British Journal of Pharmacology (2003) 139, 1135–1145
www.nature.com/bjp

The critical role of leukotriene B_4 in antigen-induced mechanical hyperalgesia in immunised rats

[1]Joice Maria Cunha, [1]Daniela Sachs, [1]Cláudio Azevedo Canetti, [2]Stephen Poole, [1]Sérgio Henrique Ferreira & *,[1]Fernando Queiroz Cunha

[1]Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil and [2]Division of Endocrinology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3 QG, England

1 We investigated the mediators responsible for mechanical hypersensitivity induced by antigen challenge in rats immunised with ovalbumin (OVA).

2 Challenge with OVA (12.5–100 μg, intraplantar) caused a dose- and time-dependent mechanical hypersensitivity, which peaked 3 h after, decreased thereafter and reached control levels 24 h later.

3 Levels of TNFα, IL-1β and cytokine-induced neutrophil chemoattractant 1 (CINC-1) were increased in paw skin after antigen challenge.

4 OVA-evoked hypersensitivity was partially inhibited (about 51%) by pretreatment with anti-TNFα , IL-1β and IL-8 sera or with IL-1 receptor antagonist (IL-1ra), but not anti-NGF serum. Pretreatment with thalidomide (45 mg kg^{-1}) or pentoxifylline (100 mg kg^{-1}) also partially inhibited the hypersensitivity at 1–3 h after challenge.

5 Pretreatment with indomethacin (5 mg kg^{-1}) or atenolol (1 mg kg^{-1}) reduced the OVA-induced hypersensitivity at 1 and 3 h, but not at 5 h after challenge, while the combination of B_1 and B_2 bradykinin receptor antagonists was ineffective over the same times.

6 Pretreatment with MK886 (5-lipoxygenase-activating protein inhibitor, 3 mg kg^{-1}), CP 105696 (LTB_4 receptor antagonist; 3 mg kg^{-1}) or dexamethasone (0.5 mg kg^{-1}) inhibited the hypersensitivity from 1 to 5 h. Furthermore, LTB_4 levels were increased in the paw skin of challenged rats.

7 In conclusion, our results suggest that the TNFα-, IL-1β- and CINC-1-driven release of prostaglandins, sympathetic amines and LTB_4 mediates the first 3 h of mechanical hypersensitivity induced by antigen challenge in rats. At 5 h after OVA administration, although TNFα has some role, LTB_4 is the critical nociceptive mediator.
British Journal of Pharmacology (2003) **139,** 1135–1145. doi:10.1038/sj.bjp.0705346

Keywords: Inflammatory pain; mechanical hyperalgesia; cytokines; LTB_4; ovalbumin

Abbreviations: BK, bradykinin; CFA, complete Freund's adjuvant; CINC-1, cytokine-induced neutrophil chemoattractant 1; DALBK, des-Arg^9-Leu^8-BK; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FLAP, 5-lipoxygenase activating protein; HOE 140, D-Arg-Arg-Pro-Hyp-Gly-3-[2-thienyl]-Ala-Ser-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-2α,3β,7$\alpha\beta$]-octahydro-1H indole-2-carbonyl-Arg; i.p., intraperitoneal; i.pl., intraplantar; IL, interleukin; IL-1β, interleukin 1 beta; IL-1ra, interleukin 1 receptor antagonist; IL-8, interleukin 8; KLH, keyhole limpet haemocyanin; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; NGF, nerve growth factor; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; s.c., subcutaneous; TNFα, tumour necrosis factor alpha

Introduction

The sensitisation of nociceptors is the common denominator of inflammatory pain and represents a functional upregulation of nociceptors that leads to a state known as hyperalgesia/allodynia. Following this event, previously mild or ineffective stimuli cause 'overt pain' in humans, or a characteristic behavioural response that is used as an end point in animal nociceptive tests (Handwerker, 1976; Perl, 1976). There are two groups of directly acting hyperalgesic mediators that satisfy the experimental and clinical criteria for agents that directly sensitise nociceptors: eicosanoids and sympathetic amines. The capacity of prostaglandins and sympathetic amines (noradrenaline and dopamine) to sensitise nociceptors has been shown in man and in animals using both behavioural and electrophysiological techniques (Hannington-Kiff, 1974; Lol & Nathan, 1978; Lol *et al*., 1980; Ferreira, 1983; Nakamura & Ferreira, 1987; Duarte *et al*., 1988).

There is a great deal of evidence in the literature that in inflammation induced by carrageenin or lipopolysaccharide (LPS) administration, the release of eicosanoids and sympathetic amines is secondary to the generation of bradykinin (BK), which stimulates TNFα production. This stimulates two pathways, each of which leads to the release of cytokines and the final nociceptive mediators that sensitise the nociceptor. The two pathways are: (i) carrageenin/LPS$\rightarrow$BK$\rightarrow$TNF$\alpha\rightarrow$

*Author for correspondence; E-mail: fdqcunha@fmrp.usp.br
Advance online publication: 23 June 2003

IL-6 → IL-1β prostaglandins (which sensitise nociceptors) and (ii) carrageenin/LPS → BK → TNFα → CINC-1 (rat IL-8-related chemokine; Watanabe *et al*., 1991) → sympathetic amines that also sensitise the nociceptors. Besides the mediators described above, platelet-activating factor leukotriene B_4 (LTB_4) and endothelin also participate in the genesis of inflammatory hyperalgesia (Bonnet *et al*., 1981; Levine *et al*., 1984; Ferreira *et al*., 1989, respectively). The LTB_4-induced hyperalgesia appears to be dependent on polymorphonuclear leukocytes, but independent of prostaglandin synthesis (Levine *et al*., 1984; Bisgaard & Kristensen, 1985).

Regarding the inflammatory reaction to antigen challenge, it is well accepted that activation of the immune system leads to the release of proinflammatory cytokines such as TNFα, IL-1β, IL-6 (Nathan, 1987; Decker, 1990), IL-18 (Gracie *et al*., 1999) and chemokines (König *et al*., 2000), which in turn, leads to the release of a series of inflammatory mediators, including eicosanoids (for review, see Bayon *et al*., 1998). Using different experimental models, it was shown that, among these mediators, TNFα (Nordahl *et al*., 2000), IL-1β (Alstergren *et al*., 1998; Kopp, 1998), prostaglandins (Portanova *et al*., 1996; Omote *et al*., 2002) and also NGF, a neurotrophic factor (Ma & Woolf, 1997), are involved in the onset of the hyperalgesia that follows challenge with antigen. However, there is no study, in a single model of inflammation following antigen challenge, of the individual contribution of the above-mentioned mediators to the onset of mechanical hyperalgesia. We have, therefore, examined the roles of BK, TNFα , IL-1β , CINC-1, NGF, prostaglandins and sympathetic amines in hyperalgesia in immunised rats, following antigen challenge. Recently, our group demonstrated that neutrophil migration induced by antigen challenge in mice depends on TNFα released by CD4 + T cells, which acts through an LTB_4-dependent mechanism (Canetti *et al*., 2001). However, since the possible involvement of LTB_4 in hyperalgesia induced by antigen challenge remained to be investigated, we also addressed this point in the present study.

Methods

Animals

Male Wistar rats (100–200 g) were housed in a temperature-controlled room, with access to water and food *ad libitum*, until use. All experiments were conducted in accordance with NIH guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the School of Medicine of Ribeirão Preto (University of São Paulo).

Procedures for active immunisation

OVA was dissolved in phosphate-buffered saline (PBS) to an appropriate concentration (2 mg ml^{-1}) and mixed with an equal volume of complete Freund's adjuvant (CFA) at a concentration of 1 mg ml^{-1} of *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate. CFA was used to augment the efficiency of the immunisation procedure (Freund, 1956) by prolonging the lifetime of injected autoantigen and by stimulating its effective delivery to the immune system; this results in altered leukocyte proliferation and differentiation (for a review, see Billiau & Matthys, 2001). Rats weighing approximately 100 g were injected subcutaneously at two different sites on their back to give a total dose of 200 μg of OVA dissolved in an emulsion containing an equal volume of PBS plus CFA. Control (sham immunised) rats were injected with this emulsion without OVA. After 21 days, the rats were challenged by the intraplantar (i.pl.) administration of OVA (at different doses dissolved in 100 μl of PBS) to one of the hindpaws.

Nociceptive test: mechanical nociceptive hypersensitivity

Mechanical nociceptive hypersensitivity was tested in rats as described previously (Ferreira *et al*., 1978). In this method, a constant pressure of 20 mmHg (measured using a sphygmomanometer) is applied (*via* a syringe piston moved by compressed air) to a 15-mm^2 area on the dorsal surface of the hindpaw, and discontinued when the rat presents a 'freezing reaction'. This reaction typically is comprised of brief apnoea, concomitant with retraction of the head and forepaws and a reduction in the escape movements that animals normally make to free themselves from the position imposed by the experimental situation. Usually, the apnoea is associated with successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction is measured before administration (zero time) and at different times after administration of the hyperalgesic agents. The intensity of mechanical hypersensitivity is quantified as the reduction in the reaction time, calculated by subtracting the value of the second measurement from the first (Ferreira *et al*., 1978). Reaction time was 30.2 ± 0.5 s (mean $\pm$ s.e.m.; $n = 15$) before injection of the hyperalgesic agents or after intraplantar injection of saline. The shortened reaction time observed after inflammatory stimulus injection is prevented by steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) (Ferreira *et al*., 1988; 1997; Cunha *et al*., 1991; 1992; Lorenzetti *et al*., 2002). This method has been used to demonstrate the contribution of eicosanoids, sympathetic amines, cAMP and cytokines to the development of peripheral inflammatory hyperalgesia (Ferreira & Nakamura, 1979a; Ferreira *et al*., 1988; 1993; Francischi *et al*., 1988; Cunha *et al*., 1991; 1992; 1999), as well as the peripheral analgesic effect of opiates (Ferreira & Nakamura, 1979b) and NSAIDs (Ferreira *et al*., 1988; Cunha *et al*., 1991; 1992). These concepts and findings have been extensively confirmed with other methodologies such as formalin-induced flinching (Vinegar *et al*., 1976; Choi *et al*., 2001; Granados-Soto *et al*., 2001), chemically induced writhing (Duarte *et al*., 1988; Follenfant *et al*., 1988; Ribeiro *et al*., 2000b), the classical Randall-Sellito method (Aley *et al*., 1995) and others (Vinegar *et al*., 1976; Safieh-Garabedian *et al*., 2002). Furthermore, this method is able to discriminate between peripheral and central analgesic effects of drugs (Ferreira *et al*., 1978; Duarte & Ferreira, 1992).

