ferreira, 1993; Fukuoka *et al.*, 1994; Watkins *et al.*, 1994). IL-1β can, in addition, induce NGF production in a number of cell types (Bandtlow *et al.*, 1987; Heumann *et al.*, 1987; Lindholm *et al.*, 1987; 1988; Matsuoka *et al.*, 1991; Yoshida & Gage, 1992). The aim of this study was to determine whether IL-1β is responsible for the upregulation of NGF during inflammation and to establish the role it and NGF play in the development of inflammatory hyperalgesia.

Methods

Animal models

Experiments were carried out on adult male Sprague Dawley rats (180–250 g). Inflammation was induced by the subcutaneous injection of 100 µl of Freunds Complete Adjuvant (CFA), (Sigma), into the plantar surface of the left hind paw under fluothane (ICI) anaesthesia (induction 4%, maintenance 2%). The CFA injection produced an area of localized erythema and oedema that did not disturb weight gain, grooming, the sleep-wake cycle or social interactions. Saline (100 µl) was injected as a control.

Behavioural tests

Mechanical hyperalgesia was assessed with a set of Von Frey hairs (4.1 to 72 g). The minimum force required to elicit a reproducible flexor withdrawal reflex on each of 3 applications of the Von Frey hairs to the dorsal surface of the toes was measured (Woolf *et al.*, 1994; Sivilotti & Woolf, 1994). Thermal hypersensitivity was assessed by the hot plate technique, measuring the time for foot withdrawal on contact with a metal plate at 50°C. Oedema was scored on a scale from 0 (no swelling) to 5 (swelling on plantar and dorsal surface of hindpaw and all toes).

Experimental procedures

Each experimental group comprised 4 or 5 animals. Behavioural measurements were made immediately before an intraplantar injection of either CFA or saline (preinjection, time 0) and 6, 24 and 48 h post injection. The animals were terminally anaesthetized with sodium pentobarbitone (Duphar) at 48 h, and the L4 dorsal root ganglion (DRG) and entire hind paw skin, ipsilateral and contralateral to the inflammation removed. The tissue samples were weighed, frozen on dry ice, stored at –70°C and subsequently processed for SP, CGRP, IL-1β and NGF determinations.

Drug administration

Based on the literature, dexamethasone (David Bull Laboratories) was administered at 120 µg kg^{-1} body weight in 100 µl saline (intramuscularly in the right thigh) in two protocols. In the first protocol (lower dose) it was given once daily –24 h and –1 h before, and +24 h post CFA or saline injection. In the second protocol (higher dose), the same dose of dexamethasone was administered three times daily at –24 h, –21 h, –18 h, –1 h, +2 h, +5 h, +24 h, +27 h and +30 h (Shive & Thrall, 1991; Thompson *et al.*, 1994). Similar experiments were performed with indomethacin (Sigma), which was administered intraperitonealy in 100 µl in 40% ethanol. In one group 2 mg kg^{-1} body weight (lower dose) (Taiwo *et al.*, 1991) was administered at time –24 h, –1 h before, and +24 h post CFA or saline injection. In another, 4 mg kg^{-1} body weight (higher dose) was administered at the same time intervals. A control group received the vehicle. Recombinant human interleukin-1β (100,000 iu μg^{-1} NIBSC) was dissolved in saline and administered by intraplantar injection (100 µl). Recombinant human interleukin-1 receptor antagonist (Synergen) was administered as a bolus of 0.625 µg in 0.1 ml saline i.v. 30 min before CFA or IL-1β injection followed by the same dose i.p. 6 h later. Nerve growth factor (Promega) was also dissolved in saline and injected in a volume of 100 µl into the hindpaw. Two anti-NGF antibodies were used, a monoclonal antibody (23c4) (Weskamp & Otten, 1987) or a polyclonal sheep antimouse antibody (Woolf *et al.*, 1994) 5 µl g^{-1} i.p., administered 1 h before the injection of CFA and again 24 h later. The antiserum was tested in a chick DRG neurite outgrowth assay and found to neutralize the

growth produced by NGF (1:4,000 10 ng ml^{-1} NGF) but not BDNF or NT3. IgG enrichment was performed by ammonium sulphate precipitation and dialysis.

Assays

Prior to assay, skin samples were homogenized in phosphate buffered saline (PBS) containing; 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin (BSA), 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI ml^{-1} aprotinin (Sigma). The supernatant was used to measure both NGF and IL-1β levels.

NGF assay

NGF was measured by a two-site enzyme-linked immunoassay (ELISA), based on the method of Weskamp & Otten (1987), with minor modifications: briefly, polystyrene high binding 96 well microtiter plates (Nunc), were coated with a polyclonal rabbit anti-NGF (raised against h.p.l.c. purified mouse NGF and shown by neurite outgrowth assays not to neutralize BDNF or NT3) in carbonate buffer (1/20000). After overnight incubation at 4°C, the plates were washed (as in subsequent steps) with PBS containing 0.05% Tween 20 and 0.4 M NaCl and then incubated with PBS-0.5% BSA for 1 h at 37°C to block non-specific binding. After washing, NGF standard (Promega) and samples (100 µl of each) were added in duplicate to the plates and incubated overnight at 4°C. After washing the plates, 100 µl of rat anti-NGF (1/20000) monoclonal antibody (23c4) (Weskamp & Otten, 1987) was added to each well and the plates incubated overnight at 4°C. Colour was developed with a biotinylated rabbit anti-rat IgG (Zymed) and peroxidase conjugated streptavidin (Dako) system. The substrate solution was prepared with 3,3',5,5-tetramethylbenzidine (TMB) (Sigma) and the optical density (OD) measured at 450 nm. Results from inflamed skin are expressed as ng/hind paw, since the weight of the inflamed paw skin can increase more than twofold.

IL-1β assay

This was measured in the same supernatant also using a two-site ELISA based upon a protocol described previously (Taktak *et al.*, 1991). Immunoaffinity purified sheep polyclonal anti-rat IL-1β antibodies, raised against recombinant rat IL-1β, (2 µg ml^{-1}) in 100 µl PBS buffer, was used to coat microtiter plates (Nunc Maxisorb). After incubation (4°C overnight) and washing the plates in assay buffer (0.01 M phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.2), 100 µl of standard (recombinant rat IL-1β a generous gift from Dr Robert Newton, DuPont-Merk, Wilmington, Delaware, U.S.A.) or sample, was added to each well and incubated overnight at 4°C. After washing the plates, 100 µl of biotinylated, immunoaffinity purified polyclonal sheep anti-rat IL-1β (1/1000) with 1% normal sheep serum, was added to the plates and incubated for 1 h at room temperature. The colour was developed by use of avidin-peroxidase (Dako) and the chromogen, orthophenylene diamine (Sigma) and the OD measured at 490 nm. Results are expressed as ng/hind paw.

