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The pivotal role of tumour necrosis factor α in the development of inflammatory hyperalgesia

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1 The hyperalgesic activities in rats of interleukin-1β (IL-1β), IL-6, IL-8, tumour necrosis factor α (TNFα) and carrageenin were investigated.

2 IL-6 activated the previously delineated IL-1/prostaglandin hyperalgesic pathway but not the IL-8/sympathetic mediated hyperalgesic pathway.

3 TNFα and carrageenin activated both pathways.

4 Antiserum neutralizing endogenous TNFα abolished the response to carrageenin whereas antisera neutralizing endogenous IL-1β, IL-6 and IL-8 each partially inhibited the response.

5 The combination of antisera neutralizing endogenous IL-1β + IL-8 or IL-6 + IL-8 abolished the response to carrageenin.

6 These results show that TNFα has an early and crucial role in the development of inflammatory hyperaglesia.

7 The delineation of the roles of TNFα, IL-1β, IL-6 and IL-8 in the development of inflammatory hyperalgesia taken together with the finding that the production of these cytokines is inhibited by steroidal anti-inflammatory drugs provides a mechanism of action for these drugs in the treatment of inflammatory hyperalgesia.

Keywords: Tumour necrosis factor; interleukin-6; inflammatory hyperalgesia

Introduction

Sensitization of pain receptors causing hyperalgesia is the common denominator of pain of various origins. Two hyperalgesic pathways have been identified in experimental animals and in man; the relative contribution of each pathway may depend upon the characteristics of the injurious stimulus (Ferreira, 1972; Hannington-Kiff, 1974; Coderre *et al.*, 1984; Nakamura & Ferreira, 1987). In both pathways the release of hyperalgesic mediators, e.g. prostaglandins and sympathomimetics, occurs subsequent to the release of the cytokines interleukin-1β (IL-1β) and IL-8, respectively (Cunha *et al.*, 1991). Thus cytokines appear to constitute a link between cellular injury or recognition of non-self and the development of local and systemic manifestations of inflammation, e.g. cell migration, oedema, fever, release of actue-phase proteins and hyperalgesia (Dinarello *et al.*, 1986; Ferreira *et al.*, 1988; Faccioli *et al.*, 1990; Dinarello, 1991; Cunha *et al.*, 1991).

A property of several cytokines is their capacity to induce their own production and that of other cytokines: tumour necrosis factor α (TNFα) induces production of IL-1 (Dinarello *et al.*, 1986) and IL-1 induces production of IL-1 (Dinarello *et al.*, 1987), IL-6 (Van Damme *et al.*, 1987) and IL-8 (Streiter *et al.*, 1989). In the present study we tested TNFα and IL-6 for their capacities to cause sensitization of pain receptors and examined the possibility that TNFα and IL-6 were involved in the IL-1/prostaglandin mediated and the IL-8/sympathetic mediated hyperalgesic pathways activated by the inflammatory agent carrageenin (Ferreira *et al.*, 1988; Cunha *et al.*, 1991).

Methods

Nociceptive test

The intensity of hyperalgesia was assessed by a modification of the Randall-Selitto test (Ferreira *et al.*, 1978). The intensity of hyperalgesia was quantified as the variation in reaction time (delta reaction time) obtained by subtracting the value measured at the time intervals indicated after administration of the hyperalgesic substance from the control reaction time (zero time).

Experimental protocol

Hyperalgesia was measured following injections of IL-6, TNFα, IL-1β and IL-8, and carrageenin into hind paws of rats (intraplantar, i.pl.). The intensity of hyperalgesia was measured before injection, 0.5–6 h and 24 h after injection of IL-6 and TNFα and before and 3 h after injection of IL-1β, IL-8 and carrageenin. Drugs were injected i.pl., 30 min before cytokines. The drugs injected were Lys-D-Pro-Thr (Ferreira *et al.*, 1988), the cyclo-oxygenase inhibitor, indomethacin (Vane, 1971) and the β-adrenoceptor antagonist, atenolol (Robertson *et al.*, 1983). The doses of indomethacin, Lys-D-Pro-Thr and atenolol used have prevously been shown to inhibit hyperalgesia evoked by IL-1β (indomethacin and Lys-D-Pro-Thr; Ferreira *et al.*, 1988; Cunha *et al.*, 1991) and IL-8 (atenolol; Cunha *et al.*, 1991). In other experiments cytokines were incubated with anti-cytokine sera for 15 min before injection of the mixture; anti-cytokine sera were injected (i.pl.) 30 min before carrageenin. Results are presented as means with s.e.mean. Formal statistical tests are not reported since for all the differences discussed, means differed by more than three times the larger s.e.mean.