The nociceptive hypersensitivity was measured at the indicated times after intraplantar injection of OVA (12.5–100 μg paw^{-1}, in 100 μl), keyhole limpet haemocyanin (KLH, 25 μg paw^{-1}, in 100 μl) or PBS (100 μl) into the hindpaw of rats. Different individuals were responsible for preparing the solutions to be injected, performing the injections and measuring the reaction times. Multiple paw treatments with PBS did not alter basal reaction time, which was similar to that observed in noninjected paws. Moreover, despite the fact that there is evidence in the literature that intraplantar administration of CFA in naive mice induces long-lasting mechanical

hypersensitivity in both paws (Ferreira *et al*., 2001), in our experimental model the subcutaneous administration of CFA to the back of the animals did not alter basal reaction times.

Experimental protocols

Hyperalgesic effect of intraplantar injection of OVA in immunised rats Mechanical hypersensitivity was measured 1, 3, 5, 7, 12 and 24 h after the administration of OVA (12.5, 25, 50 or 100 μg paw^{-1}) injected in a final volume of 100 μl into the hindpaw of immunised rats. Sham-immunised animals received i.pl. injection of OVA (100 μg paw^{-1}, in 100 μl). The OVA-challenge was performed 21 days after immunisation. As a control, OVA-immunised rats were also challenged with PBS (100 μl paw^{-1}) or KLH (25 μl paw^{-1}), an unrelated antigen.

Determination of TNFα, IL-1β and CINC-1 levels in paw skin of OVA-injected rats OVA (25 μg paw^{-1}, in 100 μl) or PBS (100 μg paw^{-1}) was injected into the hindpaw of immunised rats and 0.5, 1, 3, 5 or 24 h later, the animals were killed. The skin of the whole of the plantar area of paws was obtained and homogenised in 500 μl of the appropriate buffer containing protease inhibitors, and TNFα, IL-1β and CINC-1 levels were determined as described previously (Safieh-Garabedian *et al*., 1995; Rees *et al*., 1999) by enzyme-linked immunosorbent assay (ELISA). Briefly, microtitre plates (Nunc-Maxisorb) were coated overnight at 4°C with a rabbit anti-rat CINC-1 polyclonal antibody, sheep anti-rat IL-1β or sheep anti-rat TNFα. After blocking the plates, rat CINC-1, IL-1β or TNFα standards at various dilutions in a medium and 50 μl of samples were added in triplicate and maintained at room temperature for 2 h. Sheep anti-CINC-1, IL-1β or TNFα biotinylated polyclonal antibodies were added at a 1 : 500 dilution, followed by incubation at room temperature for 1 h. Finally, 100 μl of avidin-HRP (1 : 5000 dilution) was added to each well and, after 30 min, the plates were washed and the colour reagent OPD (40 μg, 50 μl well^{-1}) was added. After 15 min, the reaction was terminated with H_2SO_4 (1 M, 50 μl well^{-1}) and the optical density measured at 490 nm. The results were obtained by comparing the optical density with standard curves. In addition, the results were adjusted to 500 μl, the volume used to extract the cytokine from the paw skin, and were expressed as nanograms of respective cytokine per paw. As a control, the levels of these cytokines were determined in naive rats and sham-immunised animals injected with PBS (100 μg paw^{-1}) or with OVA (25 μg paw^{-1}, in a final volume of 100 μl). The cytokine levels in skin samples from control rats were determined 3 h later.

Effects of anticytokines sera or IL-1ra treatments on OVA-induced mechanical hypersensitivity Rats were treated with preimmune serum (control group), sheep anti-rat TNFα, IL-1β, NGF or sheep anti-human IL-8 (each at doses of 50 μg paw^{-1}), and 15 min later, the same paw was injected with OVA (25 μg paw^{-1}, in 100 μl). IL-1ra (300 μg paw^{-1}, in 100 μl) was injected 30 min before OVA challenge (at the same dose). The sham-immunised group received an OVA injection (also at the dose of 25 μg paw^{-1}, in 100 μl). Mechanical hypersensitivity was measured 1, 3 and 5 h after OVA injection. The effectiveness of these doses of the antisera or of IL-1ra against their respective cytokines has been demonstrated previously (Ferreira *et al*., 1988; Cunha *et al*., 1991; 1992; 2000; Lorenzetti *et al*., 2002) and was also confirmed in this study.

Effects of dexamethasone, thalidomide, pentoxifylline, HOE 140 plus des-Arg^9-Leu^8-BK (DALBK), indomethacin, atenolol, MK 886 and CP 105696 on OVA-induced mechanical hypersensitivity Immunised rats were treated with PBS (100 μl paw^{-1}; control), TRIS buffer (1 ml, i.p.;. control), 1% DMSO in PBS (1 ml, i.p.; control), 0.1% methyl cellulose in H_2O (1 ml, p.o.; control), HOE 140 dissolved in PBS (1 mg kg^{-1}, i.p.), DALBK dissolved in PBS (500 μg paw^{-1}), indomethacin dissolved in 0.1 M TRIS buffer, pH 8.0 (5 mg kg^{-1}, i.p.) or atenolol dissolved in PBS (1 mg kg^{-1}, i.p.), and 30 min later, the animals received an i.pl. injection of OVA (25 μg paw^{-1}, in 100 μl). Pretreatments with thalidomide dissolved in 1% DMSO in PBS (45 mg kg^{-1}, i.p.), pentoxifylline dissolved in PBS (100 mg kg^{-1}, i.p.), MK 886 dissolved in 0.1% methyl cellulose in H_2O (1 mg kg^{-1}, orally) or dexamethasone dissolved in PBS (0.5 mg kg^{-1}, s.c.) were performed 1 h before OVA challenge. CP 105696 dissolved in 1% DMSO in PBS (3 mg kg^{-1}, i.p.) was administered 45 min before OVA injection. Reinforcement doses of these drugs (at the same concentrations given above) or of solvents were injected 3 h after the OVA challenge. The sham-immunised group received an OVA injection (also at the dose of 25 μg paw^{-1}, in 100 μl). Mechanical hypersensitivity was evaluated 1, 3 and 5 h after the OVA challenge. The effectiveness of these doses of the drugs against their respective mediators has been demonstrated previously (Nakamura & Ferreira, 1987; Ferreira *et al*., 1988; 1997; Poole *et al*., 1999a; Ribeiro *et al*., 2000a; Canetti *et al*., 2001; Viana *et al*., 2002).

Determination of LTB_4 level in paw skin of OVA-injected rats OVA (25 μg paw^{-1}, in 100 μl) or PBS (100 μl) was injected into the hindpaws of immunised and sham-immunised rats and 3 h later the animals were killed. Total skin samples of the plantar area of paws from these groups and from naive rats (control values) were obtained and homogenised in 500 μl of the appropriate buffer containing protease inhibitors (as described previously by Safieh-Garabedian *et al*., 1995). LTB_4 levels were determined by enzyme immunoassay using a commercial kit (Biotrak™, Amersham Pharmacia Biotech, England, U.K.) according to the manufacturer's instructions. The results were obtained by comparing the optical density with standard curves. In addition, the results were adjusted to 500 μl, the volume used to extract LTB_4 from the paw skin, and were expressed as pg paw^{-1}.

Materials The following drugs were used: OVA, indomethacin, atenolol, dexamethasone, pentoxifylline, HOE 140, des-Arg^9-Leu^8-BK (DALBK), CFA (H37Ra, ATCC 25177), KLH (keyhole limpet haemocyanin) (Sigma, St Louis, MO, U.S.A.), thalidomide (RBI, MA, U.S.A.), MK 886 (5-lipoxygenase-activating protein inhibitor; Calbiochem, CA, U.S.A.), CP 105696 (LTB_4 receptor antagonist, a gift from Pfizer/Groton Laboratories, Groton, CT, U.S.A.) and IL-1ra (National Institute for Biological Standards and Control, U.K.). The following antisera were used: sheep anti-rat IL-1β, sheep anti-rat TNFα , sheep anti-rat NGF and sheep anti-human IL-8 (National Institute for Biological Standards and Control, U.K.).

Statistical analysis Results are presented as means and standard errors of the mean for groups of five animals (for *in vivo* experiments) or three animals (for *in vitro* experiments) and they are representative of two or three different experiments. The amount of variation between the different experiments was not statistically different (ANOVA) and it was not higher than 10%. The differences between the experimental groups were also compared by ANOVA and, in the case of significance, individual comparisons were subsequently made with Bonferroni's *t*-test. The level of significance was set at $P<0.05$.

