Local production of corticotropin releasing hormone is increased in experimental intestinal inflammation in rats

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Abstract

Background/Aims—Corticotropin releasing hormone (CRH) suppresses immunological functions via stimulation of the pituitary-adrenal axis, but is also found in peripheral tissues. Peripheral proinflammatory activity of CRH is suggested by increased tissue concentrations in arthritis and in vitro immunostimulatory effects. This study evaluated intestinal CRH concentrations, immunolocalisation, and synthesis in chronic enterocolitis and investigated in vitro responsiveness of lamina propria mononuclear cells to CRH.

Methods—Chronic granulomatous enterocolitis was induced by intramural injection of peptidoglycan-polysaccharide polymers in the ileocaecal region of Lewis rats. CRH protein was measured in caecal specimens by immunohistochemistry and radioimmunoassay and caecal CRH mRNA expression was analysed by reverse transcriptase polymerase chain reaction.

Results—In the chronically inflamed caecum abundant immunoreactive CRH was found in inflammatory cells, mesenchymal cells, as well as in myenteric plexi. In contrast, only a few CRH containing cells were detected in normal and HSA injected control caecums. Moreover, caecal CRH protein levels were increased during chronic enterocolitis. Local CRH synthesis as indicated by mRNA expression was considerably increased in chronic enterocolitis whereas it was undetectable or low in uninflamed caecum. In addition, CRH stimulated in vitro proliferation of lamina propria mononuclear cells and inhibited mitogen induced proliferation.

Conclusion—Increased CRH protein and mRNA expression in chronic enterocolitis and responsiveness of intestinal mononuclear cells to CRH indicate an immunomodulatory role for locally produced CRH in intestinal inflammation.

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Regulation of immune reactivity to mucosal or systemic noxious stimuli without inducing destructive chronic inflammation requires balanced interaction between the nervous, endocrine, and immune systems. The immune

system shares bi-directional communication pathways with both the nervous and endocrine networks.^{1–3} For example, peripheral and mucosal lymphoid organs have direct neuro-peptidergic innervation.^{4–5} Not only can immune cells be found in intimate association with nerve fibres,⁶ they also have specific high-affinity receptors for neuropeptides and hormones.⁷ Furthermore, hormones and neuropeptides endogenous to the gastrointestinal tract modulate the activity of immune cells isolated from gut associated lymphoid tissues and the intestinal lamina propria.^{8–9} A variety of neuropeptides can be synthesised by immune cells suggesting that neuropeptides have autocrine/paracrine actions on immune cells or that the immune system shares functional homology with the neuroendocrine system.^{3–10} Together, these findings indicate that neuropeptides and hormones can regulate the activity of immune cells in endocrine, paracrine, and possibly autocrine fashions similar to immunoregulatory activities of cytokines.

Local production of corticotropin releasing hormone (CRH) has been associated with peripheral inflammation^{11–13} suggesting that this neuropeptide may have a proinflammatory role when produced outside the brain. This is in sharp contrast with its well known immunosuppressive role as the key regulator of the hypothalamus-pituitary-adrenal axis. At present it is firmly established that the production of CRH is not confined to the hypothalamus. CRH has been demonstrated in the normal spinal cord, lung, liver, spleen, stomach, pancreas, ovaries, placenta, endometrium, and intestine with differential distribution among mammalian species.^{14–21} More importantly, in vitro studies almost unequivocally point to an immunostimulatory effect of CRH. CRH stimulates cytokine secretion,^{22–24} increases the expression of interleukin 2 receptors on T cells,²⁵ increases the FMLP induced production of reactive oxygen metabolites by macrophages,²⁶ stimulates both spontaneous and ConA induced splenocyte proliferation,^{25–27} (van Tol unpublished observations), and stimulates natural killer cell activity.²⁸

In contrast, not much is known about the role of local production of CRH in the pathogenesis of tissue inflammation although increased CRH protein or mRNA expression, or both, are found in human arthritis and rat models of bacterial cell wall induced arthritis and carrageenin induced granulomatous exudative inflammation.^{11–13} Direct evidence for the pivotal peripheral role of CRH in

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experimental inflammation came from the carrageenin model in which systemic immunoneutralisation of CRH resulted in marked suppression of inflammation.¹¹

In this study we investigated the presence and local production of CRH in a model of chronic enterocolitis. Subserosal intramural injection of poorly degradable, purified peptidoglycan-polysaccharide from group A *Streptococcus pyogenes* (PG-APS) in the caecum and distal ileum induces biphasic inflammation in genetically susceptible Lewis rats.²⁹ Granulomatous enterocolitis with associated arthritis, hepatic granulomas, anaemia, and leucocytosis spontaneously develops approximately two weeks after a single injection of PG-APS.^{29–30} Transmural granulomatous inflammation with extensive fibrosis is characteristic for the chronic phase of enterocolitis that persists for at least 16 weeks in this model.