Materials

Drugs IL-1β, IL-6, IL-8 (72 amino acids) and TNFα were NIBSC preparations coded 86/680, 88/514, 89/520 and 87/650, respectively. Indomethacin (Indo) was a gift from Merck, Sharpe & Dohme Ltd (Hoddesdon, Herts). Carrageenin was a gift from FMC Corporation (Philadelphia, U.S.A.). Atenolol (ATN) was purchased from Sigma (St. Louis, U.S.A.). Lys-D-Pro-Thr was (custom) synthesized by Cambridge Research Biochemicals (Cambridge, England)

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and characterized by the manufacturer by fast atom bombardment mass spectrometry, amino acid analysis and analytical reverse-phase high performance liquid chromatography (h.p.l.c.). The peptide was purified to $\geqslant 95\%$ by preparative h.p.l.c. at NIBSC.

Antisera The following antisera were used: sheep anti-human IL-1β (Poole *et al.*, 1989); sheep anti-rat IL-1β (Bristow *et al.*, 1991); goat anti-human IL-6 (Rafferty *et al.*, 1991); rabbit anti-rat IL-6 (Rothwell *et al.*, 1991), a generous gift from Professor J. Gauldie (McMaster University, Hamilton, Ontario, Canada); sheep anti-human IL-8 (Cunha *et al.*, 1991), kindly provided by Dr R. Thorpe, Division of Immunobiology, NIBSC; goat anti-human TNFα and sheep anti-murine TNFα (Mahadevan *et al.*, 1990), kindly provided by Dr T. Meager, Division of Immunobiology, NIBSC.

Animals Male Wistar rats, 130–180 g, housed in temperature controlled-rooms (23 ± 2°C) with water and food *ad libitum* until use.

Results

Time courses of hyperalgesic responses to IL-6 and TNFα

Injection of IL-6 or TNFα into one hindpaw (i.pl.) evoked a dose-dependent hyperalgesia in both hind paws although the effects of smaller doses were greatest in the injected paws (Figure 1). The intensity of hyperalgesia reached a plateau between 2–3 h after injection of IL-6 or TNFα; the responses to IL-6 were maintained at 6 h whereas responses to TNFα had begun to decline at 6 h after injection. Responses to both cytokines returned to pre-injection values within 24 h.

Inhibition by drugs of the hyperalgesic responses to IL-1β, IL-6, IL-8 and TNFα

Injection of IL-1β (0.5 pg) and IL-8 (100 pg) evoked hyperalgesic responses of similar magnitude to those evoked by IL-6 (1 ng) and TNFα (2.5 pg, Figure 2). Data are shown for injected paws only. Similar responses were obtained in contralateral paws as can be seen from Figure 1 for IL-6 and TNFα and in the study of Cunha *et al.* (1991) for IL-1β and IL-8. Local pretreatment (i.pl.) with indomethacin (Indo, 100 μg) or Lys-D-Pro-Thr (200 μg), 30 min before the cytokine, abolished responses to IL-1β (−91 ± 3% after Indo, −92 ± 1% after Lys-D-Pro-Thr), markedly attenuated responses to IL-6 (−74 ± 1% and −78 ± 3%) and attenuated responses to TNFα (−47 ± 3% and −50 ± 3%) but did not affect responses to IL-8 (+2 ± 4% and 0 ± 1%, Figure 2). In contrast, local pretreatment with atenolol (25 μg) markedly attenuated responses to IL-8 (−68 ± 2%) and TNFα (−66 ± 3%) but not responses to IL-1β (−6 ± 2%) and IL-6 (−3 ± 2%, Figure 2). The inhibitory effects on TNFα evoked hyperalgesia of pretreatment with indomethacin together with Lys-D-Pro-Thr were not additive (−51 ± 4%). In con-

