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Immunosuppressive activity of corticotrophin-releasing factor

Inhibition of interleukin-1 and interleukin-6 production by human mononuclear cells

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Corticotrophin-releasing factor (CRF) from the hypothalamus stimulates corticotrophin (ACTH) secretion. Concentrations of CRF in the peripheral circulation are normally low, and increase during pregnancy due to CRF secretion by the placenta [Cunnah, Jessop, Besser & Rees (1987) J. Endocrinol. **113**, 123–131], although CRF in maternal blood does not appear to stimulate the hypothalamo–pituitary–adrenal (HPA) axis [Potter, Behan, Fischer, Linton, Lowry & Vale (1991) Nature (London) **349**, 423–426]. We have examined the possibility that the placental CRF might contribute to the suppression of the maternal immune system, which is necessary to prevent rejection of the foetus, by studying endotoxin-evoked cytokine production by monocytes as a model for activation of the immune response. CRF inhibited endotoxin-evoked cytokine production from human mononuclear cells (MNCs), the fraction of peripheral blood containing monocytes. The effects of CRF were reversed by a specific CRF receptor antagonist, and were additive with glucocorticoid inhibition of cytokine secretion. Anti-interleukin-1 (IL-1) antisera inhibited endotoxin-evoked IL-6 production; however, the CRF effect was not additive, suggesting that CRF inhibition of IL-6 production may be secondary to CRF inhibition of IL-1. These results suggest a role for CRF as an immunosuppressant during pregnancy.

INTRODUCTION

A number of interactions between the immune system and the hypothalamo–pituitary–adrenal (HPA) axis have been reported, including interleukin-1 (IL-1)- and interleukin-6 (IL-6)-mediated corticotrophin-releasing factor (CRF) and corticotrophin (ACTH) release (Sapolsky *et al.*, 1987; Naitoh *et al.*, 1988), immunomodulatory effects of ACTH (Blalock, 1989) and glucocorticoids (Snyder & Unanue, 1982), CRF mediation of IL-1-induced fever (Rothwell, 1989), regulation of natural killer cell cytotoxicity (Irwin *et al.*, 1990), and inhibition of T-lymphocyte proliferation by CRF (Jain *et al.*, 1991). IL-1 and IL-6 are cytokines whose production is known to be involved in the induction of inflammation and immunity (Bendtzen, 1988). Regulators of the immune response such as glucocorticoids may act by modulating production of these cytokines from monocytes.

CRF levels in the normal peripheral circulation are low; however, during pregnancy maternal plasma CRF concentrations can rise to as much as 9 ng/ml (Cunnah *et al.*, 1987). The physiological role of these high concentrations of CRF during gestation is unknown. A CRF-binding protein in human plasma has been described (Linton *et al.*, 1988) and recently cloned, and binding to this protein inhibits the ACTH-releasing action of CRF in gestational maternal plasma (Potter *et al.*, 1991). The CRF-binding protein is thought to prevent complete plasma degradation of synthetic CRF. It has therefore been suggested that the human CRF-binding protein is involved in the regulation of the post-secretory effects of CRF, both at the pituitary gland by inhibiting the activation of the HPA axis and peripherally by increasing the biological half-life of the peptide in the circulation.

Activation of monocytes, leading to the production of inflammatory mediators including the cytokines IL-1 and IL-6, is considered to be an essential early event in the activation of the immune response (Dinarello, 1989). Therefore, in the present study, bacterial-endotoxin-evoked IL-1β and IL-6 production was used as a model for monocyte activation.

We report here that CRF inhibited endotoxin-evoked IL-1β and IL-6 production from human mononuclear cells (MNCs), and that this inhibition was reversed by a specific CRF receptor antagonist, α-helical CRF-(9–41), indicating that CRF acts via a specific cell surface receptor on monocytes, the source of most of the IL-1 and IL-6 produced by activated MNCs. The effects of CRF on MNCs were additive with glucocorticoid inhibition of cytokine secretion. Endotoxin-evoked IL-6 production was inhibited by anti-IL-1 antisera; however, this was not found to be additive with the CRF effect, thus implying that CRF inhibition of IL-6 production may be modulated by CRF inhibition of IL-1 production.