Results

Hyperalgesic effect of OVA in immunised rats

Intraplantar injection of OVA into the hindpaw of immunised rats evoked mechanical hypersensitivity in a dose- (12.5–100 μg paw^{-1}, in 100 μl) and time- (1–24 h) dependent manner when compared to sham-immunised rats injected with OVA (100 μg paw^{-1}). The mechanical hypersensitivity of immunised animals injected with OVA at doses of 25, 50 or 100 μg paw^{-1} was already significant 1 h after the OVA injection ($P<0.05$), and this peaked 3 h later, declining thereafter and reaching the control level 24 h later. In the group injected with 12.5 μg paw^{-1} of OVA, the hypersensitivity response was not significant after 1 h ($P>0.05$), but increased to significant values at 3 and 5 h ($P<0.05$) after OVA injection, declining thereafter (Figure 1). Injection of OVA (100 μg paw^{-1}, in 100 μl) into the hindpaw of sham-immunised (control) rats or, alternatively, of an unrelated antigen, KLH (25 μg paw^{-1}) in OVA-immunised animals did not alter the mechanical nociceptive threshold significantly when compared to the PBS-injected immunised animals (Figure 1).

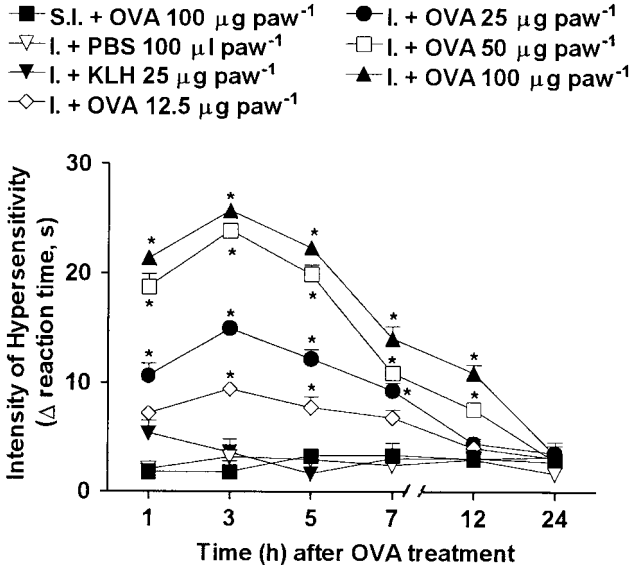

Figure 1 Hyperalgesic effect of ovalbumin (OVA) in immunised rats. The mechanical hypersensitivity was evaluated at 1, 3, 5, 7, 12 and 24 h after intraplantar injection of OVA in immunised (I.) rats at doses of 12.5, 25, 50 or 100 μg paw^{-1}, in 100 μl and in sham-immunised rats (S.I.) at a dose of 100 μg paw^{-1}. Control immunised rats were injected with PBS (100 μg paw^{-1}) or an unrelated antigen, KLH (25 μg paw^{-1}, in 100 μl). See method for immunisation protocol. Results are expressed as means ± s.e.m. in groups of five rats. *$P<0.05$ when compared to S.I. rats treated with OVA (ANOVA followed by Bonferroni's *t*-test).

OVA-induced TNFα, IL-1β and CINC-1 production in paw skin of immunised rats

Intraplantar injection of OVA (25 μg paw^{-1}, in 100 μl) induced a significant increase in the production of TNFα, IL-1β and CINC-1 in the paw skin of immunised rats when compared to OVA-treated sham-immunised rats. The patterns of cytokine production were broadly similar, although there were some differences in the time courses (Figure 2, panels a–c). Concentrations of TNFα and CINC-1 peaked 1 h after OVA

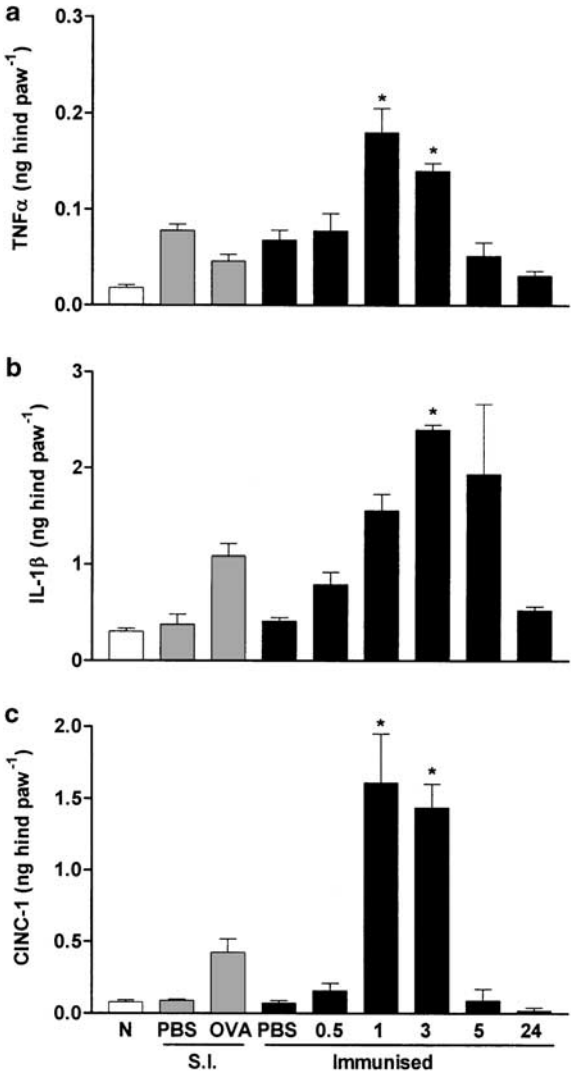

Figure 2 Concentrations of TNFα (panel a), IL-1β (panel b) and CINC-1 (panel c) in rat hindpaw injected with PBS or OVA. PBS (100 μl) or OVA (25 μg paw^{-1}, in 100 μl) was injected in the sham-immunised (S.I.) and immunised groups. Immunised rats were killed at 0.5, 1, 3, 5 or 24 h after the injection and paw skin samples were obtained for measurement of cytokines by ELISA. The cytokine levels shown for naive rats (N, open bar), PBS or OVA-treated S.I. rats (grey bars) represent values obtained 3 h after the treatments. Results are expressed as means ± s.e.m. of three samples for each group. *$P<0.05$ when compared to OVA-injected S.I. rats (ANOVA followed by Bonferroni's *t*-test).

injection ($P<0.05$), remaining significantly elevated 3 h later ($P<0.05$) and decreasing to control levels at 5 h (Figure 2, panels a and c). The concentration of IL-1β peaked 3 h after OVA challenge ($P<0.05$), decreasing thereafter and reaching the control level 24 h later (Figure 2, panel b). The injection of PBS (100 μl paw^{-1}) in immunised rats did not induce a significant increase in TNFα, IL-1β and CINC-1 levels in rat paw skin when compared to PBS-injected sham-immunised rats.

Antinociceptive effect of cytokine antisera on OVA-induced nociceptive hypersensitivity

Next, we used anticytokine sera to investigate whether the cytokines released in the paw after OVA administration mediated the OVA-evoked nociceptive hypersensitivity in immunised rats (Figure 3). The control group was treated with preimmune serum. Pretreatment with anti-rat TNFα serum (50 μl paw^{-1}, 15 min before OVA injection) significantly reduced the nociceptive hypersensitivity induced by OVA (25 μg paw^{-1}), when evaluated 1, 3 or 5 h after challenge

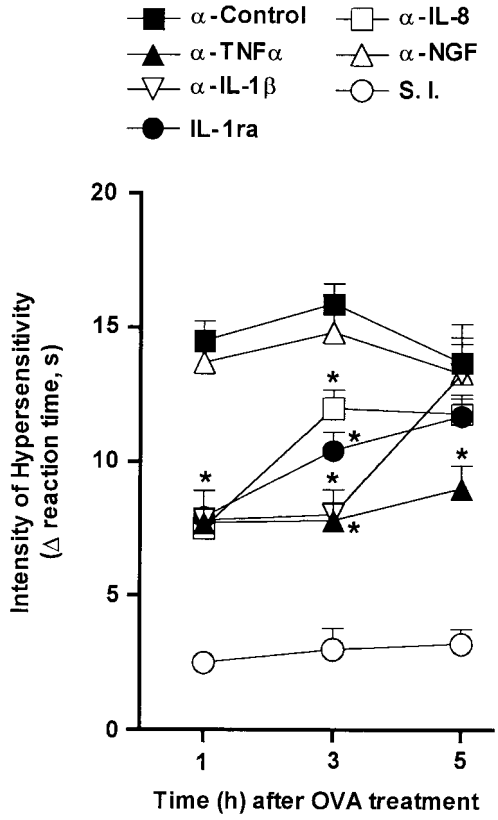

Figure 3 Effect of anticytokine sera or interleukin 1 receptor antagonist (IL-1ra) on OVA-evoked mechanical hypersensitivity. Preimmune serum (control group; α-Control), sheep anti-rat TNFα (α-TNFα, 50 μl), IL-1β (α-IL-1β, 50 μl) serum, sheep anti-human IL-8 serum (α-IL-8, 50 μl) or sheep anti-rat NGF serum (α-NGF, 50 μl) were administered 15 min before intraplantar injection of OVA (25 μg paw^{-1}). IL-1ra (300 μg paw^{-1}, in 100 μl) was administered 30 min before OVA challenge. The sham-immunised group (S.I.) received an intraplantar injection of OVA (25 μg paw^{-1}). The mechanical hypersensitivity was assessed 1, 3 and 5 h after OVA challenge. Results are expressed as means $\pm$ s.e.m. in groups of five rats. *$P<0.05$ when compared to preimmune serum-treated rats (ANOVA followed by Bonferroni's t-test).