SP and CGRP assay

These peptides were extracted from the DRG samples into 0.05 M acetic acid in polypropylene tubes in a boiling water bath for 15 min. The samples were homogenized, centrifuged and the supernatant lyophilized. An inhibition ELISA was used to measure SP and CGRP (Stjernschatz *et al.*, 1982). To coat the plates with SP, the peptide was conjugated to poly D-glutamate, using 1-ethyl-3-(dimethylaminopropyl)-carbodiimide. High binding microtiter plates (Beckman) were coated with CGRP (0.02 µg ml^{-1}) and conjugated SP (0.02 µg ml^{-1}) in carbonate buffer (100 µl) and incubated overnight at 4°C. To block non-specific binding, these plates, after washing with PBS/Tween, were incubated with PBS-1% BSA for 1 h at 37°C. The lyophilized samples were reconstituted in PBS/Tween buffer, containing 0.1% gelatin and 0.001% aprotinin (Sigma). This buffer was also used to prepare the serially diluted standard curve. In another set of low binding, polypropylene plates (Costar), 60 µl of standard (SP or CGRP) or sample were incubated with 60 µl of polyclonal rabbit anti-SP; 1/100000 (Sera Lab) or polyclonal rabbit anti-CGRP, 1/120000 (Cambridge Research Biochemicals) antisera at 4°C overnight. The following day, 100 µl from each well in the low binding plate was transferred to the corresponding wells in the high binding plates, coated with the corresponding peptide and blocked for non-specific binding. The wells were incubated at 4°C overnight, washed and 100 µl of biotinylated anti-rabbit IgG was added to each well. The plates were incubated for 2 h at 37°C and subsequently the streptavidin-peroxidase system used to obtain colour reaction as described above.

Statistical analysis

Data are presented as the mean ± s.e.mean. Differences between experimental groups and controls were analysed by one-way analysis of variance (ANOVA) followed by Dunnet's Multiple Comparison test or the Repeated Measure ANOVA followed by Tukey-Kramer Multiple Comparison test.

Results

Behaviour

Naive and saline-injected control animals exhibited a high mechanical threshold for initiating paw withdrawal (69.3 ± 2.7 g) and required over 20 s contact to withdraw from the 50°C hot plate (21.6 ± 1.9 s). CFA-induced inflammation of the hindpaw produced both mechanical and thermal hyperalgesia (Figure 1) manifesting as a substantial reduction in the Von Frey hair mechanical threshold for eliciting withdrawal (9.4 ± 0.9 g, at 48 h, $P < 0.0001$) and in the hot plate paw withdrawal latency (8.2 ± 0.7 s at 48 h, $P < 0.0001$).

Dexamethasone injection, using the lower and higher dose protocols in animals given intraplantar saline, had no effect on any behavioural measurement. The lower dose dexamethasone procotol failed to change significantly the thermal or mechanical hyperalgesia produced by the CFA-induced inflammation, although a decrease in the swelling score (at 48 h) occurred (1.9 ± 0.1 vs 3.2 ± 0.2 in CFA treatment without steroid). The higher dose dexamethasone protocol significantly decreased the CFA-induced mechanical hyperalgesia (Figure 1) and further reduced the swelling score to 1.0 ± 0.2. Thermal hyperalgesia was not significantly reduced except to a limited extent at 48 h ($P < 0.01$) (Figure 1).

Indomethacin injection (lower dose and higher dose protocol) in saline-injected animals, had no effect in any of the behavioural tests. The lower dose indomethacin protocol had no significant affect on the mechanical or thermal hyperalgesia or swelling, produced in the ipsilateral hind paw by an intraplantar CFA injection (Figure 1). The higher dose indomethacin protocol significantly reduced CFA-induced mechanical and thermal hyperalgesia (Figure 1) and reduced the swelling score to 0.8 ± 0.15 in the ipsilateral hind paw. Indomethacin vehicle injections were without effect.

NGF levels in the hind paw

Both dexamethasone and indomethacin at the lower and higher dose levels, when administered to animals injected with intraplantar saline, had no effect on the baseline NGF levels measured in the hind paw 48 h post-saline injection. CFA injected on its own produced a significant increase in NGF levels in the skin of the ipsilateral hind paw 48 h post injection (Figure 2). Dexamethasone in the lower dose protocol did not prevent the CFA-induced increase in NGF levels but the

higher dose dexamethasone protocol did (Figure 2). Similar results were obtained with indomethacin. The higher dose protocol prevented the CFA-induced increase in NGF levels (Figure 2).

IL-1β levels in the hind paw

CFA-induced inflammation resulted in a substantial increase in IL-β levels in the ipsilateral hind paw 48 h postinjection (Figure 3). Dexamethasone treatment at the lower and higher dose levels significantly reduced the elevated IL-β levels in the ipsilateral hind paw in a non dose-dependent fashion but did not eliminate it (Figure 3). Indomethacin in the lower dose protocol did not prevent the elevation in IL-1β level but at the higher dose level there was a significant reduction in the levels of this cytokine (Figure 3).

SP and CGRP levels

Dexamethasone and indomethacin administration at the two dose levels had no effect on neuropeptide levels in the DRG of saline-injected animals. CFA caused a significant increase, 48 h after injection, in SP and CGRP in the ipsilateral DRG, when compared with the ipsilateral DRG of animals that received saline only (Figure 4) or the levels in the contralateral DRG. Dexamethasone and indomethacin at both dose levels prevented the CFA-induced increase in SP and CGRP (Figure 4).