MATERIALS AND METHODS

Materials

CRF (GMP grade) and α-helical CRF-(9–41) were obtained from Sigma Chemical Co., Poole, Dorset, U.K., and contained < 50 pg (0.35 units) of endotoxin/mg of CRF in the Limulus Amoebocyte Lysate (LAL) test.

The international standard for endotoxin, ampoules coded 84/650, and cytokines were provided by NIBSC. Polyclonal anti-IL antisera were raised in sheep (Poole *et al.*, 1989), and were collected and stored in sterile pyrogen-free conditions. Neither antiserum showed detectable cross-reactivity with IL-6.

All other reagents and plasticware were sterile and pyrogen-free, and glassware was baked at 250 °C for 1 h.

Preparation of mononuclear cells

Human MNCs were isolated from buffy coat residues of heparinized blood from healthy donors using Ficol Histopaque gradient centrifugation (Poole *et al.*, 1989). MNCs were seeded into 24-well plates at 10^6 cells/0.5 ml of RPMI medium containing 2% foetal calf serum (FCS; heat-inactivated, pyrogen-free). RPMI/2% FCS (0.2 ml) containing α-helical CRF-(9–41), anti-IL-1 antiserum, dexamethasone or controls, as described in

Abbreviations used: CRF, corticotrophin-releasing factor; ACTH, corticotrophin; MNCs, mononuclear cells; IL-1, interleukin-1; IL-6, interleukin-6; HPA, hypothalamo–pituitary–adrenal; FCS, foetal calf serum; PBS, phosphate-buffered saline (0.1 M-sodium phosphate (pH 7.4)/0.15 M-NaCl).

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appropriate Figure legends, was added, followed by 0.2 ml of RPMI/2% FCS containing CRF or controls and then 0.1 ml of RPMI/2% FCS containing endotoxin. After incubation for 24 h (37 °C, 5% CO_2), supernatants were removed and stored frozen prior to assay for cytokines. For the intracellular fraction, settled plus adherent cells were lysed by the addition of 300 μl of water and freeze–thawing, followed by addition of 700 μl of RPMI/2.86% FCS. Samples were stored frozen prior to assay.

Assay for IL-1β

IL-1β was assayed using a two-site immunoradiometric assay which employed an affinity-purified goat anti-IL-1β antibody preparation both as capture and detector antibody, as reported by Rafferty *et al.* (1991). Briefly, detector antibody was prepared by radiolabelling a portion (10 μg/10 μl) with 200 μCi of $Na^{125}I$ (Amersham International) by the chloramine T method, followed by purification using Dowex 1X8 (Sigma). Flexible 96-well microtitre plates (Falcon 3911; Marathon Labs., London, U.K.) were coated with 100 μl of affinity-purified antibody [1 μg/ml in phosphate-buffered saline (PBS)]. PBS containing 5% dried milk was used as a blocking agent. Standard and test samples were diluted in PBS/5% dried milk, and 50 μl portions were incubated with 50 μl (200000 c.p.m.) of radiolabelled detector antibody for 4 h. After washing, the radioactivity bound to the wells was detected using a γ-radiation counter and corrected for non-specific binding. The intra-assay and inter-assay coefficients of variation were 7.8 ± 0.4% and 12.4 ± 0.1% respectively for the IL-1β assay.