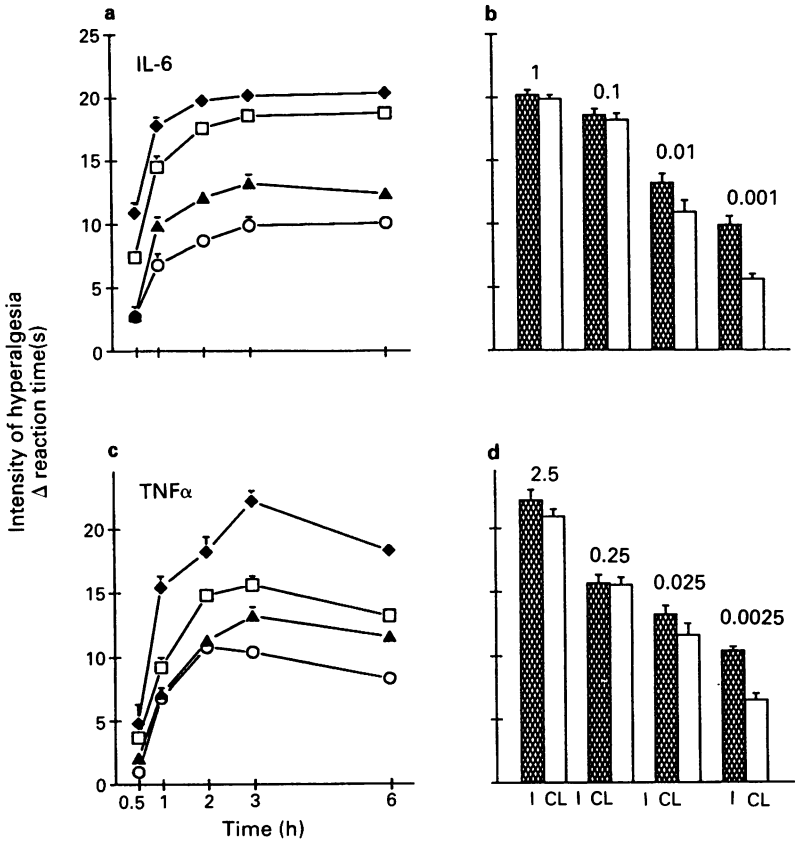

Figure 1 Time course of the development of hyperalgesia to interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) injected into rat paws. Panel (a) shows the intensity of hyperalgesia in injected paws 0.5–6 h after injection of IL-6 (0.001 (○); 0.01 (▲); 0.1 (□) and 1.0 (◆) ng in 100 μl i.pl.). Panel (b) shows the intensity of hyperalgesia in injected paws (I, cross-hatched columns) and in contralateral paws (CL, open columns) 3 h after injection of IL-6 (at the doses indicated above the columns, ng/paw). Panel (c) shows the intensity of hyperalgesia in injected paws 0.5–6 h after injection of TNFα (0.0025 (○); 0.025 (▲); 0.25 (□) and 2.5 (◆) pg in 100 μl i.pl.). Panel (d) shows the intensity of hyperalgesia in injected paws (I, cross-hatched columns) and in contralateral paws (CL, open columns) 3 h after injection of TNFα (at the doses indicated above the columns, pg/paw). Vertical bars are s.e.mean in groups of 5 rats.