($P<0.05$). Pretreatment with IL-1ra (300 μg paw^{-1}, 30 min before OVA injection), sheep anti-rat IL-1β or sheep anti-human IL-8 (50 μl paw^{-1}, 15 min before the OVA injection) significantly inhibited the nociceptive hypersensitivity induced by OVA (25 μg paw^{-1}, in 100 μl) when evaluated 1 or 3 ($P<0.05$), but not 5 h ($P>0.05$) after OVA challenge. Pretreatment with sheep anti-rat NGF (50 μl paw^{-1}, 15 min before OVA injection) did not alter the nociceptive effect of OVA (25 μg paw^{-1}) in immunised rats ($P>0.05$). As observed previously, injection of OVA (25 μg paw^{-1}) in the sham-immunised group did not cause hypersensitivity (Figure 3). The doses of the antisera or of IL-1ra inhibit the nociceptive hypersensitivity induced by the respective cytokines by more than 90% (data not shown).

Effect of HOE 140 plus DALBK, dexamethasone, thalidomide, pentoxifylline, indomethacin and atenolol on OVA-induced nociceptive hypersensitivity

Immunised rats were treated twice (30 min before and 3 h after intraplantar OVA injection) with the combination of B_1 and B_2 bradykinin receptor antagonists (HOE 140 plus DALBK), cytokine synthesis inhibitors (dexamethasone, thalidomide and pentoxifylline), a cyclooxygenase inhibitor (indomethacin) and a beta adrenoceptor antagonist (atenolol). We found that treatment with HOE 140 (1 mg kg^{-1}, i.p.) plus DALBK (500 ng paw^{-1}) did not inhibit the hyperalgesic effect of OVA in immunised rats when this was evaluated 1, 3 and 5 h after OVA challenge ($P>0.05$, Figure 4). HOE 140 (at the same doses) or DALBK (at the same doses) administered individually also did not inhibit the hyperalgesic effect of OVA (data not shown). Pretreatment with thalidomide (45 mg kg^{-1}, i.p.) or pentoxifylline (100 mg kg^{-1}, i.p.) significantly reduced the OVA-induced nociceptive hypersensitivity when evaluated 1 and 3 ($P<0.05$, Figure 4), but not 5 h after OVA challenge ($P>0.05$, Figure 4). Dexamethasone (0.5 mg kg^{-1}, s.c.) eliminated the nociceptive hypersensitivity induced by OVA (25 μl paw^{-1}) when this was evaluated 1, 3 or 5 h after OVA challenge ($P<0.05$, Figure 4). Pretreatment with indomethacin (5 mg kg^{-1}, i.p.), atenolol (1 mg kg^{-1}, i.p.) or atenolol plus indomethacin (at the same doses given above) significantly inhibited the nociceptive hypersensitivity induced by OVA when evaluated 1 and 3 ($P<0.05$) but not 5 h after OVA challenge ($P>0.05$, Figure 5). The intraplantar pretreatment with dexamethasone (5 μg paw^{-1}), indomethacin (100 μg paw^{-1}) or atenolol (25 μg paw^{-1}) produced results similar to those obtained with the systemic treatments described above (data not shown). Furthermore, the doses of HOE 140, DALBK and atenolol inhibited the nociceptive hypersensitivity induced by BK and dopamine, respectively, by more than 90% (data not shown). The doses of indomethacin, dexamethasone, thalidomide and pentoxifylline inhibited the nociceptive hypersensitivity induced by carrageenin by 50, 95, 90 and 88%, respectively (data not shown).

Inhibition of OVA-induced nociceptive hypersensitivity by 5-lipoxygenase activating protein (FLAP) inhibitor (MK 886) or LTB_4 receptor antagonist (CP105696)

Immunised rats were pretreated with CP 105696 (3 mg kg^{-1}) or MK 886 (3 mg kg^{-1}), 45 min or 1 h before OVA challenge, respectively. Reinforcement doses of CP 105696 (at the same

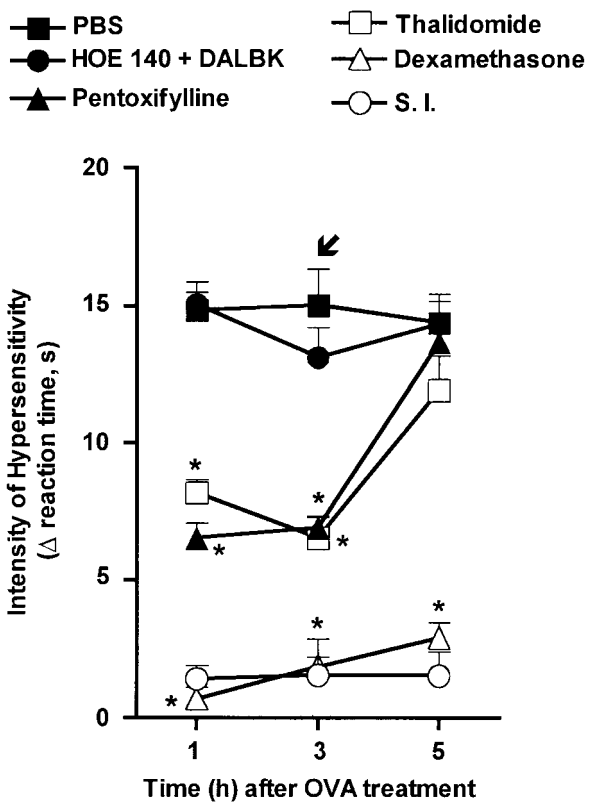

Figure 4 Effect of thalidomide, dexamethasone, pentoxifylline or HOE 140 plus DALBK on OVA-evoked mechanical hypersensitivity. PBS (equivalent volume), dexamethasone (0.5 mg kg^{-1}), pentoxifylline (100 mg kg^{-1}, i.p.) or thalidomide (45 mg kg^{-1}, i.p.) were administered 1 h before and 3 h after (indicated by arrow) OVA challenge and HOE 140 (1 mg kg^{-1}, i.p.) plus des-Arg^9-Leu^8-bradykinin (DALBK, 500 μg paw^{-1}) were administered 30 min before and 3 h after (indicated by arrow) OVA treatment. The sham-immunised group (S.I.) received an intraplantar injection of OVA (25 μg paw^{-1}). The mechanical hypersensitivity was assessed at the time indicated after OVA challenge. Results are expressed as means $\pm$ s.e.m. in groups of five rats. *$P<0.05$ when compared to PBS-treated rats (ANOVA followed by Bonferroni's *t*-test).

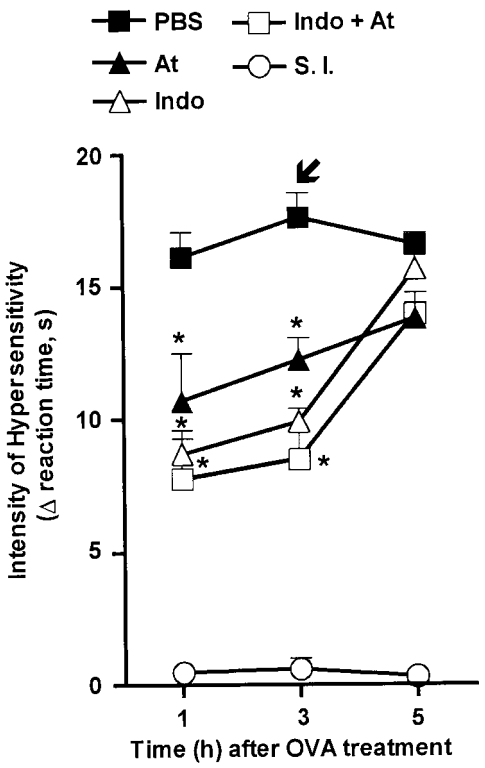

Figure 5 Effect of atenolol and indomethacin treatment on the OVA-evoked mechanical hypersensitivity. PBS (equivalent volume), indomethacin (5 mg kg^{-1}, i.p.), atenolol (1 mg kg^{-1}, i.p.) or indomethacin plus atenolol (at the same doses) were administered 30 min before and 3 h after (indicated by arrow) OVA challenge. The sham-immunised group (S.I.) received an intraplantar injection of OVA (25 μg paw^{-1}). The mechanical hypersensitivity was assessed at the time indicated after OVA challenge. Results are expressed as means $\pm$ s.e.m. in groups of five rats. *$P<0.05$ when compared to PBS-treated rats (ANOVA followed by Bonferroni's *t*-test).

dose) or MK 886 (at the same dose) were given 3 h after intraplantar injection of OVA (25 μg paw^{-1}). The treatment of immunised rats with MK 886 or CP 105696 significantly reduced the OVA-induced hypersensitivity when this was evaluated 1, 3 or 5 h after OVA challenge ($P<0.05$, Figure 6, panel a). The dose of CP 105696 inhibited the nociceptive hypersensitivity induced by LTB_4 by 95% (data not shown in figure).

Increase in LTB_4 levels in paw skin of immunised rats stimulated by OVA

OVA (25 μg paw^{-1}, in 100 μl) and PBS (100 μl paw^{-1}) were injected into the hindpaws of immunised or sham-immunised (control) rats. The animals were killed 3 h later and skin samples were obtained for the determination of LTB_4 levels. The injection of OVA into the hindpaws of immunised rats increased LTB_4 levels three-fold when compared to the sham-immunised rats stimulated with OVA (at the same dose; $P<0.05$), immunised rats injected with PBS or naive rats (Figure 6, panel b).