In this and other experimental models of inflammation Lewis rats have been shown to be a highly susceptible strain.^{29–31} Part of this genetic susceptibility in Lewis rats underlying the development of exaggerated inflammation may involve a blunted hypothalamic CRH response to bacterial cell wall products, cytokines, and neurotransmitters.^{31–33}

Here we hypothesise that Lewis rats have increased peripheral CRH production associated with chronic inflammation, and that local proinflammatory effects of CRH contribute to the development or perpetuation of chronic intestinal inflammation, or both, in a susceptible host.

Methods

Bacterial cell wall preparation

Sterile PG-APS polymers with the molecular weight of 5×10^6 kDa to 5×10^8 kDa were prepared from group A, type 3 strain D58 *Streptococcus pyogenes* as described previously³⁴ and provided by Dr J Schwab and R Brown, Department of Immunology and Microbiology, University of North Carolina at Chapel Hill. The preparation was sonicated to disperse aggregates immediately before use and the final concentration was calculated based on rhamnose content.³⁵

Induction of enterocolitis

Female, inbred specific pathogen free (SPF) Lewis rats (140–160 g) were obtained from Charles Rivers Breeding Laboratories (Raleigh, NC) and kept in a SPF facility with free access to food and water. All experiments were performed in compliance with the criteria outlined by the University of North Carolina Institutional Animal Care and Use Committee. Animals were anaesthetised with 1.3 ml/kg body weight Innovar (Pitman-Moore Co, Washington Crossing, NJ), the intestines were exposed by aseptic laparotomy and subserosally injected with PG-APS (12.5 µg rhamnose/g body weight) or the same dose of human serum albumin (HSA, Baxter Health Care) as described previously.²⁹ A total volume

of 0.45 ml was divided over seven injection sites including the junction of the mesentery and the distal ileum (two injections $\times 0.05$ ml), two distal ileal Peyer's patches (2×0.05 ml), the caecal tip (lymphoid aggregate, 0.05 ml), and the mid and upper caecum (two sites $\times 0.1$ ml). Rats in the acute inflammation group were killed 48 hours after injections whereas rats in the chronic inflammation group were killed 29, 33, or 85 days after injections. All animals were killed by overdose inhalation of CO₂. Gross inflammation was scored by a single blinded observer according to criteria developed and validated for this model.²⁹ Values of 0–4 were assigned to the number of caecal nodules, contraction of mesentery, number and severity of adhesions, and caecal wall thickening, with a maximal possible summed gross gut score (GGS) of 16.

Tissue collection and processing

Caecal tissue samples were snap frozen in liquid nitrogen and stored at -80°C for isolation of RNA and protein. The caecal tip was oriented in a plastic container with OCT (Miles Inc, Elkhart, IN), snap frozen in isopentane, and stored at -80°C for immunohistochemistry studies. Total RNA was isolated from samples of caecal tissue from PG-APS, HSA injected, and normal Lewis rats using a standard procedure.²⁹ RNA concentration and purity was quantified by absorbance at 260 nm and A260/280 ratios. The integrity of RNA was verified by electrophoresis of samples in 1.4% agarose gels containing ethidium bromide.