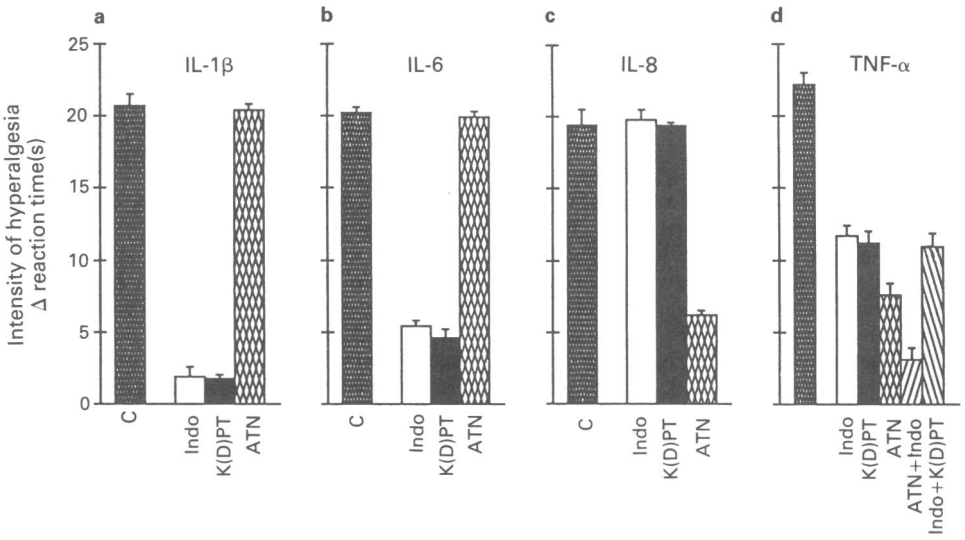

Figure 2 Inhibition of the hyperalgesic effects of (a) interleukin-1β (IL-1β), (b) IL-6, (c) IL-8 and (d) tumour necrosis factor α (TNFα). Responses were measured 3 h after injection (in 100 μl, i.pl.) of (a) IL-1β (0.5 pg); (b) IL-6 (1 ng); (c) IL-8 (100 pg) and (d) TNFα (2.5 pg). Pretreatments were given (100 μl, i.pl.) 30 min before cytokines: C = saline, Indo = indomethacin, 100 μg; K(D)PT = Lys-D-Pro-Thr, 200 μg; ATN = atenolol, 25 μg. Vertical bars are s.e.mean in groups of 5 rats.

trast, the effects of indomethacin together with atenolol were additive: this combination abolished TNFα-evoked hyperalgesia (−86 ± 4%, Figure 2d).

Indomethacin, Lys-D-Pro-Thr and atenolol injected alone were without effect in injected and contralateral paws and had no effect on responses to cytokines measured in contralateral paws: data not shown but published previously (Ferreira *et al.*, 1988; Cunha *et al.*, 1991).

Inhibition by anti-cytokine sera of the hyperalgesic responses to IL-1β, IL-6, IL-8 and TNFα

The hyperalgesic responses to human IL-1β were abolished when it was incubated (for 15 min) and injected with sheep anti-human IL-1β serum but not by incubation and injection with other antisera (Figure 3a). Hyperalgesic responses to human IL-6 were abolished by incubation and injection with goat anti-human IL-6 serum or sheep anti-rat IL-1β serum but not with other antisera (Figure 3b). Hyperalgesic responses to human IL-8 were abolished by incubation with sheep anti-human IL-8 serum but not with other antisera (Figure 3c). Hyperalgesic responses to human TNFα were abolished by incubation and injection with goat anti-human TNFα serum but not with preimmune serum. Antisera neutralising rat IL-1β, IL-6 and IL-8 each attenuated responses to human TNFα (−59 ± 3%, −61 ± 4%, −67 ± 5%). The inhibitory effects on TNFα evoked hyperalgesia of antisera neutralising rat IL-1β and IL-6 were not additive (−58 ± 2%); in contrast, the inhibitory effects of antisera neutralizing rat IL-1β and IL-8 were additive (−89 ± 3%), as were those of antisera neutralizing rat IL-6 and IL-8 (−87 ± 2%, Figure 3d).