IL-1β and IL-1ra

Intraplantar injections of IL-1β (1, 100 pg and 1 ng) produced a transient reduction in the withdrawal time to the hotplate test with a relative decrease in reaction time of $-20 \pm 9.8\%$ (1 pg), $-30 \pm 6.2\%$ (100 pg) and $-24 \pm 3.5\%$ (1 ng) at 3 h and -14.3 ± 11, -16 ± 5.3 and $-12.2 \pm 5\%$ respectively at 6 h. For these three doses the thermal reaction time had returned to baseline levels by 24 and 48 h. IL-1β (10 ng) produced a decrease of $-44.7 \pm 6.4\%$ at 3 h with maintained reductions at 6 h of $-25.8 \pm 10\%$, at 24 h of $-22 \pm 10.2\%$ and at 48 h, $-26 \pm 10.9\%$. IL-1β did not, at any of these doses, produce mechanical hyperalgesia. The thermal hypersensitivity produced by IL-1β (1 ng) was completely blocked by prior i.v. administration of 0.625 μg IL-1ra. IL-1ra administered by itself had no effect on behavioural sensitivity (data not shown).

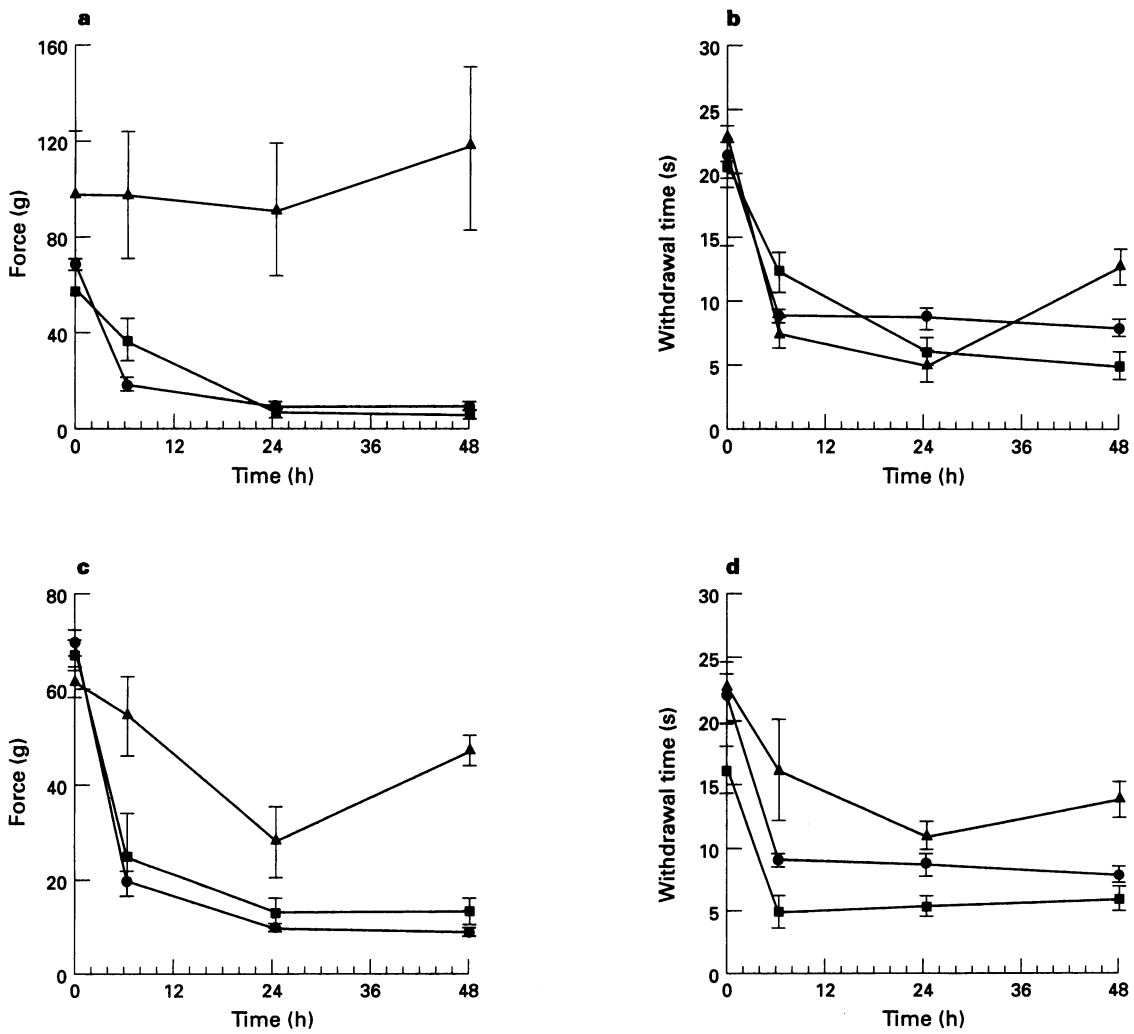

Figure 1 Changes in mechanical (a, c) and thermal (b, d) sensitivity produced by CFA-induced inflammation (●) in the hindpaw of rats and the effects of the lower (■) and higher (▲) dose regimens of dexamethasone (a, b) or indomethacin (c, d) treatment. The lower dose of dexamethasone was (120 μg kg^{-1}, daily) the higher (120 μg kg^{-1} 8 h^{-1}), the lower dose of indomethacin was (2 mg kg^{-1}, daily) and the higher (4 mg kg^{-1}, daily). The higher dose of dexamethasone significantly reduced mechanical hyperalgesia measured by Von Frey mechanical threshold for eliciting the flexion withdrawal reflex, ($P < 0.0001$) without any significant effect on thermal hyperalgesia measured by the hot-plate test except at 48 h. The higher dose indomethacin reduced mechanical hyperalgesia ($P < 0.0001$) and thermal hyperalgesia ($P < 0.005$). Data are mean ± s.e.mean of 5 experiments.

An intraplantar injection of IL-1β (1 ng) produced a significant elevation in NGF levels in the skin, measured at 48 h, which was prevented by IL-1ra administration (0.625 μg i.v., 30 min before and 0.625 μg i.p. 6 h after the IL-1β injection) (Figure 5), IL-1ra by itself had no effect on basal levels of NGF.