Immunohistochemistry

Indirect immunohistochemical staining for rat CRH was done using the avidin-biotin peroxidase complex (ABC) kit (Vectastain, Vector Lab, Burlingame, CA). Sections of brain from a longterm adrenalectomised Lewis rat were used as controls because immunostaining of CRH is increased in the brain due to lack of corticosteroid feedback inhibition. Ten µm frozen sections were cut from the brain of the adrenalectomised rat and from fresh frozen caecal tips collected from PG-APS or HSA injected or normal Lewis rats. Sections were post-fixed in 4% paraformaldehyde with 10% sucrose for five minutes and washed in phosphate buffered saline (PBS). All incubations were performed at room temperature unless otherwise stated. Sections were treated with methanol and 0.6% H₂O₂ for 45 minutes to exhaust endogenous peroxidase activity, incubated with 2% normal goat blocking serum in PBS for 10 minutes, then incubated overnight with either the primary rabbit antirat CRH antiserum (1:500–1:1000 dilutions, Peninsula Lab, Belmont, CA), normal rabbit serum, rabbit IgG, or 2% normal goat serum at 4°C. The sections were washed in PBS, and incubated with 2% normal goat serum blocking solution for 10 minutes, followed by incubation with biotinylated goat anti-rabbit IgG (1:400 dilution, Organon Teknika,

Durham, NC) for 45 minutes, washed in PBS, and incubated with the A and B components (1:400) of the Vectastain kit for 45 minutes. Coloured precipitation product was developed by immersing sections in 0.05 M TRIS-HCL buffer pH 7.4 containing 0.075% 3,3 diamino-benzidine tetrahydrochloride (DAB, Aldrich Chemical, Milwaukee, WI) and 0.02% H₂O₂. After washing in TRIS buffer and PBS, the staining was intensified with evaporating OsO₄ (2% in dH₂O) for five minutes, washed in PBS, counterstained with 0.05% toluidine blue in 30% ethyl alcohol, dehydrated through graded alcohols and xylene, mounted with Permount (Fisher Scientific, Pittsburgh, PA), and cover slipped.

Radioimmunoassay (RIA) and non-collagen protein determinations

For extraction of immunoreactive CRH (irCRH), total protein, and collagen the tissues were homogenised in 1 ml of 0.1 N acetic acid for 30 seconds using a Tissuemizer. The homogenates were boiled for five minutes, cooled on ice for five minutes followed by centrifugation for 10 minutes at 2000×g. The supernatants were collected and stored at -20°C. CRH concentrations were determined using a specific radioimmunoassay for rat/human CRH (Peninsula Lab, Belmont, CA). Total protein concentrations in the supernatants were determined using a microprotein assay (Sigma, St Louis, MO).

Collagen measurements

An 100 µl aliquot of the tissue homogenate was lyophilised and subsequently hydrolysed with 6N HCL in vacuo under N₂ at 110°C for 22 hours. The hydrolysate was dried by a speed vacuum concentrator, dissolved in distilled water, filtered with 0.22 µm membrane, and subjected to amino acid analysis using a Varian 5500 HPLC system.³⁶ The relative amount of collagen in the total protein fraction was determined using a value of 100 residues of hydroxyproline per 1000 for a typical fibrillar collagen standard.³⁶

RT-PCR

For the reverse transcriptase polymerase chain reaction (RT-PCR) analysis of CRH mRNA, 1 µg of total RNA isolated from intestinal tissues and brain controls was reverse transcribed to cDNA using random hexamers in a reaction volume of 50 µl containing 200 units of Superscript Reverse Transcriptase RNase H- (Gibco BRL, Gaithersburg, MD), 50 mM TRIS-HCL (Gibco), 75 mM KCL (Gibco), 3 mM MgCL₂ (Gibco), 10 mM DTT (Gibco), 1 mM dNTP (Perkin Elmer/Applied Biosystems, Foster City, CA), 1 µM random hexamers (Perkin Elmer), and 0.5 Units/µl RNase inhibitor (Perkin Elmer). The reaction mixture was incubated for 45 minutes at 45°C, boiled for five minutes to stop the reaction, cooled on ice for three minutes, and stored at -20°C. All PCR reactions were done in a 50