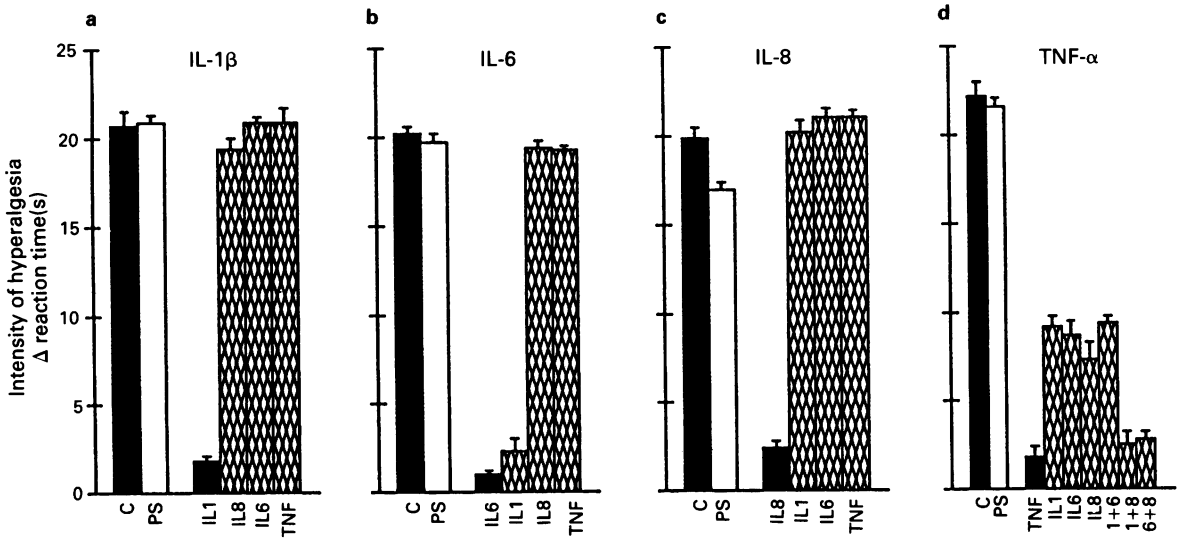

Figure 3 Effects of various antisera on the hyperalgesic effects of (a) interleukin-1β (IL-1β), (b) IL-6, (c) IL-8 and (d) tumour necrosis factor α (TNFα). Responses were measured 3 h after injection (in 100 μl, i.pl.) of (a) IL-1β (0.5 pg); (b) IL-6 (1 ng); (c) IL-8 (100 pg) and (d) TNFα (2.5 pg). Antisera neutralizing the cytokines indicated on the x-axis (100 μl) were incubated for 15 min with the cytokine before injection. Vertical bars are s.e.mean in groups of 5 rats. C = control; PS = pre-immune serum.

Inhibition by anti-cytokine-sera of the hyperalgesic responses to carrageenin

Carrageenin-evoked hyperalgesia was inhibited by pretreatment with sheep anti-rat IL-1β serum (50 μl: −47 ± 5% and 150 μl: −57 ± 3%) and with rabbit anti-rat IL-6 serum (50 μl: −44 ± 4% and 150 μl: −56 ± 2%); the effects of the antisera (50 μl of each: −43 ± 2%) were not additive (Figure 4). Carrageenin-evoked hyperalgesia was also inhibited by a serum neutralizing rat IL-8 (sheep anti-human IL-8 serum, 50 μl: −64 ± 3% and 150 μl: −62 ± 2%). The inhibitory effects of the anti-IL-8 serum (50 μl) were additive with the inhibitory effects of sheep anti-rat IL-1β serum (50 μl: −93 ± 1%) and with rabbit anti-rat IL-6 serum (50 μl: −90 ± 2%, Figure 4). Carrageenin-evoked hyperalgesia was inhibited by a serum neutralising rat TNFα (sheep anti-murine TNFα, 50 μl: −66 ± 3% and 150 μl: −90 ± 2%) and the inhibitory effects of the smaller dose of anti-TNFα serum (50 μl) were additive with the inhibitory effects of each of the three antisera (50 μl) neutralising rat IL-1β (−94 ± 2%), IL-6 (−88 ± 4%) and IL-8 (−84 ± 2%, Figure 4).

Antisera injected alone were without effect in injected and contralateral paws and had no effect on responses to cytokines and carrageenin measured in contralateral paws: data not shown.

Discussion

TNFα and IL-6 have been shown to cause dose-dependent hyperalgesia in rats, like IL-1β (Ferreira *et al.*, 1988) and IL-8 (Cunha *et al.*, 1991). All four cytokines evoked hyperalgesia in both hind paws when injected into one hind paw, suggesting systemic distribution of the injected cytokines. This notion is supported by the findings that smaller doses of cytokines evoked consistently smaller effects in contralateral paws and that intraplantar administration of antagonists and antisera inhibited local hyperalgesia but not hyperalgesia in contralateral paws. Also, the onset of hyperalgesia was slower in contralateral paws and it was possible to restrict hyperalgesic responses to one of the cytokines, IL-1β, to the injected paws by repeated injections of very small doses (Ferreira: unpublished data).