IL-1ra and CFA inflammation

Pretreatment with IL-1ra (0.625 μg) i.v., 30 min before intraplantar CFA administration significantly reduced the inflammatory mechanical hyperalgesia at 3 and 6 h (Figure 6). The treatment had no significant effect on the thermal hy-

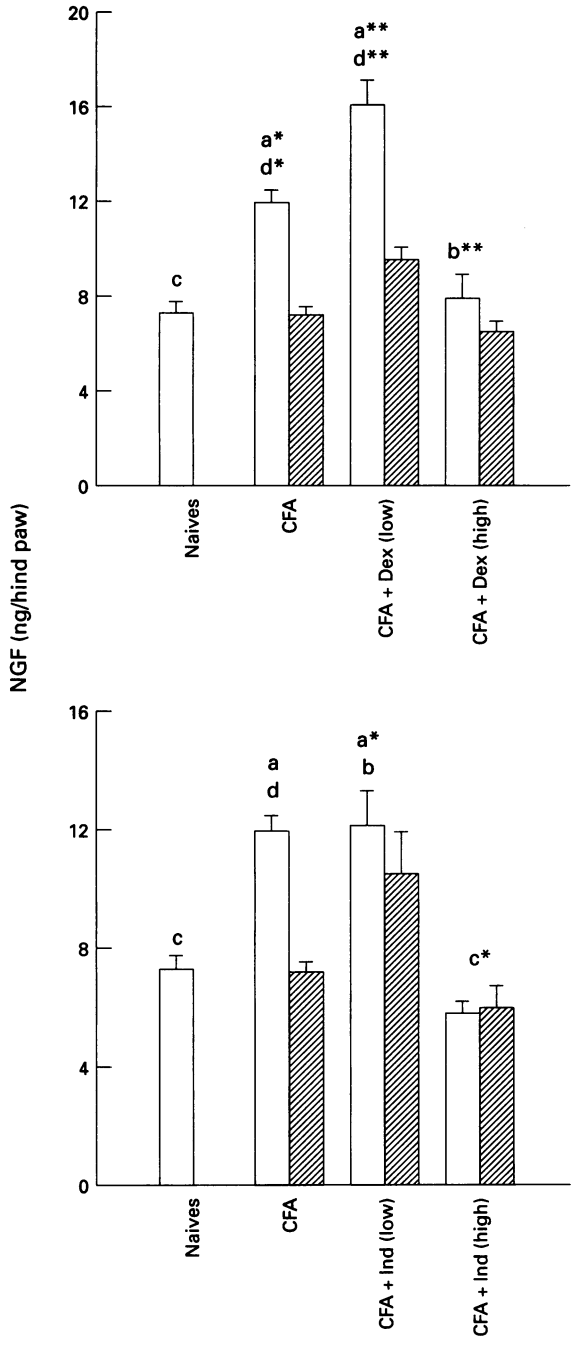

Figure 2 The inflammation generated by intraplantar CFA injection caused a significant increase in nerve growth factor (NGF) levels, measured 48 h after the CFA injection, in the ipsilateral hind paw (open columns) compared with contralateral hindpaw (hatched columns). The lower dose dexamethasone (Dex) treatment had no significant effect on NGF level but the higher does regime prevented the increase (Upper panel). The lower dose indomethacin (Ind) had no significant effect on NGF levels in the ipsilateral hindpaw, but the higher dose prevented the increase (lower panel). Data are mean ± s.e.mean of 5 experiments. $^{a}P<0.05$, $^{a**}P<0.01$ and $^{a***}P<0.001$ vs naive; $^{b}P<0.05$, $^{b**}P<0.001$ low vs high doses; $^{c}P<0.05$, $^{c*}P<0.01$ vs CFA; $^{d*}P<0.01$, $^{d**}P<0.001$ ipsi vs contra.

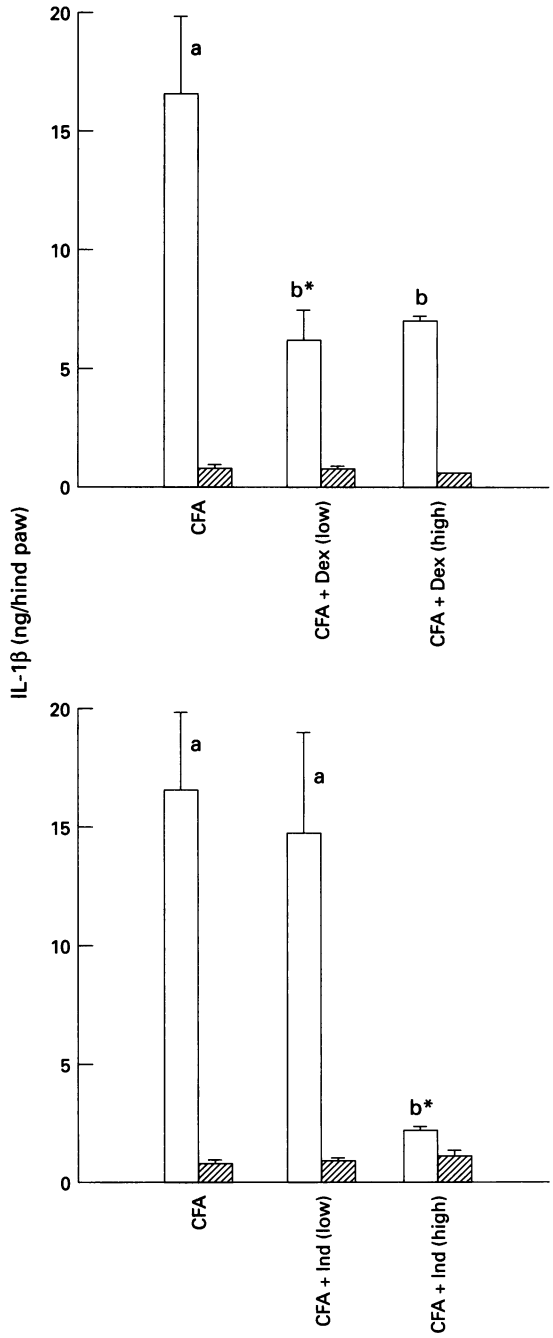

Figure 3 The inflammation generated by intraplantar CFA injection caused a significant increase in interleukin-1β (IL-1β) level in the ipsilateral paw (open column) at 48 h post CFA injection compared with the contralateral hindpaw (hatched column). Both the lower and higher dose dexamethasone (Dex) treatments significantly reduced IL-1β level in the ipsilateral hind paw (upper panel). The lower dose indomethacin (Ind) has no significant effect on IL-1β level but the higher dose level, significantly reduced the level of this cytokine in the ipsilateral paw (lower panel). Data are mean ± s.e.means of 5 experiments. $^{a}P<0.001$ ipsi vs contra; $^{b}P<0.05$, $^{b*}P<0.01$ vs ipsi CFA.

persensitivity produced by the CFA (data not shown). The elevation of NGF produced by the CFA, measured at 48 h, was blocked by the IL-1ra administration (Figure 6). When IL-1ra was administered for the first time 3 days after the CFA injection, when mechanical and thermal hypersensitivity were fully established, it produced a temporary (1.5 h) blip in the mechanical sensitivity with an increase in mechanical threshold from 10.9±0.9 to 28.6±10 g ($P<0.05$) with return at 3 and 6 h to the normal high levels of mechanical sensitivity. No significant change in thermal responsiveness was detected.