Assay for IL-6

Extracellular levels of IL-6 were measured by a specific enzyme-linked immunoabsorbent assay (Taktak *et al.*, 1991). Briefly, 96-well microtitre plates (Nunc-Immuno Plate MaxiSorp) were coated with immunoaffinity-purified goat anti-IL-6 antibodies (1 μg/ml in 100 μl of PBS) by incubation overnight at 4 °C. Samples or standards (100 μl) were added and incubated for 2 h. After washing, biotinylated immunoaffinity-purified goat anti-IL-6 antibodies (0.014 μg/100 μl per well) were added and incubated for 1 h. The plates were washed three times and incubated with avidin–horseradish peroxidase (1:500 dilution; 100 μl; Dako Ltd., High Wycombe, Bucks., U.K.) for 15 min. The plates were washed three times and incubated with *o*-phenylenediamine [0.2 mg/ml containing 0.4 μl of 30% (v/v) H_2O_2/ml (Sigma)] for 15 min. The reaction was quenched with 150 μl of 1 M-H_2SO_4 and absorbances were determined at 490 nm. The intra-assay and inter-assay coefficients of variation were 6.8 ± 0.9% and 10.4 ± 0.3% respectively for the IL-6 assay.

RESULTS

Bacterial endotoxin at concentrations between 10 and 1000 pg/ml dose-dependently stimulated human peripheral blood MNCs to produce increased levels of IL-1β and IL-6 (Fig. 1). Concentrations of intracellular IL-1β, extracellular IL-1β and IL-6 from MNCs stimulated with 100 pg of endotoxin/ml for 24 h were typically 2.4 ng/ml, 1.8 ng/ml and 3.2 ng/ml respectively (Figs. 1*a*, 1*b* and 1*c*). Typically, production of both intracellular and extracellular IL-1β in response to endotoxin was linear over the concentration range studied (10–1000 pg/ml). Secretion of IL-6, however, typically reached a maximum at 500 pg of endotoxin/ml. CRF (5–80 ng/ml) dose-dependently inhibited intracellular IL-1β, extracellular IL-1β and IL-6 production from stimulated MNCs at all concentrations of endotoxin (10–1000 pg/ml). At 100 pg of endotoxin/ml, CRF (80 ng/ml) resulted in 42%, 47% and 42% inhibition of intracellular IL-1β, extracellular IL-1β and IL-6 production respectively. Concentrations of CRF above 80 ng/ml did not result in further inhibition (results not shown).

α-Helical CRF-(9–41) is a specific CRF receptor antagonist that competitively inhibits CRF-stimulated ACTH secretion (Rivier *et al.*, 1984). The inhibitory effect of CRF (80 ng/ml) on intracellular IL-1β, extracellular IL-1β and IL-6 production evoked by 100 pg of endotoxin/ml was dose-dependently reversed by α-helical CRF-(9–41) (Fig. 2). Complete reversal was obtained with 10 μg of α-helical CRF-(9–41)/ml.

Dexamethasone (0.1–10 nM) dose-dependently inhibited endotoxin-stimulated extracellular IL-1β production by human MNCs (Fig. 3). CRF (80 ng/ml), dexamethasone (10 nM), or CRF and dexamethasone in combination, inhibited extracellular