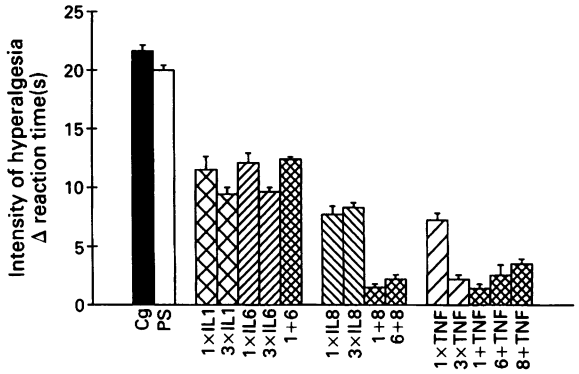

Figure 4 Inhibition of the hyperalgesic effects of carrageenin by antisera neutralising endogenous, i.e. rat, cytokines. Responses were measured 3 h after injection of carrageenin (100 μg in 50 μl, i.pl.). Antisera were injected (50 μl = 1 × and 150 μl = 3 × in a total volume of 150 μl) 30 min before carrageenin. Cg = carrageenin; PS = preimmune serum; 1 × IL1 = 50 μl sheep anti-rat IL-1β serum; 3 × IL1 = 150 μl sheep anti-rat IL-1β serum; 1 × IL6 = 50 μl rabbit anti-rat IL-6 serum; 3 × IL6 = 150 μl rabbit anti-rat IL-6 serum; 1 + 6 = 50 μl sheep anti-rat IL-1β serum + 50 μl rabbit anti-rat IL-6 serum; 1 × IL8 = 50 μl sheep anti-human IL-8 serum; 3 × IL8 = 150 μl sheep anti-human IL-8 serum; 1 + 8 = 50 μl sheep anti-rat IL-1β serum + 50 μl sheep anti-human IL-8 serum; 6 + 8 = 50 μl rabbit anti-rat IL-6 serum + 50 μl sheep anti-human IL-8 serum: 1 × TNF = 50 μl sheep anti-murine TNFα serum; 3 × TNF = 150 μl sheep anti-murine TNFα serum; 1 + TNF = 50 μl sheep anti-rat IL-1β serum + 50 μl sheep anti-murine TNFα serum; 6 + TNF = 50 μl rabbit anti-rat IL-6 serum + 50 μl sheep anti-murine TNFα serum. 8 + TNF = 50 μl sheep anti-human IL-8 serum + 50 μl sheep anti-murine TNFα serum. Vertical bars are s.e.mean in groups of 5 rats.

The doses of the four cytokines that evoked maximum hyperalgesic effects were: IL-1β (0.5 pg), TNFα (2.5 pg), IL-8 (100 pg) and IL-6 (1 ng), giving an order of potency of IL-1β>TNFα>>IL-8>>IL-6 for these human sequence cytokines in rats. Whether this order of potency reflects the order of potency of the endogenous cytokines of the rat is unknown. The rat sequence cytokines were not available for this study and certain cytokines are known to exhibit species preference or specificity (Lumpkin, 1987; Morstyn & Burgess, 1988).

Antagonism of IL-1β-evoked hyperalgesia by indomethacin and Lys-D-Pro-Thr and antagonism of IL-8 evoked hyperalgesia by antenolol confirm previous results that hyperalgesia evoked by IL-1β and IL-8 are effected via prostaglandin-mediated and sympathetic mediated pathways, respectively (Ferreira *et al.*, 1988; Cunha *et al.*, 1991). The sensitivity of IL-6-evoked hyperalgesia to blockade by indomethacin suggests that this response, like that to IL-1β, was mediated by cyclo-oxygenase products, e.g. prostaglandins. The sensitivity of responses to IL-6 to blockade by the IL-1β related peptide Lys-D-Pro-Thr and sheep anti-rat IL-1β serum suggests that IL-6 was causing hyperalgesia via a pathway common with IL-1β. In this pathway it would appear that IL-6 induced production of IL-1β. Although the converse, i.e. IL-1 induced production of IL-6, occurs (Van Damme *et al.*, 1987) we were unable to demonstrate IL-6 induced production of IL-1β *in vitro* in human blood mononuclear cells (Poole: unpublished data); also, IL-6 suppressed endotoxin-induced and TNF-induced IL-1 production in these cells (Schindler *et al.*, 1990). However, it is possible that *in vivo*, in the presence of the full repertoire of cells and mediators involved in inflammatory hyperalgesia, IL-6 has the capacity to induce IL-1 production. This possibilty was suggested recently by Rothwell (1991). Further experiments will be required to elucidate the precise sequence of events in the IL-1β/IL-6/prostaglandins hyperalgesic pathway.