NGF and anti-NGF

Intraplantar NGF (2, 200, 2000 ng) produced an increase in thermal and mechanical hypersensitivity at 6 but not 24 h, with a % reduction in the force required to elicit a flexion reflex of −31.6±12 for 2 ng, −69.1±4.2 for 200 ng and −49.6±12.5% for 2 μg and a reduction in the thermal reaction time of −28.8±3.8, −65.5±4.7 and −55.8±7.2% for these doses respectively at 6 h. NGF injection (2 μg) produced no change in IL-1β levels (890±67 vs 570±50 pg/hindpaw for a saline injection, measured at 48 h).

As reported before (Woolf *et al.*, 1994), the behavioral effects of the NGF could be prevented by prior administration of an anti-NGF antibody (5 μl g^{-1} i.p., administered 1 h before the injection of NGF). This anti-NGF antibody also prevented the behavioural effects of intraplantar IL-1β (1 ng), but had no effect on the elevation in NGF levels produced by the IL-1β (Figure 5). We have previously reported that this polyclonal anti-NGF antibody substantially reduces the mechanical and thermal hypersensitivity produced by CFA-induced inflammation (Woolf *et al.*, 1994) and we now report that it fails to reduce the elevation in the levels of IL-1β 48 h after CFA (5.55±1.1 ng/hindpaw ipsilateral to the CFA and 0.62±0.06 ng/hindpaw contralateral). In order to confirm that the effect on the CFA-induced hyperalgesia was due to

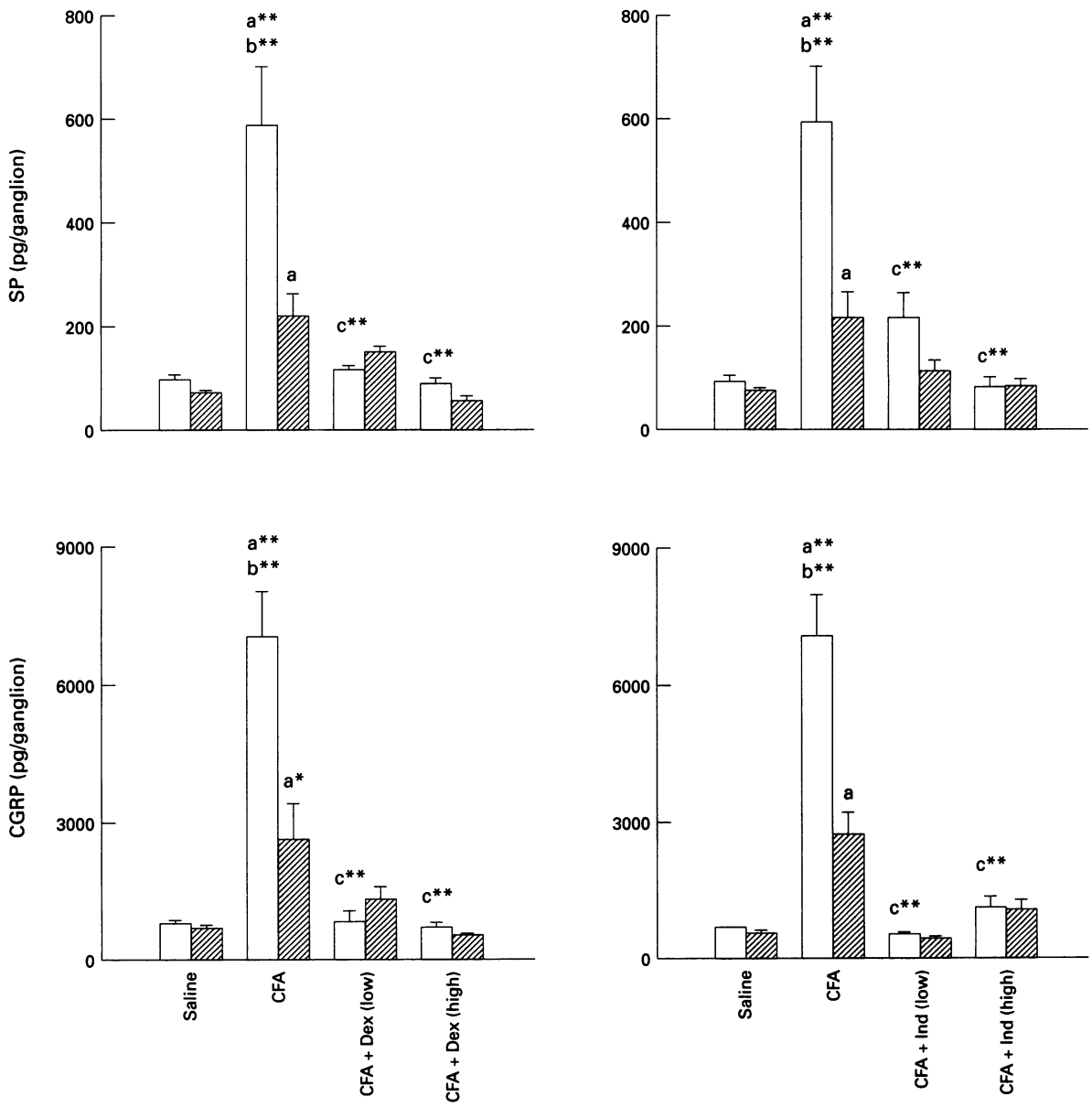

Figure 4 CFA induced inflammation caused a significant increase in substance P (SP, upper panels) and calcitonin gene-related peptide (CGRP, lower panels) levels in the ipsilateral L4 DRG (open columns) measured at 48 h, compared with the contralateral side (hatched columns) or with animals receiving vehicle instead of CFA . Both the lower dose and the higher dose dexamethasone (Dex, left hand columns) and indomethacin (Ind) treatment (right hand columns) prevented the CFA-induced increase in SP and CGRP levels. Data are mean±s.e.means of 5 experiments. $^{a}P<0.05$, $^{a**}P<0.001$ vs saline; $^{b**}P<0.001$ ipsi vs contra; $^{c**}P<0.001$ vs CFA.