Discussion

In this study, we investigated the individual contribution of cytokines, BK, prostaglandins, leukotrienes, sympathetic amines and NGF, a neurotrophic factor, to the mechanical hypersensitivity exhibited following antigen challenge. The intraplantar injection of antigen (OVA) induced a dose- and time-dependent mechanical hypersensitivity, which peaked 3 h after challenge, declined thereafter and reached control levels 24 h later. This response was an antigen-specific immune reaction, because the same injection of OVA did not induce hypersensitivity in sham-immunised or naive animals. Furthermore, injection of KLH, a protein to which the rats had not been immunised, did not induce hypersensitivity in rats immunised to OVA.

The first 3 h of OVA-induced mechanical hypersensitivity was related to the endogenous release of TNFα, IL-1β and CINC-1. The OVA-induced hypersensitivity was significantly inhibited at 1 and 3 h by specific anti-rat TNFα, IL-1β and anti-human IL-8 (which cross-reacts with rat CINC-1) antisera (Lorenzetti *et al*., 2002) or IL-1ra treatments. Although NGF causes thermal and mechanical hyperalgesia (Lewin *et al*., 1993; 1994), it is not involved in the OVA-evoked mechanical hypersensitivity, because intraplantar pretreatment with antiserum that neutralises this cytokine had no effect on OVA-induced hypersensitivity. Consistent with the involvement of

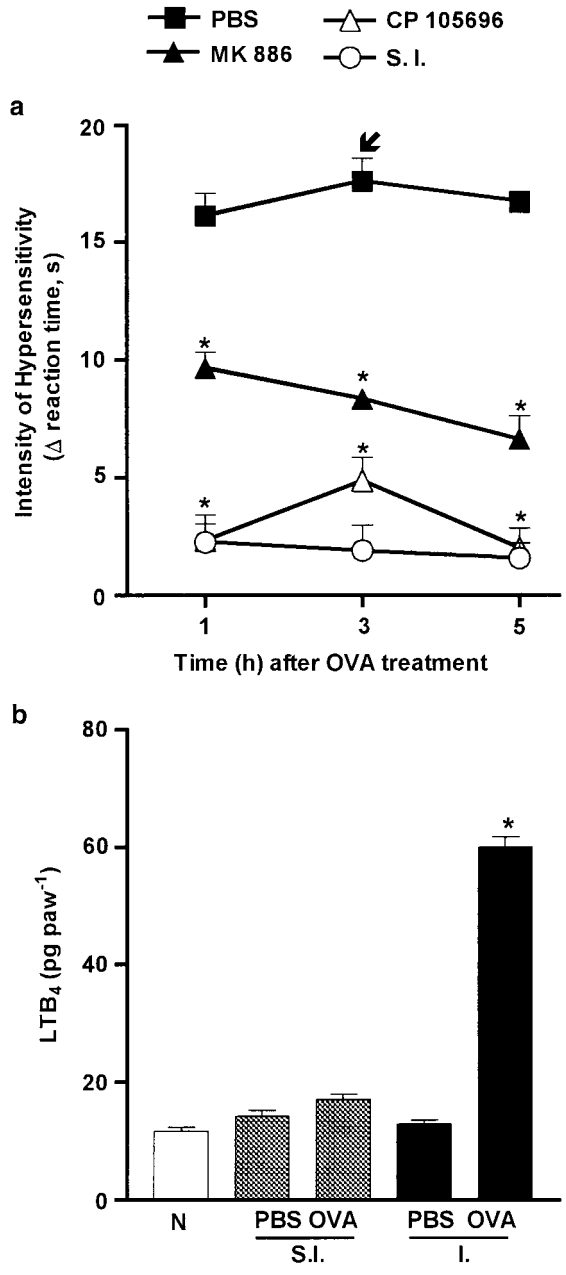

Figure 6 Participation of LTB_4 in OVA-evoked mechanical hypersensitivity. Panel (a) Effect of LTB_4 synthesis inhibitor (MK 886) and LTB_4 receptor antagonist (CP 105696) on OVA-induced mechanical hypersensitivity. PBS (equivalent volume, i.p.) or CP 105696 (3 mg kg^{-1}, i.p.) was administered 45 min before and 3 h after (indicated by arrow) OVA injection (25 μg paw^{-1}). MK 886 (1 mg kg^{-1}, orally) was administered 1 h before and 3 h after (indicated by arrow) OVA injection. The sham-immunised group (S.I.) received an i.pl. injection of OVA (25 μg paw^{-1}). The mechanical hypersensitivity was assessed at the time indicated after OVA treatment. Results are expressed as means $\pm$ s.e.m. in groups of five rats. *$P<0.05$ when compared to PBS-treated rats (ANOVA followed by Bonferroni's t test). Panel (b) Levels of LTB_4 in paw skin of immunised, sham-immunised and naive rats. The sham-immunised group (S.I.) and immunised group (I) received an intraplantar injection of PBS (100 μl paw^{-1}) or OVA (25 μg paw^{-1}) and, 3 h later, rats were killed and paw skin samples were obtained for measurement of LTB_4 levels by EIA. The levels of LTB_4 in paw skin samples from naive rats were determined for control values. Results are expressed as means $\pm$ s.e.m. of three samples from each group. *$P<0.05$ when compared to OVA injected sham-immunised group (S.I.), PBS-treated immunised rats or naive animals (ANOVA followed by Bonferroni's *t*-test).

endogenous TNFα, IL-1β and CINC-1 in OVA-evoked hypersensitivity, levels of these mediators were found to be elevated in rat paw skin subsequent to the intraplantar injection of the same dose of OVA that evoked mechanical hypersensitivity. Moreover, the pattern of cytokine expression in the paw was similar to the time course of OVA-evoked mechanical hypersensitivity. The involvement of these cytokines in mechanical hyperalgesia has also been shown in carrageenin- or LPS-induced inflammation (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; 1992; Lorenzetti *et al.*, 2002) and in the nociceptive acetic acid- or zymosan-induced writhing response in mice (Ribeiro *et al.*, 2000b). Furthermore, there is evidence that TNFα mediates the development of temporomandibular joint pain in patients with chronic connective tissue disease (Nordahl *et al.*, 2000) and that IL-1β, IL-8 and IL-18 are involved in the pathogenesis of rheumatoid arthritis (Eastgate *et al.*, 1988; Endo *et al.*, 1991; Gracie *et al.*, 1999, respectively).

The conclusion that endogenous TNFα, IL-1β and CINC-1 are involved in the first 3 h of OVA-evoked hypersensitivity in immunised animals is reinforced by our finding that thalidomide and pentoxifylline significantly reduced the hypersensitivity response at 1 and 3 h, but not at 5 h after OVA-challenge. These drugs, among other effects, inhibit the synthesis of TNFα and IL-1β (Strieter *et al.*, 1988; Zabel *et al.*, 1989; Sampaio *et al.*, 1991; Bienvenu *et al.*, 1992; Schandené *et al.*, 1992; Weinberg *et al.*, 1992; Moreira *et al.*, 1993). Furthermore, dexamethasone inhibited OVA-induced hypersensitivity at 1, 3 and also at 5 h after the challenge. This difference in the pattern of inhibition among thalidomide, pentoxifylline and dexamethasone can be explained by the fact that thalidomide and pentoxifylline did not inhibit the synthesis of LTB_4, which is inhibited by dexamethasone (Hirata *et al.*, 1980), and, as discussed below, LTB_4 mediates the later phase of OVA-evoked hypersensitivity.

During the last decade, investigations of the mechanism by which the mechanical hyperalgesia following LPS or carrageenin injection is induced by cytokines have shown that they constitute a link between these stimuli and the release of the final hyperalgesic mediators, that is, the prostaglandins and sympathetic amines (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; 1992; Lorenzetti *et al.*, 2002). TNFα , IL-1β and CINC-1 act sequentially: TNFα is released first after the injury and stimulates the production of IL-1β , which, in turn, stimulates the production of cyclooxygenase metabolites (Ferreira *et al.*, 1988; Cunha *et al.*, 1992) and of CINC-1, which stimulates the release of sympathetic amines (Lorenzetti *et al.*, 2002). In the present study, indomethacin, a nonsteroidal anti-inflammatory drug that inhibits prostaglandin synthesis, and atenolol, a β_1-adrenoceptor antagonist, significantly inhibited the OVA-induced hypersensitivity in the first 3 h but were ineffective at 5 h after the OVA-challenge. These profiles of inhibition were similar to those observed with antisera against the above-mentioned cytokines, or with thalidomide and pentoxifylline, suggesting that, as with carrageenin- and LPS-induced inflammation, it is the cytokine-driven release of prostaglandins and sympathetic amines that mediates the early phase of OVA-induced hypersensitivity in immunised animals. The fact that atenolol, a selective β_1 adrenoceptor antagonist, inhibits the OVA-induced hypersensitivity suggests that the sympathetic amines released after the OVA challenge are activating the β_1-adrenoceptor on the sensitive neurone. In previous

studies, it was demonstrated that in carrageenin- or LPS-induced inflammation, sympathetic amines are also released and activate the β_1 (Nakamura & Ferreira, 1987) or β_2 (Levine *et al*., 1988) adrenoceptor. Our results do not exclude the possibility that the β_2 adrenoceptor is also activated after OVA challenge.