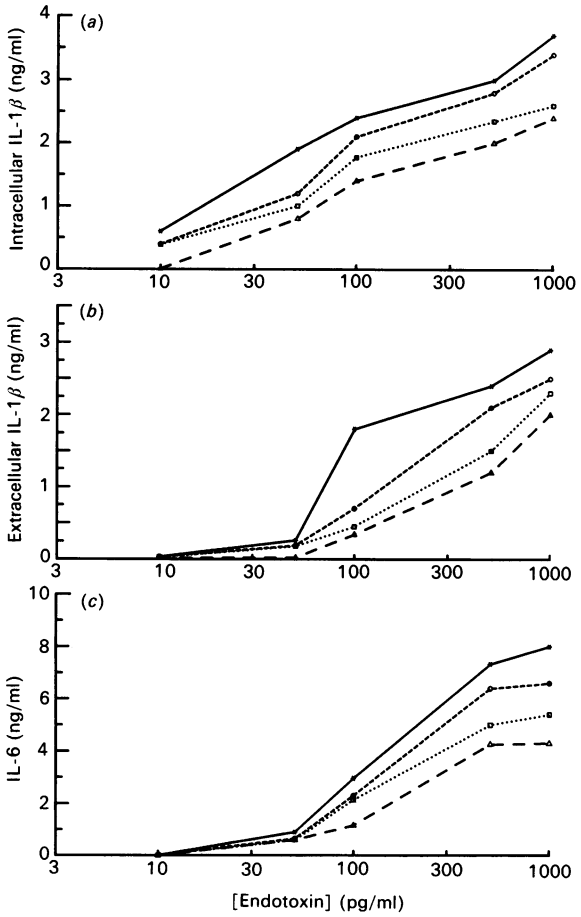

Fig. 1. Inhibition of endotoxin-stimulated cytokine secretion by CRF

MNCs were prepared and incubated with endotoxin at the concentrations shown plus or minus CRF, as described in the Materials and methods section. △, CRF (80 ng/ml); □, CRF (20 ng/ml); ○, CRF (5 ng/ml); *, control. CRF (80 ng/ml) given alone did not evoke production of detectable intracellular IL-1β, extracellular IL-1β or IL-6. Settled plus adherent cells were lysed as described for the determination of intracellular IL-1β (*a*). Supernatants were assayed to determine extracellular IL-1β (*b*) and IL-6 (*c*). IL-1β and IL-6 were determined by immunoradiometric assay and e.l.i.s.a. respectively as described. Data points represent the means of triplicate incubations assayed in triplicate. The variation between replicate incubations was generally not greater than the intra-assay coefficient of variation for either cytokine assay. Errors (not shown) were therefore calculated as the overall S.E.M. for each point ($n = 9$). The average value for the S.E.M. calculated in this way was 12.45 ± 1.8%.

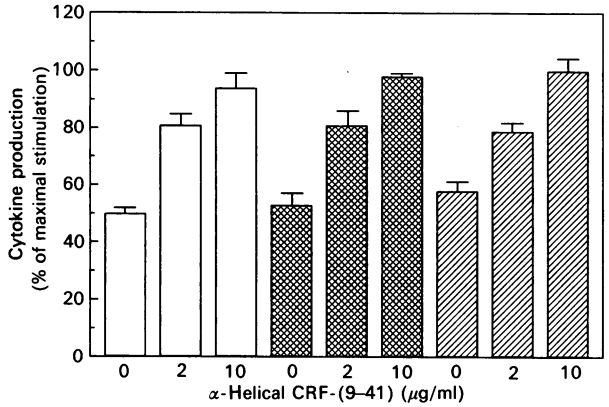

Fig. 2. Reversal by a CRF receptor antagonist of CRF inhibition of endotoxin-induced cytokine production

MNCs were prepared and incubated with endotoxin (100 ng/ml) plus CRF (80 ng/ml) and α-helical CRF-(9–41) (0, 2 or 10 μg/ml as indicated), as described in the Materials and methods section. Cell contents or supernatants were assayed for intracellular IL-1β (□), extracellular IL-1β (⊠) or IL-6 (▨) as described in the Materials and methods section. In each case data are represented as the percentage of cytokine production evoked by endotoxin (100 ng/ml) in the absence of CRF or CRF antagonist (intracellular IL-1β, 3.95 ng/ml; extracellular IL-1β, 1.52 ng/ml; IL-6, 5.89 ng/ml). Data points represent the means ± S.E.M. of triplicate incubations assayed in triplicate ($n = 9$), calculated as described in the legend to Fig. 1.