the neutralization of NGF, we administered a monoclonal anti-NGF antibody (23c4) (Weskamp & Otten, 1987) (5 μl g^{-1}, i.p. in a concentration that inhibited NGF induced dorsal root ganglion neurite outgrowth at 1:10000) and this too completely prevented the establishment of mechanical hyperalgesia (control mechanical thresholds 67±4.5 g, 24 h post CFA without anti-NGF 8±0.4 g, with anti-NGF 63±4.6 g). Thermal hypersensitivity was also eliminated by the monoclonal anti-NGF (control thermal reaction time 21.6±1.8 s, 24 h post CFA without anti-NGF 6±0.7 s, with anti-NGF 19±1.5 s).

Discussion

The acute inflammation in the hindpaw generated by intraplantar CFA injection results in a significant increase in IL-1β and NGF levels in the inflamed tissue with a relatively much greater change in the former. The inflammation also results in an elevation in the levels of neuropeptides in the primary sensory neurones innervating the inflamed hindlimb

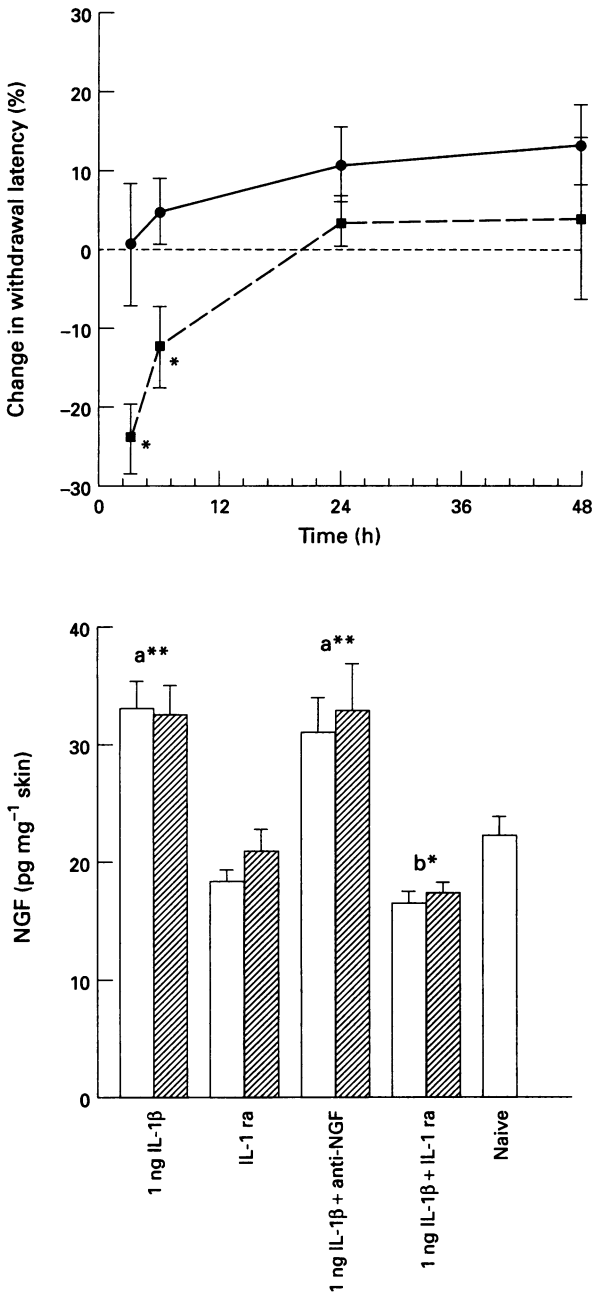

Figure 5 Upper panel: an intraplantar injection of 1 ng interleukin 1β (IL-1β, ■) produced a significant increase in thermal sensitivity 3 and 6 h post injection which was completely prevented by administration of 5 μl g^{-1} of anti-nerve growth factor (antiNGF) i.p. 1h before (●). The asterisks represent significant differences of $P<0.01$. Lower panel: intraplantar IL-1β elevated NGF levels in the skin both ipsi- (open columns) and contralateral (hatched columns) to the injection site when measured 48 h later. This was prevented by prior i.v. administration of 0.625 μg IL-1 receptor antagonist (IL-1ra). Anti-NGF administration (5 μl g^{-1}, i.p. 1 h before) did not affect the increase in NGF levels. Data are mean±s.e.means of 4 experiments. $^{a}**P<0.001$ vs naive; $^{b}*P<0.01$ vs IL-1β.