It seems that TNFα has a role in hypersensitivity 5 h after OVA administration, a conclusion supported by the significant inhibition of the hypersensitivity obtained at this time point with a specific anti-TNFα antiserum. However, this TNFα effect is not mediated by the sequential release of IL-1β/eicosanoids and of CINC-1/sympathetic amines, because neither antisera against these cytokines nor indomethacin and atenolol affected the OVA-evoked hypersensitivity at this time point. Furthermore, thalidomide and pentoxifylline were also ineffective on OVA-induced hypersensitivity at this time point. The conclusion that TNFα participates in OVA-induced hypersensitivity at 5 h seems to contradict the observation that soluble TNFα was not detected in the paw skin at this time point after OVA challenge. A possible explanation for these apparently contradictory results is that, at this time point, the hyperalgesic effect of the membrane-associated form of TNFα predominates, which is antagonised by antiserum, but not detected by ELISA. In fact, we recently showed that LPS-stimulated macrophages release soluble TNFα and express the membrane-associated form of this cytokine, which is not detected by ELISA (Crossara-Alberto *et al*., 1997). However, if this hypothesis is correct, thalidomide did not inhibit the expression of this form of TNFα, since the pretreatment of the rats with this drug did not inhibit the OVA-induced hypersensitivity at this time point. Further experiments are needed to clarify this point.

It was proposed that BK acting synergistically on B_1 and B_2 receptors stimulates the release of TNFα involved in carrageenin- or LPS-induced mechanical hyperalgesia (Ferreira *et al*., 1993; Poole *et al*., 1999a). To investigate whether BK generation also preceded the release of cytokine involved in the genesis of OVA-induced hypersensitivity, we used a combination of B_1 and B_2 receptor antagonists. It was found that pretreatment of the immunised rats with DALBK plus HOE 140, selective B_1 and B_2 receptors antagonists, respectively, did not significantly inhibit OVA-induced mechanical hypersensitivity. Despite this observation, it has been reported in the literature that there is a substantial increase in the expression of the B_2 receptor in the lumbar dorsal root ganglion neurones in antigen-induced arthritis in the rat knee and that this increase is relevant for the generation of acute and chronic inflammatory pain (Segond von Banchet *et al*., 2000). The use of different experimental models could explain these apparently contradictory results.

The role of endogenous LTB_4 in the genesis of OVA-induced-mechanical hypersensitivity was evaluated using MK 886, an LTB_4 synthesis inhibitor (FLAP inhibitor; Miller *et al*., 1990) and CP 105696, an LTB_4 receptor antagonist (Koch *et al*., 1994). It was observed that this eicosanoid, together with prostaglandins and sympathetic amines, mediates the first 3 h of OVA-induced hypersensitivity. Moreover, LTB_4 also mediates the OVA-induced hypersensitivity at 5 h after the challenge. This is supported by the observation that OVA-evoked hypersensitivity was significantly inhibited by MK 886 and CP 105696 at 1, 3 and also 5 h after challenge. Confirming the involvement of LTB_4 in the process, a significant increase in LTB_4 levels was observed in the paw of sensitised rats after intraplantar injection of OVA. Mechanical and thermal hyperalgesic effects of LTB_4 in rats and humans have been described previously and appear to be dependent on polymorphonuclear leukocytes (Levine *et al*., 1984; Bisgaard & Kristensen, 1985). The possible participation of neutrophils in the onset of the OVA-evoked hypersensitivity is under investigation. Although this finding is the first demonstration that LTB_4 mediates the hyperalgesia observed in antigen-induced hyperalgesia, a number of studies have reported significant levels of LTB_4 in human inflammatory diseases in which hyperalgesia occurs, such as rheumatoid arthritis (Davidson *et al*., 1983; Ahmadzadeh *et al*., 1991; Gursel *et al*., 1997). Taken together, these results suggest that LTB_4 antagonists could be useful for the treatment of pain observed in immune inflammatory diseases. Whether the release of LTB_4, which participates in the OVA-induced hypersensitivity, is mediated by cytokines was not investigated in the present study. However, there is evidence in the literature that TNFα, which was increased during the first 3 h after the OVA challenge, is capable of inducing LTB_4 production (Meyer *et al*., 1988; Camussi *et al*., 1989; Canetti *et al*., 2001).

Recently, Feitosa *et al*. (2002), investigating the mediators involved in OVA-induced oedema in sensitised rats, demonstrated that dexamethasone and serotonin antagonists, but not indomethacin, MK 886, thalidomide or pentoxifylline, inhibited oedema formation significantly. Similarly, we also demonstrated, in our experimental model, that OVA-induced paw oedema is not inhibited by the pretreatment of the animals with indomethacin, atenolol, thalidomide, pentoxifylline or MK 886 (results not shown), treatments that inhibited the hyperalgesia (Figures 4–6). Dexamethasone treatment reduced both OVA-induced oedema and mechanical hyperalgesia (Figure 4). These results clearly showed that there are different mechanisms involved in the genesis of oedema and hyperalgesia. Therefore, it may be that the oedema formation induced by OVA challenge in immunised rats depends on the release of serotonin and kinins that promote an increase in venous permeability. On the other hand, sensitisation of primary sensory neurones by eicosanoids or sympathetic amines depends on the release of cytokines by resident cells (Cunha *et al*., 2000). In line with this idea, we have shown that the administration of TNFα, IL-1β and IL-8, at doses that caused hyperalgesia, did not cause oedema (Poole *et al*., 1999b). Moreover, thalidomide, a drug that inhibits the release of TNFα, also reduces mechanical hyperalgesia, but not oedema formation induced by intraplantar administration of carrageenin (Ribeiro *et al*., 2000a).

It is well known that cAMP and protein kinases A and C are involved in molecular events in the primary sensory neurone associated with acute and persistent hyperalgesia triggered by inflammatory stimuli, as well as by final hyperalgesic mediators such as prostanoids and sympathetic amines (Taiwo & Levine, 1991; Aley & Levine, 1999; Cunha *et al*., 1999; Aley *et al*., 2000). However, the involvement of these intracellular mediators in the mechanical hypersensitivity induced by antigen challenge is not yet fully understood, and this remains an aim of our future studies.

In conclusion, our results suggest that the cytokine (TNFα, IL-1β and CINC-1)-driven release of prostaglandins, sympathetic amines and LTB_4, but not of bradykinin, mediates the first 3 h of mechanical hypersensitivity induced by OVA in

immunised rats. At 5 h after OVA administration, although TNFα has a minor role, LTB_4 is the key nociceptive mediator. Therefore, inhibition of the synthesis of this eicosanoid could be beneficial for the control of hyperalgesia in immune inflammatory diseases.

We gratefully acknowledge the technical assistance of Ieda R. dos Santos Schivo, Giuliana Bertozi Francisco and Sérgio Roberto Rosa. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Pesquisa (CNPq) and Programa de Núcleos de Excelência (PRONEX).

References

AHMADZADEH, N., SHINGU, M., NOBUNGA, M. & TAWARA, T. (1991). Relationship between leukotriene B_4 and immunological parameters in rheumatoid synovial fluids. *Inflammation,* **15,** 497–503.

ALEY, K.O., GREEN, P.G. & LEVINE, J.D. (1995). Opioid and adenosine peripheral antinociception are subject to tolerance and withdrawal. *J.Neurosci.,* **15,** 8031–8038.

ALEY, K.O. & LEVINE, J.D. (1999). Role of protein kinase A in the maintenance of inflammatory pain. *J. Neurosci.,* **19,** 2181–2186.

ALEY, K.O., MESSING, R.O, MOCHLY-ROSEN, D. & LEVINE, J.D. (2000). Chronic hypersensitivity for inflammatory nociceptor sensitization mediated by the ε isozyme of protein kinase C. *J. Neurosci.,* **20,** 4680–4685.

ALSTERGREN, P., ERNBERG, M., KVARNSTROM, M. & KOPP, S. (1998). Interleukin-1 in synovial fluid from arthritic temporomandibular joint and its relation to pain, mobility, and anterior open bite. *J. Oral Maxillofac. Surg.,* **56,** 1059–1065.

BAYON, Y., ALONSO, A., HERNANDEZ, M., NIETO, M.L. & SANCHEZ-CRESPO, M. (1998). Mechanisms of cell signalling in immune-mediated inflammation. *Cytokines Cell Mol. Ther.,* **4,** 275–286.

BIENVENU, J., COULON, L., BARBIER, Y., BARBIER, M., DOCHE, C., LEPAPE, A. & GUENOUNOU, M. (1992). Study of pentoxifylline induced modulation of TNF alpha and interleukin-6 secretion in healthy and septic patients by the use of an *ex-vivo* model on whole blood. *Nouv. Rev. Fr. Hematol.,* **34,** S65–S67.

BILLIAU, A. & MATTHYS, P. (2001). Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *J. Leuk. Biol.,* **70,** 849–860.

BISGAARD, H. & KRISTENSEN, J.K. (1985). Leukotriene B_4 produces hyperalgesia in humans. *Prostaglandins,* **30,** 791–797.

BONNET, J., LOISEAU, A.M., ORVOEN, M. & BESSIN, P. (1981). Platelet-activating factor acether (PAF-acether) involvement in acute inflammatory and pain processes. *Agents Actions,* **11,** 559–562.

CAMUSSI, G., TETTA, C., BUSSOLINO, F. & BAGLIONI, C. (1989). Tumour necrosis factor stimulates human neutrophils to release leukotriene B_4 and platelet-activating factor. Induction of phospholipase A_2 and acetyl-CoA:1-alkyl-*sn*-glycero-phosphocholine O2-acetyltransferase activity and inhibition by antiproteinase. *Eur. J. Biochem.,* **182,** 6661–6666.