µl reaction volume with 1 µl of the first strand cDNA and 50 mM KCl, 20 mM TRIS-HCL, 2.5 µM MgCL₂, 0.1–0.5 mM dNTPs, 0.1–0.25 mM of each antisense and sense primer, and 1.5–2.5 U of Taq polymerase (Perkin Elmer). The primers used for rat CRH were sense 5' CAAGTACGTTGAGAACT-GAAG and antisense 5' CCGAGCAGCGGG-ACTTCTGT for the first amplification and the same sense primer with antisense 5' GG-GCTGCTCCGGTTGCAAG for the semi-nested primer second amplification run. For the nested primer amplification, 1 µl of the first PCR product obtained after 35 cycles was diluted 1:250 and used as a template in a fresh reaction mix for a second amplification of 32 cycles. For rat β-actin¹² sense 5' ACCACAGC-TGAGAGGGAAATCG and antisense 5' AG-AGGTCTTTACGGATGTCAACG primers were used for 25–30 cycles amplification. All primers were constructed to span an intron to enable assessment of contaminating genomic DNA. Conditions for amplification of rat CRH were denaturation at 95°C for five minutes, and amplification cycles, 94°C for 45 seconds, 58°C for one minute, 72°C for 1.5 minutes, and for rat β-actin 94°C for 45 seconds, 60°C for one minute, and 72°C for 1.5 minutes. Rat brain cDNA was used as a positive control and to optimise amplification conditions for CRH mRNA. Amplifications without cDNA were run as negative controls. The PCR products were analysed on a 1.5% agarose TAE gel containing ethidium bromide. The specificity of the amplification product was confirmed by size and by obtaining fragments of predicted size (245 and 62 bp) after digestion with restriction enzyme BamH1 (Boehringer Mannheim).

Lamina propria mononuclear cell (LPMC) isolation and in vitro stimulation

LPMC were isolated from ileal tissue from normal Lewis rats. Tissue was washed in calcium and magnesium free Hank's balanced salt solution (CMF-HBSS) with antibiotics. The tissue was cut longitudinally, washed, cut into smaller pieces, and incubated in CMF-HBSS with 10 mM DTT (Sigma) and antibiotics for 20 minutes at room temperature.

After washing, the tissue was transferred to prewarmed CMF-HBSS with 0.8 mM ethylenediamine tetraacetic acid (Fischer Scientific, Pittsburgh PA) and additives for four to five sequential incubations. After epithelium depletion the tissue pieces were cut smaller and incubated twice for one hour in RPMI with 10% FCS, antibiotics, and Dispase grade I (0.08 mg/ml, Boehringer Mannheim) and DNase grade II (0.075 mg/ml, Boehringer Mannheim) at 37°C on a shaking table. After enzymatic digestion the supernatants were filtered through a mesh (Nitex, Tetko Inc, Elmsford, NY), washed, and mononuclear cells were isolated by density gradient centrifugation (Histopaque, 1.083 g/l, Sigma). Then the cells were washed, counted, and cultured at 2×10⁶ cells/ml in 200 µl of RPMI supplemented with 10% FCS, antibiotics and

synthetic CRH 1–41 (Sigma) or 0.25 µg/well ConA (Sigma), or both, in 96 well microtitre plates. The plates were incubated in 5% CO₂ at 37°C for 72 hours and cells were pulse labelled with 50 µCi/well [³H]-thymidine (Amersham, Arlington Heights, IL) 18 hours before harvesting and proliferation was measured by liquid scintillation counting.

Statistics

The data are presented as mean (SEM). Groups were compared using the unpaired two tailed Student's *t* test. In the proliferation studies dose dependency of the CRH incubations was analysed by multiple regression analysis followed by the Wilcoxon's signed rank sum test to determine differences between incubations.

Results

Assessment of inflammation

To confirm induction of chronic intestinal inflammation, the gross gut score (GGS) for enterocolitis was determined. The score, which includes adhesions, mesenteric contractions, caecal serosal nodules, and caecal wall thickening, was significantly increased during the acute phase after two days (6.7 (0.4) (PG-APS) *v* 0.3 (0.3) (HSA), *p*=0.0001). This increase in the GGS was even more pronounced during the chronic phases of inflammation, as shown at 26 days (8.7 (0.6) (PG-APS) *v* 0.6 (0.3) (HSA), *p*=0.0001) and 29 days (9.3 (1.6) (PG-APS) *v* 1.3 (0.7) (HSA), *p*=0.0012). The HSA injected control animals showed no signs of active intestinal inflammation at all evaluated time points.

Immunohistochemistry

Specificity of the anti-CRH serum was tested on sections of the hypothalamus from an adrenalectomised rat. Strong immunoreactive CRH staining was visible in the external layer of the median eminence at the base of the third ventricle (data not shown). Incubations with normal rabbit serum, purified rabbit IgG, as well as normal goat serum were found to be uniformly negative.