TNFα evoked hyperalgesia by activation of both the IL-1β/IL-6/prostaglandin and IL-8/sympathetic mediated hyperalgesic pathways since local administration of indomethacin, Lys-D-Pro-Thr and atenolol all attenuated responses to TNFα while the combination of indomethacin and atenolol abolished responses to this cytokine. Further evidence for the involvement of both hyperalgesic pathways in the mediation of response to TNFα comes from the finding that antisera neutralizing rat IL-1β, IL-6 and IL-8 each attenuated responses to TNFα. The inhibitory effects of antisera neutralizing rat IL-1β and IL-8 were additive as were those of antisera neutralizing rat IL-6 and IL-8.

Antagonism of carrageenin-evoked hyperalgesia by indomethacin, Lys-D-Pro-Thr, atenolol and antiserum neutralizing rat IL-8 confirms previous results showing that carageenin-evoked hyperalgesia is mediated via the IL-1β/IL-6/prostaglandins and the IL-8/sympathetic pathways (Ferreira *et al.*, 1988; Cunha *et al.*, 1991). The inhibition of responses to carrageenin by antisera neutralizing rat IL-1β and IL-6 and the abolition of responses to carrageenin by antisera neutralizing rat TNFα are new findings, as are the results of experiments in which combinations of antisera were used. The lack of additive inhibitory effects against carrageenin of antisera neutralizing rat IL-1β and IL-6 supports the notion that IL-6 was causing hyperalgesia via a pathway common with IL-1β. The additive effects of antisera neutralizing rat IL-8 with antisera neutralizing rat IL-1β and IL-6 supports previous work indicating the involvement of the IL-1β/prostaglandins and IL-8/sympathetic pathways in carrageenin evoked hyperalgesia (Cunha *et al.*, 1991). The capacity of the larger dose of antiserum neutralising rat TNFα to abolish carrageenin-evoked hyperalgesia and the finding that the smaller dose of this antiserum given together with antisera

neutralizing rat IL-1β, IL-6 or IL-8 also abolished responses to carrageenin suggest that TNFα plays an early and crucial role in the development of hyperalgesia in response to carrageenin. This role is consistent with the early role of TNFα in the development of fever in response to bacterial endotoxin (Dinarello *et al.*, 1986; Rothwell, 1991).

The above experiments show that TNFα activates a cascade of cytokine release. The induction of IL-8 by TNFα leads to the development of sympathetic hyperalgesia. The induction of IL-1β and IL-6 by TNFα leads to the development of hyperalgesia mediated by cyclo-oxygenase products. In inflammatory hyperalgesia evoked by carrageenin, TNFα has a pivotal role since a single injection of this cytokine mimicked the response to carrageenin by inducing production of IL-1β, IL-6 and IL-8 and a single injection of antiserum neutralizing endogenous TNFα abolished the response to carrageenin.

The delineation of the roles of IL-1, IL-6, IL-8 and TNFα in the development of inflammatory hyperalgesia (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; and in the present study) adds to our understanding of the mechanisms underlying the potent anti-inflammatory effects of glucocorticoid drugs. It has been known for many years that this class of drugs inhibit both the early and late changes that contribute to the inflammatory process. A proportion of this anti-inflammatory activity can be accounted for by the inhibition by glucocorticoids of production of prostaglandins, leukotrienes, thromboxanes and related mediators (Blackwell *et al.*, 1980; Hirata *et al.*, 1980). The more recent findings that steroidal anti-inflammatory drugs inhibit production of cytokines (Lew *et al.*, 1988; Waage & Baake, 1988; Barton *et al.*, 1991; Seitz *et al.*, 1991) provide an additional mechanism of action for glucocorticoids in the treatment of inflammatory hyperalgesia.

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