CANETTI, C.A., SILVA, J.S., FERREIRA, S.H. & CUNHA, F.Q. (2001). Tumour necrosis factor-alpha and leukotriene B_4 mediate the neutrophil migration in immune inflammation. *Br. J. Pharmacol.,* **134,** 1619–1628.

CHOI, S.S., LEE, J.K. & SUH, H.W. (2001). Antinociceptive profiles of aspirin and acetaminophen in formalin, substance P and glutamate pain models. *Brain Res.,* **921,** 233–239.

CROSSARA-ALBERTO, D.P., CUNHA, F.Q. & FERREIRA, S.H. (1997). Bradykinin stimulates the production of membrane-associated and soluble tumour necrosis factor-α. In: *4th International Congress on the Immune Consequences of Trauma, Shock and Sepsis* Munich, Germany, 4–8 March. ed. Faist, E., pp. 333–337 Monduzzi Editore. Bologna, Italy.

CUNHA, F.Q., POOLE, S., LORENZETTI, B.B. & FERREIRA, S.H. (1991). Interleukin-8 as a mediator of sympathetic pain. *Br. J. Pharmacol.,* **104,** 765–767.

CUNHA, F.Q., POOLE, S., LORENZETTI, B.B. & FERREIRA, S.H. (1992). The pivotal role of tumour necrosis factor α in the development of inflammatory hyperalgesia. *Br. J. Pharmacol.,* **107,** 660–664.

CUNHA, F.Q., TEIXEIRA, M.M. & FERREIRA, S.H. (1999). Pharmacological modulation of secondary mediator systems-cyclic AMP and cyclic GMP-on inflammatory hyperalgesia. *Br. J. Pharmacol.,* **127,** 671–678.

CUNHA, J.M., CUNHA, F.Q., POOLE, S. & FERREIRA, S.H. (2000). Cytokine-mediated inflammatory hyperalgesia limited by interleukin-1 receptor antagonist. *Br. J. Pharmacol.,* **130,** 1418–1424.

DAVIDSON, E.M., RAE, S.A. & SMITH, M.J. (1983). Leukotriene B_4, a mediator of inflammation present in synovial fluid in rheumatoid arthritis. *Ann.Rheum. Dis.,* **42,** 677–679.

DECKER, K. (1990). Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur. J. Biochem.,* **192,** 245–261.

DUARTE, I.D.G. & FERREIRA, S.H. (1992). The molecular mechanism of central analgesia induced by morphine or carbachol and the L-arginine–nitric oxide–cGMP pathway. *Eur. J. Pharmacol.,* **221,** 171–174.

DUARTE, I.D.G., NAKAMURA, M. & FERREIRA, S.H. (1988). Participation of the sympathetic system in acetic acid-induced writhing in mice. *Braz. J.Med. Biol. Res.,* **21,** 341–343.

EASTGATE, J.A., SYMONS, J.A., WOOD, N.C., GRILINTON, F.M., GIOVINE, F.S. & DUFF, G.W. (1988). Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis. *Lancet,* **2,** 706–709.

ENDO, H., AKAHOSHI, T., TAKAGISHI, K., KASHIWAZAKI, S. & MATSUSHIMA, K. (1991). Elevation of interleukin-8 (IL-8) levels in joint fluids of patients with rheumatoid arthritis and the induction by IL-8 of leukocytes infiltration and synovitis in rabbit joint. *Lymphokine Cytokine Res.,* **10,** 245–252.

FEITOSA, R.F., MELCIADES, G.B., ASSREY, A.M., ROCHA, M.F., RIBEIRO, R.A. & LIMA, A.A. (2002). The pharmacological profile of ovalbumin-induced paw oedema in rats. *Mediators Inflamm.,* **11,** 155–163.

FERREIRA, J., CAMPOS, M.M., PESQUERO, J.B., ARAUJO, R.C., BADER, M. & CALIXTO, J.B. (2001). Evidence for the participation of kinins in Freund's adjuvant-induced inflammatory and nociceptive responses in kinin B_1 and B_2 receptor knockout mice. *Neuropharmacology,* **41,** 1006–1012.

FERREIRA, S.H. (1983). Prostaglandins: peripheral and central analgesia. In: *Advances In Pain Research and Therapy* ed. Bonica, J.J., Lindblom, U. & Iggor, A., Vol 5. pp 627–634. New York: Raven Press.

FERREIRA, S.H., CUNHA, F.Q., LORENZETTI, B.B., MICHELIN, M.A., PERRETTI, M., FLOWER, R.J. & POOLE, S. (1997). Role of lipocortin-1 in the anti-hyperalgesic actions of dexamethasone. *Br. J. Pharmacol.,* **121,** 883–888.

FERREIRA, S.H., LORENZETTI, B.B., BRISTOW, A.F. & POOLE, S. (1988). Interleukin-1β as a potent hyperalgesic agent antagonised by a tripeptide analogue. *Nature,* **334,** 698–700.

FERREIRA, S.H., LORENZETTI, B.B. & CORREA, F.M.A. (1978). Central and peripheral antialgesic action of aspirin like drug. *Eur. J. Pharmacol.,* **53,** 39–48.

FERREIRA, S.H., LORENZETTI, B.B., CUNHA, F.Q. & POOLE, S. (1993). Bradykinin release of TNFα plays a key role in the development of inflammatory hyperalgesia. *Agents Actions,* **38**, Special Conference Issue.

FERREIRA, S.H. & NAKAMURA, M.I. (1979a). Prostaglandin hyperalgesia, a cAMP/Ca^{2+} dependent process. *Prostaglandins,* **18,** 179–190.

FERREIRA, S.H. & NAKAMURA, M.I. (1979b). III – Prostaglandin hyperalgesia: relevance of the peripheral effect for the analgesic action of opioid-antagonists. *Prostaglandins,* **18,** 201–208.

FERREIRA, S.H., ROMITELLI, M. & DE NUCCI, G. (1989). Endothelin-1 participation in overt and inflammatory pain. *J. Cardiovasc. Pharmacol.,* **13,** S220–S222.

FOLLENFANT, R.L., HARDY, G.W., LOWE, L.A. & SCHNEIDER, C. (1988). Antinociceptive effects of the novel opioid peptide BW443C compared with classical opiates; peripheral versus central actions. *Br. J.Pharmacol.,* **93,** 85–92.

FRANCISCHI, J.N., LORENZETTI, B.B. & FERREIRA, S.H. (1988). Interleukin-1 mimics the hyperalgesia induced by a factor obtained by macrophage lysis. *Braz. J. Med. Biol. Res.,* **21,** 321–331.

FREUND, J. (1956). The mode of action of immuno-pharmacological adjuvants. *Adv. Tuberc. Res.,* **1,** 130–148.

GRACIE, J.A, FORSEY, R.J., CHAN, W.L., GILMOR, A., LEUNG, B.P., GREER, M.R., KENNEDY, K., CARTER, R., WEI, XQ., XU, D., FIELD, M., FOULIS, A., LIEW, F.Y. & MCINNES, I.B. (1999). A proinflammatory role for IL-18 in rheumatoid arthritis. *J. Clin. Invest.,* **104,** 1393–1401.

GRANADOS-SOTO, V., ALONSO-LOPEZ, R., ASOMOZA-ESPINOSA, R., RUFINO, M.O., GOMES-LOPEZ, L.D. & FERREIRA, S.H. (2001). Participation of COX, IL-1 beta and TNF alpha in formalin-induced inflammatory pain. *Proc. West Pharmacol. Soc.,* **44,** 15–17.

GURSEL, T., FIAT, S. & ERCAN, Z.S. (1997). Increase serum leukotriene B_4 level in the active stage of rheumatoid arthritis in children. *Prostaglandins Leukot. Essent. Fatty Acids,* **56,** 205–207.

HANDWERKER, H.O. (1976). Influences of algogenic substances on the discharges of unmyelinated cutaneous nerve fibres identified as nociceptor. In: *Advances in Pain Research and Therapy* ed. Bonica, J.J. & Albe-Fessard, D. pp 41–45. New: York: Raven Press.

HANNINGTON-KIFF, J.G. (1974). Intravenous regional sympathetic block with guanethidine. *Lancet,* **1,** 1019–1020.

HIRATA, F., SCHIFFMANN, E., VENKATASUBRAMANAIN, K., SALOMON, D. & AXEROLD, J. (1980). A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. U.S.A.,* **77,** 2533–2536.

KOCH, K., MELVIN JR, L.S., REITER, L.A., BIGGERS, M.S., SHOWELL, H.J., PETTIPHER, E.R., CHENG, J.B., MILICI, A.J., BRESLOW, R., CONKLYN, M.J., SMITH, M.A., HACKMAN, B.C., DOHERTY, N.S., SALTER, E., FARREL, C.A. & SCHULTE, G. (1994). (+)1-(3*S*,4*R*)-[3-(4-phenylbenzyl)-4-hydroxychroman-7-yl]-cyclopentane carboxylic acid, a highly potent, selective leukotriene B_4 antagonist with oral activity in the murine collagen-induced arthritis model. *J. Med. Chem.,* **37,** 3197–3199.

KÖNIG, A., KRENN, V., TOKSOY, A., GERHARD, N. & GILLITZER, R. (2000). Mig, GROα and RANTES messenger RNA expression in lining layer, infiltrates and different leukocyte populations of synovial tissue from patients with rheumatoid arthritis, psoriatic arthritis and osteoarthritis. *Virchows Arch.,* **436,** 449–458.

KOPP, S. (1998). The influence of neuropeptides, serotonin, and interleukin 1 beta on temporomandibular joint pain and inflammation. *J. Oral Maxillofac. Surg.,* **56,** 189–191.