The histological appearance of chronic colitis in the caecal tip consists of active granulomatous inflammation in a transmural distribution with extensive fibrosis and evidence of an acute inflammatory component; the submucosa is particularly involved. Abundant presence of CRH immunoreactive cells in the inflamed caecum was observed in chronic enterocolitis 85 days after PG-APS injection (Fig 1A). Anti-CRH immunostaining of caecal tip sections of normal Lewis caecum and of HSA injected control animals at 33 days (Fig 1B), showed a pattern of rarely scattered CRH immunoreactive cells in the submucosa, which might be of neuronal origin and weak staining of plexi in the external muscle layer. No staining was seen in crypt epithelial or neuroendocrine cells. During the chronic phase of

inflammation several different cell types were found to contain irCRH. Similar to what was found in the acutely inflamed caecal tissues (two days, data not shown), CRH immunoreactivity was seen in inflammatory cells with a predominant macrophage-like appearance in the chronic phase of PG-APS induced enterocolitis (Fig 1C). CRH was also detected within the myenteric plexi, which appeared to be hyperplastic (Fig 1D). In addition, mesenchymal cells lining the periphery of granulomas were positive for irCRH (Fig 1E). Control incubations of the chronically inflamed caecum with normal rabbit serum, purified rabbit IgG, normal goat serum (Fig 1F) as well as serial dilutions of the primary CRH antiserum confirmed specificity of the immunostaining for CRH in gut tissue and the absence of endogenous peroxidase activity.

Tissue concentrations of CRH by radioimmunoassay

Whole caecal tissue CRH concentrations were significantly increased in the chronic phase of inflammation in PG-APS-injected rats as detected by specific RIA (Fig 2A). Smaller amounts of irCRH could also be detected in caecal tissues of HSA injected control animals. Because the increase in irCRH in chronically inflamed caecum is accompanied by a relatively large increase in the total protein content per gram of wet tissue due to the inflammation, CRH contents were normalised based on the amount of protein extracted (Fig 2B). In this model of granulomatous enterocolitis however, there is abundant deposition of collagen in the chronic phase of inflammation as revealed by Masson's trichrome staining.³⁰ Based on amino acid analysis it was also determined that the collagen fraction comprised a significantly larger proportion of the total caecal protein content in the PG-APS injected group compared with HSA injected control animals (25% *v* 8%, *p*<0.05). Adjusting the tissue concentration of CRH based on non-collagenous protein demonstrated a 2.2-fold increase in the mean concentration of irCRH in chronically inflamed tissues compared with the HSA injected control caecal tissue (Fig 2C).

RT-PCR analysis

We were unable to detect CRH mRNA in caecal tissue samples from PG-APS or HSA injected Lewis rats by northern blot hybridisation using a cDNA probe (data not shown). However, we were able to detect CRH mRNA, which is constitutively expressed in the hypothalamus, in normal Lewis rat brain samples by this method. Therefore we used RT-PCR to detect the relatively low transcript levels of intestinal CRH mRNA. The RT-PCR amplification conditions using a semi-nested primer amplification technique were optimised using the normal Lewis rat brain as a positive control and were used to evaluate CRH mRNA expression during different phases of chronic inflammation in the caecum. This semi-quantitative assessment of CRH mRNA

Figure 1: Immunohistochemical staining for CRH in caecal tissues derived from Lewis rats after intramural injections of PG-APS, 85 days (A, C-F) or HSA, 33 days (B). CRH immunoreactivity is visualised in inflammatory and mesenchymal cells in the submucosa, particularly surrounding a granuloma (arrow) and in nervous tissue between the hypertrophic external muscle layers (arrowhead) (A, original magnification $\times 40$). Only a few CRH immunoreactive cells (arrows), possibly of nervous tissue origin, are found in control tissues 33 days after HSA injection (B, original magnification $\times 100$). CRH containing macrophage-like cells are found in focal areas of mononuclear cellular infiltrate during chronic inflammation (C, original magnification $\times 400$). CRH immunoreactivity is also present within the myenteric plexus (D, original magnification $\times 200$) and in mesenchymal cells comprising the peripheral rim of a submucosal granuloma (E, original magnification $\times 100$). Negative staining was obtained in control incubations of the same PG-APS induced chronically inflamed caecal specimen using normal goat serum (F, original magnification $\times 100$).

expression was done by normalising for constitutive β -actin mRNA levels in each individual sample. Although this does not yield exact data on the fold increase of mRNA message it clearly showed high CRH mRNA expression at all time points during chronic caecal inflammation at 29, 33, and 85 days after PG-APS injection (Fig 3). In contrast, the CRH mRNA message was undetectable or in some cases very low in uninjected or control HSA injected caecal tissues compared with the inflamed tissue samples.