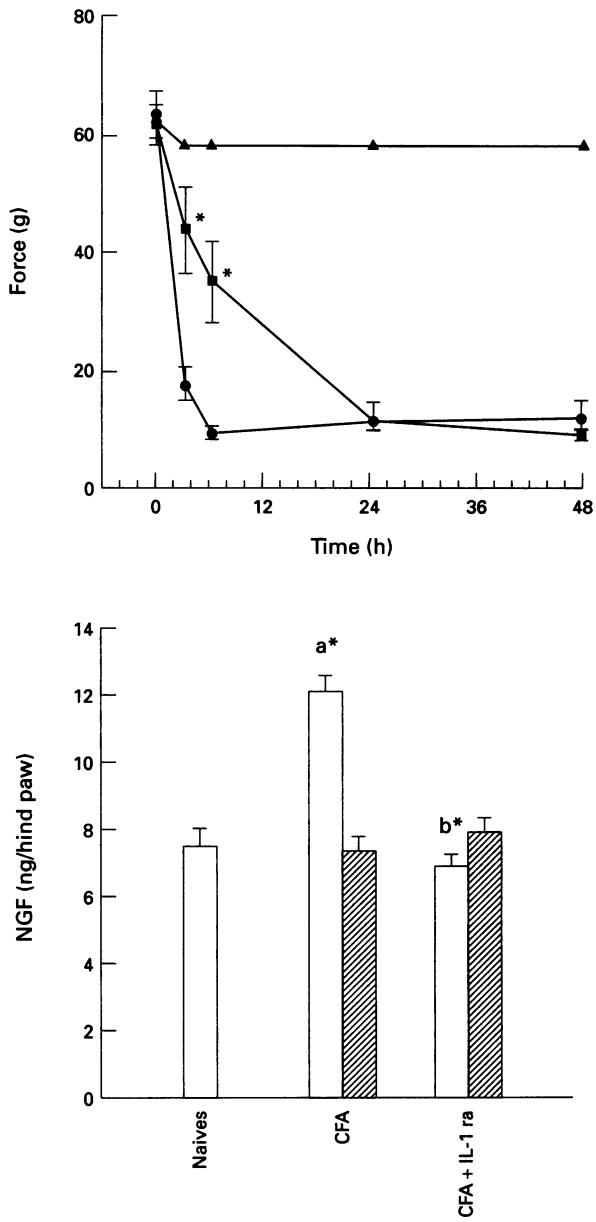

Figure 6 Upper panel: Interleukin-1 (IL-1) receptor antagonist (IL-1ra), 0.625 μg i.v. 30 min before and i.p. 6 h after intraplantar CFA (■) reduced the mechanical hyperalgesia at 3 and 6 h produced by CFA-induced inflammation (●). The receptor antagonist by itself had no effect on mechanical sensitivity (▲). The symbols represent significant differences of $*P<0.01$. Lower panel: IL-1ra pretreatment prevented the elevation in nerve growth factor (NGF) levels produced by CFA injection; open columns, ipsilateral; hatched columns, contralateral. $^{a}*P<0.01$ vs naive, $^{b}*P$ vs CFA.

and in substantial thermal and mechanical hyperalgesia. A key issue is whether these changes are causally related.

Several cell types have been shown to produce IL-1β including blood monocytes, tissue macrophages, blood neutrophils, endothelial cells, smooth muscle cells, fibroblasts, dermal dendritic cells, keratinocytes, T and B lymphocytes (Libby *et al.*, 1986; Dinarello, 1991); also, IL-1β levels are raised during inflammation (Arai *et al.*, 1990; Dawson *et al.*, 1993), which we now confirm. Systemic IL-1β produces a thermal hyperalgesia reported to be non-prostaglandin dependent and mediates the hyperalgesia generated by lipopolysaccharide (Watkins *et al.*, 1994). IL-1β administration into the hindpaw has been reported to produce a dose-dependent increase in sensitivity to low intensity mechanical pressure in rats measured by a modified Randall-Sellito test (Ferreira *et al.*, 1988). This finding differs from our own in that we could show thermal hyperalgesia but no change in the mechanical threshold for eliciting a flexion withdrawal reflex after intraplantar IL-1β. This difference is probably related to the different nature of the tests and endpoints used. We have demonstrated, though, that blockade of IL-1β action during the establishment of CFA inflammation reduces acute mechanical hyperalgesia with a smaller effect on established inflammatory hyperalgesia. The mechanism of IL-1β-induced changes in sensitivity has been variously argued to act via prostaglandin production (Ferreira *et al.*, 1988; McQuay *et al.*, 1988; Crestani *et al.*, 1991, but see Follenfant *et al.*, 1989; Watkins *et al.*, 1994), via the induction of B_1 bradykinin receptors (Perkins & Kelly, 1994), or as a result of a direct activation of nociceptors (Fukuoka *et al.*, 1994). Another possibility is that IL-1β produces its effects by upregulating an active intermediary such as NGF. IL-1β up-regulates NGF in cultured fibroblasts (Lindholm *et al.*, 1987; 1988; Matsuoka *et al.*, 1991) and NGF has been shown to produce hyperalgesia when administered either systemically (Lewin *et al.*, 1993) or locally (Woolf *et al.*, 1994). We have now shown that IL-1β can induce NGF levels in the skin and that neutralizing the action of NGF blocks the hyperalgesic actions of the IL-1β. IL-1β regulates substance P levels in sympathic ganglion cells (Hart *et al.*, 1991) through the induction of a neuropoietic cytokine leukaemia inhibitory factor (LIF) (Shadiack *et al.*, 1993). IL-β could alter neuropeptide expression in primary sensory neurones secondary to an increase in NGF levels since NGF has well known actions on the regulation of both substance P and CGRP in adult DRG neurones (Lindsay & Harmar, 1989; Lindsay *et al.*, 1989; Woolf *et al.*, 1994). Certainly anti-NGF antibodies decrease the elevation in substance P and CGRP seen with CFA-inflammation (Donnerer *et al.*, 1992; Woolf *et al.*, 1994), even though they do not block IL-1β upregulation.

NGF levels are substantially elevated during inflammation (Weskamp & Otten, 1987; Varilek *et al.*, 1992; Donnerer *et al.*, 1992; Woolf *et al.*, 1994). The specific signalling mechanisms and cell types responsible are not known, although, our data points to a major role for IL-1β in increasing NGF production in that IL-1ra (Dinarello, 1991; Eisenberg *et al.*, 1990) significantly reduced the elevation in NGF produced both by IL-1β injections and CFA inflammation. A number of other growth factors/inflammatory mediators may also be important. Platelet-derived growth factor (PDGF), acidic and basic fibroblast growth factor (a and bFGF), tumour necrosis factor (TNFα), epidermal growth factor (EGF), and transforming growth factor (TGFα and β) all increase NGF production in fibroblasts *in vitro* (Matsuoka *et al.*, 1991; Hattori *et al.*, 1993). The increase in NGF during CFA-induced inflammation (Donnerer *et al.*, 1992; Woolf *et al.*, 1994) is important in mediating hyperalgesia because a polyclonal anti-NGF anti-serum injected before or after CFA, significantly reduced the increase in sensitivity seen when CFA is administered on its own (Woolf *et al.*, 1994). We have now extended these observations by showing that a monoclonal anti-NGF antibody produces the same anti-hypersensitivity effects.