LEVINE, J.D., CODERRE, T.J., HELMS, C. & BASBAUM, A.I. (1988). Beta 2- adrenergic mechanisms in experimental arthritis. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 4553–4556.

LEVINE, J.D., LAU, W., KWIAT, G. & GOETZL, E.J. (1984). Leukotriene B_4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. *Science,* **225,** 743–745.

LEWIN, G.R., RITTER, A.M. & MENDELL, L.M. (1993). Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. *J. Neurosci.,* **13,** 2136–2148.

LEWIN, G.R., RUEFF, A. & MENDELL, L.M. (1994). Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur. J. Neurosci.,* **6,** 1903–1912.

LOL, L. & NATHAN, P.W. (1978). Painful peripheral states and sympathetic blocks. *J. Neurol. Phychiatry,* **41,** 664–671.

LOL, L., NATHAN, P.W., SCHOTT, G.D. & WILSON, P.G. (1980). Effects of regional guanethidine infusion in certain painful states. *J. Neurol. Psychiatry,* **43,** 446–451.

LORENZETTI, B.B., VEIGA, F.H., CANETTI, C.A., POOLE, S., CUNHA, F.Q. & FERREIRA, S.H. (2002). CINC-1 mediates the sympathetic component of the inflammatory mechanical hypersensitivity in rats. *Eur. Cytokine Netw.,* **13,** 1–6.

MA, Q.P. & WOOLF, C.J. (1997). The progressive tactile hyperalgesia induced by peripheral inflammation is nerve growth factor dependent. *NeuroReport,* **8,** 807–810.

MEYER, J.D., YURT, R.W., DUHANEY, R., HESSE, D.G., TRACEY, K.J., FONG, Y.M., VERMA, M., SHIRES, G.T., DINEEN, P., LOWRY, S.F. & DAVIS, J.M. (1988). Tumour necrosis factor-enhanced leukotriene B_4 generation and chemotaxis in human neutrophils. *Arch. Surg.,* **123,** 1454–1458.

MILLER, D.K., GILLARD, J.W., VICKERS, P.J., SADOWSKI, S., LÈVEILLÉ, C., MANCINI, J.A., CHARLESON, P., DIXON, R.A.F., FORD-HUTCHINSON, A.W., FORTIN, R., GAUTHIER, J.Y., RODKEY, J., ROSEN, R., ROUZER, C., SIGAL, I.S., STRADER, C.D. & EVANS, J.F. (1990). Identification and isolation of membrane protein necessary for leukotriene production. *Nature,* **343,** 278–281.

MOREIRA, A.L., SAMPAIO, E.P., ZMUIDZINAS, A., FRINDT, P., SMITH, K.A. & KAPLAN, G. (1993). Thalidomide exerts its inhibitory action on tumour necrosis factor alpha by enhancing mRNA degradation. *J. Exp. Med.,* **177,** 1675–1680.

NAKAMURA, M. & FERREIRA, S.H. (1987). A peripheral sympathetic component in inflammatory hyperalgesia. *Eur. J. Pharmacol.,* **135,** 145–153.

NATHAN, C.F. (1987). Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes.. *J. Clin. Invest.,* **80,** 1550–1560.

NORDAHL, S., ALSTERGREN, P. & KOPP, S. (2000). Tumour necrosis factor-alpha in synovial fluid and plasma from patients with chronic connective tissue disease and its relation to temporomandibular joint pain. *J. Oral Maxillofac Surg.,* **58,** 525–530.

OMOTE, K., KAWAMATA, T., NAKAYAMA, Y., YAMAMOTO, H., KAWAMATA, M. & NAMIKI, A. (2002). Effects of a novel selective agonist for prostaglandin receptor subtype EP_4 on hyperalgesia and inflammation in monoarthritic model. *Anesthesiology,* **97,** 170–176.

PERL, E.R. (1976). Sensitization of nociceptors and its relation to sensation. In *Advances in Pain Research and Therapy* ed. Bonica , J.J. & Albe-Fessard, D. pp. 41–45. New York: Raven Press.

POOLE, S., CUNHA, F.Q. & FERREIRA, S.H. (1999b). Hyperalgesia from subcutaneous cytokines. In *Cytokines and Pain* ed. Watkins, L.R., Maier, S.F. pp. 59–87. Basel, Switzerland: Birkhäuser Verlag.

POOLE, S., LORENZETTI, B.B., CUNHA, J.M., CUNHA, F.Q. & FERREIRA, S.H. (1999a). Bradykinin B_1 and B_2 receptors, tumour necrosis factor α and inflammatory hyperalgesia. *Br. J. Pharmacol.,* **126,** 649–656.

PORTANOVA, J.P., ZHANG, Y., ANDERSON, G.D., HAUSER, S.D., MASFERRER, J.L., SEIBERT, K., GREGORY, S.A. & ISAKSON, P.C. (1996). Selective neutralization of prostaglandin E_2 blocks inflammation, hyperalgesia, and interleukin 6 production *in vivo*. *J. Exp. Med.,* **184,** 883–891.

REES, G., GEE, C.K., WARD, H.L., BALL, C., TARRANT, G.M., POOLE, S. & BRISTOW, A.F. (1999). Rat tumour necrosis factor-α: expression in recombinant picia pastoris, purification, characterisation and development of a novel ELISA. *Eur. Cytokine Netw.,* **10,** 383–392.

RIBEIRO, R.A., VALE, M.L., FERREIRA, S.H. & CUNHA, F.Q. (2000a). Analgesic effect of thalidomide on inflammatory pain. *Eur. J. Pharmacol.,* **391,** 97–103.

RIBEIRO, R.A., VALE, M.L., THOMAZZI, S.M., PASCHOALATO, A.B., POOLE, S., FERREIRA, S.H. & CUNHA, F.Q. (2000b). Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *Eur. J. Pharmacol.,* **387,** 111–118.

SAFIEH-GARABEDIAN, B., POOLE, S., ALLCHORNE, A., WINTER, J. & WOOLF, C.J. (1995). Interleukin-1 beta contributes to the inflammation-induced increase in nerve-growth factor levels and inflammatory hyperalgesia. *Br. J. Pharmacol.,* **115,** 1265–1275.

SAFIEH-GARABEDIAN, B., POOLE, S., HADDAD, J.J., MASSAAD, C.A., JABBUR, S.J. & SAADE, N.E. (2002). The role of the sympathetic efferents in endotoxin-induced localized inflammatory hyperalgesia and cytokine upregulation. *Neuropharmacology,* **42,** 864–872.

SAMPAIO, E.P., SARNO, E.N., GALILLY, R., KOHN, Z.A. & KAPLAN, Z. (1991). Thalidomide selectively inhibits tumour necrosis factor-α production by stimulated human monocytes. *J. Exp. Med.,* **173,** 699–703.

SCHANDENÉ, L., VANDENBUSSCHE, P., CRUSIAUX, A., ALÈGRE, M.L., ABRAMOWICZ, D., DUPONT, E., CONTENT, J. & GOLDMAN, M. (1992). Differential effects of pentoxifylline on the production of tumour necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) by monocytes and T cells. *Immunology,* **76,** 30–40.

SEGOND VON BANCHET, G.G., PETROW, P.K., BRAUER, R. & SCHAIBLE, H.G. (2000). Monoarticular antigen-induced arthritis leads to pronounced bilateral upregulation of the expression of neurokinin 1 and bradykinin 2 receptors in dorsal root ganglion neurons of rats. *Arthritis Res.,* **2,** 424–427.

STRIETER, R.M., REMICK, D.G., WARD, P.A., SPENGLER, R.N., LYNCH, J.P., LARRICK, J. & KUNKEL, S.L. (1988). Cellular and molecular regulation of TNF-alpha production by pentoxifylline. *Biochem. Biophys. Res. Commun.,* **155,** 1230–1236.

TAIWO, Y.O. & LEVINE, J.D. (1991). Further confirmation of the role of adenyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neuroscience,* **44,** 131–135.

VIANA, C.F., MELO, D.H., CARNEIRO-FILHO, B.A., MICHELIN, M.A., BRITO, G.A., CUNHA, F.Q., LIMA, A.A. & RIBEIRO, R.A. (2002). Pro-inflammatory effects of cholera toxin: role of tumor necrosis factor alpha. *Toxicon,* **40,** 1487–1494.

VINEGAR, R., TRUAX, J.F. & SELPH, J.L. (1976). Quantitative comparison of the analgesic and anti-inflammatory activities of aspirin, phenacetin and acetaminophen in rodents. *Eur. J. Pharmacol.,* **37,** 23–30.

WATANABE, K., KOIZUMI, F., KURASHIGE, Y., TSURUFUJI, S. & NAKAGAWA, H. (1991). Rat CINC, a member of the interleukin-8 family, is a neutrophil-specific chemoattractant *in vivo*. *Exp. Mol. Pathol.,* **55,** 30–37.

WEINBERG, J.B., MASON, S.N. & WORTHAM, T.S. (1992). Inhibition of tumour necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) messenger RNA (mRNA) expression in HL-60 leukemia cells by pentoxifylline and dexamethasone: dissociation of acivicin-induced TNFα and IL-1β mRNA expression from acivicin-induce monocytoid differentiation. *Blood,* **79,** 3337–3343.

ZABEL, P., SCHONHARTING, M.M., WOLTER, D.T. & SCHADE, U.F. (1989). Oxpentoxifylline in endotoxaemia. *Lancet,* **2,** 1474–1477.

(Received February 25, 2003
Revised April 5, 2003
Accepted April 25, 2003)