Proliferation studies

CRH stimulated the proliferation of LPMC isolated from normal Lewis rat ileum in a dose dependent fashion (Fig 4A). Incubation of the cells with ConA ($0.25 \mu\text{g}/\text{well}$) stimulated pro-

liferation to a similar extent as was obtained with the higher concentration of CRH in the non-mitogenic stimulation experiment (Fig 4B). However, co-incubation of ConA stimulated cells with different concentrations of CRH inhibited proliferation of LPMC (Fig 4B).

Discussion

It is now firmly established that CRH is also present outside the brain in peripheral tissues and immune cells. This study shows increased local CRH synthesis during PG-APS induced chronic intestinal inflammation. Increased concentrations of irCRH were detected by radioimmunoassay in the chronically inflamed caecum of Lewis rats compared with low constitutive values in HSA injected control animals. Tissue CRH concentrations were

Figure 2: Radioimmunoassay determinations of CRH tissue concentration in caecal homogenates 29 days after PG-APS or control HSA injections measured in the extracted tissue supernatants (A), normalised for the total protein content in the extracts (B) as well as corrected for the proportion of collagen in the extracted total protein fraction (C). Significantly increased CRH concentrations were detected in the PG-APS induced chronically inflamed caecal tissues compared with the HSA injected control specimens, * $p < 0.05$.

Figure 3: PCR analysis of reverse transcribed RNA isolated from caecal tissue of PG-APS or HSA injected and normal Lewis rats. Primers specific for rat CRH generated a 307 bp fragment after semi-nested primer amplification. cDNA transcribed from Lewis brain RNA samples and PCR reaction samples without cDNA added were included as positive and negative controls respectively. Control amplification of the samples using primers specific for rat β actin generated the predicted 281 bp fragment and confirmed equal loading. The data have been consistently reproduced in more than 10 separate assays of randomly selected chronically inflamed and control tissues from rats in other chronic phase experiments.

Figure 4: Effect of synthetic CRH on proliferation of LPMC isolated from normal Lewis rat ileum. Spontaneous (A) or ConA induced (B) proliferation after 72 hours LPMC culture presented as the mean cpm of ^3H -thymidine incorporation. Dose dependency of CRH induced stimulation of spontaneous proliferation (A, $p = 0.018$) or inhibition of ConA stimulated proliferation (B, $p = 0.019$) was analysed by multiple regression with CRH as an independent variable. $n = 6$, * $p < 0.05$ compared with control media incubation.

measured in total caecal homogenates and do not accurately reflect local concentrations, which may reach substantially higher values in focal areas of inflammation or near nerve endings. Although local CRH production cannot be accurately measured, it is quite likely that some intestinal inflammatory cells are exposed to CRH concentrations capable of modulating their activity. The ability of intestinal immune cells to respond to CRH is indicated by dose dependent effects on proliferation of LPMC.

Immunohistochemistry was performed to determine the cellular source of location of increased irCRH demonstrated by RIA. Infiltrating mononuclear cells, (myo)fibroblast-like cells surrounding granulomas, and myenteric plexi stained for CRH in the chronically inflamed caecum in contrast with only sparsely scattered positive cells in the normal or HSA injected controls. This staining pattern is very similar to that described in other models of experimental inflammation in rats.^{11 12} However, our findings in the rat intestine do not match the few reports of CRH in the normal human gastrointestinal tract. Nieuwenhuijzen Kruseman *et al*¹⁴ demonstrated CRH in mucosal cells of the antrum and small intestine whereas Kawahito *et al*¹⁹ found that irCRH colocalised with 5-HT in normal colonic enterochromaffin cells, and found abundant CRH protein in colonic lamina propria macrophages of patients with active ulcerative colitis.³⁷ We also found irCRH staining in macrophage-like cells in the inflamed caecum but not in normal or inflamed rat colonic epithelial cells. The reason for these species differences is unclear.