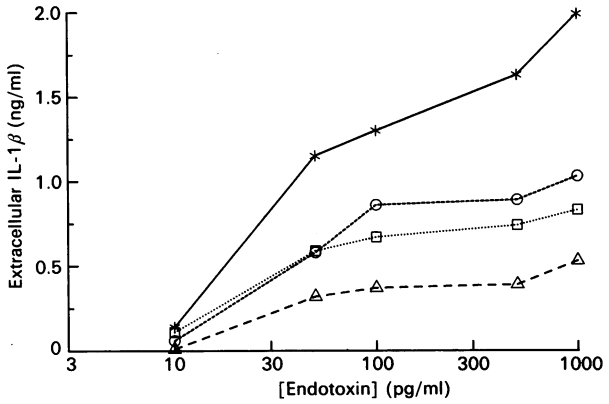

Fig. 3. Inhibition of endotoxin-stimulated cytokine secretion from human MNCs by dexamethasone and CRF

MNCs were prepared and incubated with endotoxin at the concentrations shown plus CRF, dexamethasone or controls as described in the Materials and methods section. *, Controls; □, 10 nM-dexamethasone; ○, CRF (80 ng/ml); △, dexamethasone plus CRF. CRF (80 ng/ml) and dexamethasone (10 nM) given alone or together did not evoke production of detectable intracellular IL-1β or extracellular IL-1β or IL-6. Supernatants were assayed for extracellular IL-1β as described in the Materials and methods section. The mean value for the S.E.M. ($n = 9$, calculated as described in the legend to Fig. 1) was 13.28 ± 4.4%.

IL-1β production (by 48%, 70% and 80% respectively) compared with control values for 100 pg of endotoxin/ml. Similar data were obtained for intracellular IL-1β and IL-6 production (results not shown).

A combination of anti-IL-1α and anti-IL-1β polyclonal antisera, at neutralizing concentrations (Burrows *et al.*, 1991), inhibited endotoxin-evoked IL-6 secretion (Table 1). The extent

Table 1. Inhibition of endotoxin-stimulated IL-6 production from human MNCs by specific anti-IL-1 antisera

MNCs were prepared and incubated as described in the Materials and methods section with endotoxin (500 ng/ml) plus CRF, neutralizing anti-IL-1 sera or controls. Anti-IL-1 sera consisted of a mixture of sheep anti-IL-1β and sheep anti-IL-1α, and were used at a final dilution of 1:400 of each serum. Non-immune serum controls contained a 1:200 dilution of endotoxin-free normal sheep serum. All concentrations refer to the final incubation volume of 1 ml. Supernatants were assayed for IL-6 by e.l.i.s.a. as described. Data are the means ± S.E.M. of triplicate incubations, assayed in triplicate ($n = 9$), calculated as described in the legend to Fig. 1.

Inhibitor	IL-6 secretion (ng/ml)
No CRF	13.70 ± 0.42
Non-immune serum	12.50 ± 0.18
CRF (40 ng/ml)	9.54 ± 0.31
CRF (80 ng/ml)	7.26 ± 0.28
Sheep anti-IL-1 sera	5.12 ± 0.24
Sheep anti-IL-1 sera + CRF (80 ng/ml)	4.94 ± 0.37

of inhibition (62%) was somewhat greater than with CRF at 40 and 80 ng/ml (30% and 47% respectively), and the inhibitory effects of CRF (80 ng/ml) and anti-IL-1 antisera in combination (67%) were not significantly additive.

DISCUSSION

Two forms of IL-1 exist: IL-1α, which remains mostly cell-bound, and IL-1β, which is the predominant secreted form (Oppenheim *et al.*, 1986). Regulation of IL-1β secretion, however, is not fully understood. The IL-1β precursor lacks a conventional hydrophobic leader sequence, and secretion appears to involve a membrane-associated protease (March *et al.*, 1985). Factors which regulate IL-1 production have been shown to affect synthesis and secretion differentially (Arend & Massoni, 1986). In the present study, therefore, both intracellular and extracellular levels of IL-1β were measured. The IL-6 precursor contains a secretory hydrophobic sequence (Hirano *et al.*, 1986), and there is no evidence for a significant intracellular pool. CRF inhibited production of intracellular and extracellular IL-1β and of IL-6. In the case of IL-6, where the response to endotoxin reaches a maximum above 500 pg/ml, CRF-inhibited dose–response curves occurred in the same endotoxin dose range, and showed a decreased maximum response, typical of non-competitive inhibition. Similar analysis of CRF-inhibited IL-1β production is not possible, since neither intracellular nor extracellular IL-1β production reaches a maximum at 1000 pg of endotoxin/ml. However, it would appear, particularly in the case of extracellular IL-1β, that CRF-inhibited dose–response curves are shifted to higher endotoxin concentrations, typical of competitive inhibition.