Whether the increase in peptides in the sensory neurones generated during inflammation actually contributes to the behavioural sensory changes is not known. Nociceptive inputs by virtue of the release of substance P and CGRP, have the capacity to increase the excitability of spinal neurones changing their response characteristics and this phenomenon, called central sensitization, has been shown to play a major role in the generation of post-injury pain hypersensitivity states (Woolf, 1983; 1991). NGF, by increasing the level of these peptides in the sensory neurone, may increase the capacity of afferents innervating inflamed tissue to produce central sensitization. The changes in peptide expression seen in the DRG during inflammation (Noguchi *et al.*, 1988; Donaldson *et al.*, 1992; Donnerer *et al.*, 1992; Smith *et al.*, 1992; Woolf *et al.*, 1994) are reflected in an increase in levels in the dorsal horn (Oku *et al.*, 1987; Schaible *et al.*, 1990) and by an increase in peptide receptor/binding sites in this area (Kar *et al.*, 1993; Schafer *et al.*, 1993; Stucky *et al.*, 1993). The fact that dose regimes of both dexamethasone and indomethacin that did not prevent hyperalgesia nevertheless suppressed peptide levels in the DRG, implies that the changed level of peptides in the DRG may not directly reflect acute behavioural sensitivity changes. A caveat must be that levels in the DRG reflect a dynamic interplay between production and transport from the soma. A similar argument could be made about the contribution of IL-1β to inflammatory hypersensitivity. The lower dose regime of dexamethasone substantially reduced (but did not eliminate) the IL-1β increase that followed CFA, but had no effect on the behavioural hypersensitivity. One correlation in the present study that appeared to be consistent was a decrease in inflammatory mechanical hypersensitivity and a reduction in NGF, which was achieved by the higher dose dexamethasone and indomethacin regimes and IL-1ra. This may reflect both a 'ceiling effect' of IL-1β on the upregulation of NGF and that thermal and mechanical hypersensitivity are independent of each other.

Both dexamethasone and indomethacin reduced peptide levels at doses below those which affected the increase in NGF in the inflamed tissue which may reflect multiple actions of these drugs including some directly on the sensory neurones themselves. One possibility is that the drugs interfere with the intracellular signalling action of NGF once it has bound to *trk* and is retrogradely transported to the soma by, for example, altering the levels of transcription factors. There is evidence that dexamethasone can alter neuropeptide levels in these neurones. In rat cultured neonatal sensory neurones, for example, corticosterone reduces substance P content (MacLean *et al.*, 1989) while *in vivo*, dexamethasone treatment has been shown to reduce substance P and CGRP content in dental nerves (Hong *et al.*, 1993) and adrenalectomy results in an increase in substance P and CGRP content in rat DRG (Smith *et al.*, 1991). The recent demonstration of a coexistence of neuropeptides and glucocorticoid receptors in the rat spinal and trigeminal ganglia indicates how the glucocorticoids could regulate substance P and CGRP levels without necessarily interfering with the production of target-related growth factors/cytokines (DeLeon *et al.*, 1994). Less data is available on NSAID actions on peptide expression in DRG neurones but substance P levels are reduced in human ocular aqueous humor by indomethacin treatment (Kieselbach *et al.*, 1993).

An inhibitory effect of glucocorticoids on IL-1β production is fairly well characterized (Snyder & Unanue, 1982; Lee *et al.*, 1988; Dawson *et al.*, 1993). One study has reported no effect by indomethacin on IL-1β production by macrophages obtained from tissue chambers implanted in mice (Dawson *et al.*, 1993) and an effect was only found in this study with a very high dose. There is a complex interaction amongst second messenger pathways, steroid hormones, and protooncogenes of the *fos* and *jun* families (Barnes & Adcock, 1993). These may converge on the regulation of the NGF gene and several investigators have reported that in cultured fibroblasts, glucocorticoids have an inhibitory effect on NGF production (Siminoski *et al.*, 1987; MacLean *et al.*, 1989; Lindholm *et al.*, 1990). Indomethacin acts by inhibiting cyclo-oxygenase and subsequently prostaglandin E_2 (PGE_2) synthesis (Rome &

Lands, 1975). PGE_2 has been found to elicit a dose-dependent increase in both NGF protein and mRNA levels in rat hippocampal cell cultures (Friedman *et al.*, 1990), but an involvement of eicosanoids in NGF production in peripheral tissues has not been reported. The lower dose of indomethacin would be expected to have substantially and irreversibly inhibited cyclo-oxygenase (Salmon *et al.*, 1983) but it had minimal actions on behavioural sensitivity or IL-1β/NGF levels, implying that the effects seen with the higher dose may be due to some other action of indomethacin.

The dexamethasone and indomethacin treatments were surprisingly ineffective in modifying the CFA-induced hyperalgesia at therapeutic doses (Salmon *et al.*, 1983). Inflammation results in an upregulation of the inducible COX-2 isoform of cyclo-oxygenase (Vane *et al.*, 1994) which is readily induced by cytokines including IL-1 (Maier *et al.*, 1990). Indomethacin is relatively specific for COX-1 (Mitchell *et al.*, 1993) while dexamethasone would be expected to prevent the protein synthesis-dependent induction of COX-2 during inflammation (Masferrer *et al.*, 1992; Vane *et al.*, 1994). In other words we should have blocked both the inducible and the constitutive forms of COX with the two treatments but failed in either case to modify substantially behavioural hypersensitivity, at least compared to anti-NGF. Whether this means that eicosanoids have a limited role in the pathogenesis of the sensory hypersensitivity needs to be further explored. That a potent NSAID and steroid were relatively ineffective in preventing inflammatory hyperalgesia and the upregulation of IL-1β or NGF may turn out to be related. The only doses of dexamethasone and indomethacin showing anti-hyperalgesic effects were those that prevented an increase in NGF levels supporting a key role for NGF in mediating inflammatory hypersensitivity.

The relationship between the upregulation of IL-1β and NGF during inflammation and changes in neuropeptide levels or hyperalgesia are complex and a number of apparent mismatches between different sets of data still need to be explained, such as the production of thermal but not mechanical hyperalgesia by intraplantar IL-1β injections and a reduction of mechanical but not thermal hypersensitivity by IL-1ra administration to inflamed animals. This may be the result of complex synergistic interactions between a number of cytokines acting together in concert during inflammation, where one, such as IL-1β, may be necessary but not sufficient for producing a particular change, a situation quite different from administration of a single agent to naive animals. Nevertheless the data point to IL-1β being involved in the elevation in NGF levels during inflammation and that NGF has a particular role in the generation of inflammatory hyperalgesia. Unravelling the interactions between inflammation and the nervous system must help the understanding of the changes that the former produces in the latter. From such an understanding it may be possible to design more effective drugs to prevent or inhibit the production of abnormal pain sensitivity. Drugs which interfere with the production or action of NGF may offer a new class of inflammation-specific analgesics.

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