To establish the relative contributions of CRH derived from immune cells versus nervous tissues in PG-APS induced arthritis, Crofford *et al*¹² treated rats with dexamethasone. Discordant effects of treatment on synovial CRH production and mononuclear cell infiltration and early increases in CRH staining prior to cellular infiltration suggested that the nervous system may contribute to the increased CRH concentrations in inflamed synovium. Similarly, CRH production by the enteric nervous system may contribute to PG-APS induced chronic enterocolitis. Wolter¹⁶ demonstrated that perikarya and nerve fibres in the myenteric plexus and submucosal nerve fibres of normal rat duodenum contain CRH. We found increased CRH staining in neuronal plexi in the chronically inflamed caecum of Lewis rats. In addition, neuropeptides can be produced by enteric glial cells, which secrete increased amounts of substance P when stimulated by IL1.³⁸ IL1 is also a potent stimulus of CRH secretion in the brain³⁹ and is considerably increased during chronic PG-APS induced enterocolitis.²⁹ Interactions between cytokines and the enteric nervous system may be important in the regulation of CRH secretion and in the perpetuation of inflammation.

Increased CRH mRNA expression during different phases of chronic granulomatous enterocolitis clearly demonstrates local

production rather than transport of preformed CRH from remote production sites to the intestine. Crofford *et al* detected CRH mRNA in the synovium of a Lewis rat with adjuvant induced arthritis but results could not be compared with healthy joints because of unsuccessful RNA isolation from control synovium.¹²

A previous report of CRH gene expression in human colonic inflammation has been difficult to reconcile with the authors' own evidence of increased CRH staining in lamina propria cells by immunohistochemistry.¹⁹⁻³⁷ CRH mRNA values detected by PCR analysis were higher in normal than in ulcerative colitis mucosa³⁷ whereas *in situ* hybridisation signals were confined to scattered crypt epithelial cells and did not differ among normal and inflamed biopsy specimens.¹⁹⁻³⁷ In our studies increased caecal CRH mRNA expression consistently correlated with the development of inflammation and irCRH in tissues as measured either by RIA or immunohistochemistry. Moreover, CRH mRNA values in the chronically inflamed caecum at 29, 33, or 85 days were always high compared with normal and HSA injected control caecum (Fig 3). In most control tissues CRH mRNA were undetectable by RT-PCR, but occasionally we found low constitutive CRH gene expression in normal and HSA injected caecal control samples, consistent with the constitutive CRH peptide values in control tissues as measured by RIA (Fig 2).

The *in vitro* LPMC response to CRH further supports the hypothesis that locally produced CRH has an immunomodulatory role in intestinal inflammation. In preliminary studies we found that CRH regulates proliferation of splenocytes and LPMC as well as cytokine production.⁴⁰ The opposite effect of CRH on spontaneous and mitogen induced proliferation is of unclear significance. Immune cell responses to neuropeptide stimulation depend on their activation state, for example, intracellular second messenger levels. Moreover, ConA is a non-specific stimulus and may not appropriately reproduce the *in vivo* immunophysiological activation state of LPMC in PG-APS induced enterocolitis. These results however are consistent with other studies describing stimulatory effects of CRH on cytokine secretion, IL2 receptor expression, reactive oxygen metabolite production by macrophages, lymphocyte proliferation, and natural killer cell activity.²²⁻²⁸ Of relevance to this study, the lymphoproliferative effect of CRH is higher in B lymphocytes from gut associated lymphoid tissue than in peripheral B cells.²⁷ Only a few reports indicate suppressive effects of CRH on IL6 secretion and mitogen induced antibody production.⁴¹⁻⁴² These findings in conjunction with studies showing CRH synthesis by immune cells,⁴³⁻⁴⁴ high affinity receptors for CRH on macrophages, T and B lymphocytes,⁴⁵⁻⁴⁸ and autoreactive T cell responses against CRH,⁴⁷ support the concept of a local immunomodulatory role for CRH in inflammation.

In summary, our data show increased intestinal CRH mRNA expression and tissue

concentrations in chronic experimental colitis suggesting a role for CRH in intestinal inflammation. More detailed *in vitro* studies with isolated mucosal immune cells will provide further insight into the immunostimulatory activities of locally produced CRH and its contribution to chronic disease in genetically susceptible hosts.

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