CRF inhibited production of all three cytokines in the dose range 5–80 ng/ml. CRF levels in non-pregnant human peripheral blood are low (5–28 pg/ml) (Linton *et al.*, 1987). During pregnancy, however, maternal plasma CRF concentrations can rise to as much as 9 ng/ml (Cunnah *et al.*, 1987). Since this CRF is produced by the placental and foetal membranes, local concentrations at the maternal–foetal interface will be significantly higher, and likely to be of the same order as concentrations of CRF shown in our studies to inhibit cytokine production from monocytes.

The reversal of CRF inhibition of monocyte activation by the specific CRF receptor antagonist α-helical CRF-(9–41) (Fig. 2) suggests that the inhibitory actions of CRF on MNCs are mediated through a specific cell surface CRF receptor. This receptor is probably present on monocytes, the source of most of the IL-1 and IL-6 produced from activated MNCs (Taktak *et al.*, 1991).

Glucocorticoids have significant immunosuppressive and anti-inflammatory properties, including inhibition of endotoxin-stimulated cytokine production (Snyder & Unanue, 1982) and gene expression (Lee *et al.*, 1988), and it has been proposed that a feedback mechanism exists whereby glucocorticoids, produced by IL-1 activation of the HPA axis, inhibit monocyte cytokine production (Besedovsky *et al.*, 1986). During pregnancy, maternal plasma concentrations of cortisol are elevated (Nolten & Rueckert, 1981) and may be involved with CRF in the regulation of monocyte activation. The additive inhibitory effects of CRF and glucocorticoids suggest, therefore, that during gestation physiological concentrations of glucocorticoids and CRF, acting in combination, may provide almost complete inhibition of cytokine production from monocytes.

The mechanism of inhibition by CRF of monocytic cytokine production and its site of action are not known. Inhibition of both intracellular IL-1β, which would be expected to be largely a precursor (March *et al.*, 1985), and extracellular IL-1β, suggests that inhibition is at a stage earlier than secretion. Activation of immune cells results in the production of several peptide mediators such as IL-1 and IL-6 which regulate homeostasis and host defence reactions. Regulation of this production is complex, and production of each cytokine may be modulated by the levels of other cytokines from the same cell type (Dinarello *et al.*, 1987; Wong & Clarke, 1988). Inhibition of endotoxin-stimulated IL-6 production by immunoneutralization of all secreted IL-1 activity, using a combination of neutralizing anti-IL-1β and anti-IL-1α antisera (Burrows *et al.*, 1991) (Table 1) suggests that IL-6 production by monocytes is dependent on extracellular IL-1 production. The inhibitory effects of CRF on IL-6 production seen in these studies may therefore be explained by CRF inhibition of monocytic IL-1 secretion.

The physiological roles of CRF during gestation and its immunosuppressive actions are not known. Despite high circulating concentrations, placental CRF does not stimulate the maternal HPA axis (Potter *et al.*, 1991), possibly because of a circulating CRF-binding protein. However, it is possible that the high levels of CRF produced by the placenta in pregnancy, acting alone or in combination with other agents such as glucocorticoids, contribute to the suppression of the maternal immune system, and this may act as a local immunosuppressant at the maternal–foetal interface to prevent rejection of the foetus.

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