

# Expression of interleukin 1 $\beta$ and interleukin 1 $\beta$ converting enzyme by intestinal macrophages in health and inflammatory bowel disease

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## Abstract

**Background**—In the lipopolysaccharide (LPS) stimulated peripheral blood monocyte, the precursor form of interleukin 1 $\beta$  (IL-1 $\beta$ , 31 kD) is processed by IL-1 $\beta$  converting enzyme (ICE) to the mature, bioactive form (17 kD). IL-1 $\beta$  is a proinflammatory cytokine which is likely to have a role in the pathogenesis of inflammatory bowel disease (IBD).

**Aims**—To investigate the expression and processing of IL-1 $\beta$  and ICE by tissue macrophages from normal and IBD colonic mucosa.

**Methods**—Mucosal biopsy specimens and lamina propria cells from normal and IBD colons were studied by reverse transcription polymerase chain reaction (RT-PCR), western blot analysis, and ELISA (enzyme linked immunosorbent assay).

**Results**—Normal colonic macrophages synthesised only the precursor form of IL-1 $\beta$  whereas in IBD the mature form was also produced. Similarly, cells from normal colonic mucosa synthesised ICE as the precursor (p45) only, whereas macrophages from IBD colons produced active (p20) ICE. Ac-Tyr-Val-Ala-Asp-CHO, a specific peptide aldehyde inhibitor of ICE, significantly reduced the amount of mature IL-1 $\beta$  released by isolated IBD macrophages (from a median of 1.2 (range 0.78–4.42) ng/ml to 0.43 (0.21–1.6) ng/ml;  $p < 0.01$ ).

**Conclusions**—Exposure of normal colonic macrophages to LPS only induces the production of the precursor form of IL-1 $\beta$ , because the cells fail to activate ICE. In contrast, IBD colonic macrophages are able to activate ICE and hence release mature IL-1 $\beta$  in a manner similar to circulating monocytes. This is consistent with IBD macrophages being recently recruited from the circulating monocyte population. Targeted inhibition of ICE may represent a novel form of therapy in IBD.

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Keywords: interleukin 1 $\beta$ ; interleukin 1 $\beta$  converting enzyme; macrophages; lipopolysaccharide; ulcerative colitis; Crohn's disease

Interleukin 1 $\beta$  (IL-1 $\beta$ ), a cytokine released predominantly by mononuclear phagocytes, is a key mediator in immunoregulatory and inflammatory processes.<sup>1,2</sup> The processing and

release of human IL-1 $\beta$  is best characterised in lipopolysaccharide (LPS) stimulated peripheral blood monocytes. These cells synthesise IL-1 $\beta$  initially as a 31 kD biologically inactive propeptide which is released as the mature active (17 kD) peptide following cleavage by IL-1 $\beta$  converting enzyme (ICE).<sup>3,4</sup> ICE is a cysteine protease which is synthesised as an inactive 45 kD proenzyme (p45). Its enzymatic activity is mediated by two subunits, p20 (20 kD) and p10 (10 kD), which are cleavage products of the proenzyme.<sup>5</sup>

In the mucosa affected by active inflammatory bowel disease (IBD), a large amount of mature IL-1 $\beta$  is produced.<sup>6,7</sup> Studies of mucosal expression<sup>8</sup> and on isolated cells<sup>9,10</sup> have shown that IL-1 $\beta$  is produced almost exclusively by intestinal macrophages. Cells isolated from normal colonic mucosa produce very little mature IL-1 $\beta$ ,<sup>10</sup> despite stimulation with LPS.<sup>9</sup> The inability of normal intestinal macrophages to produce mature IL-1 $\beta$  could result from regulation at one or more steps from gene activation to post-translational processing of the propeptide by ICE and release of the mature peptide. To investigate this further, we have studied the expression of messenger RNA (mRNA) transcripts and both precursor and processed forms of IL-1 $\beta$  and ICE in lamina propria macrophages isolated from normal and IBD colonic mucosa.

## Methods

### COLONIC MUCOSAL BIOPSY SPECIMENS

Biopsy specimens were taken during colonoscopy from the sigmoid colon of adult patients with histologically normal mucosa (n=13; eight males; median age 57 years, range 30–85) and those with active IBD (10 ulcerative colitis (UC), two Crohn's disease (CD); seven males; median age 50 years, range 32–66). Activity was defined macroscopically as grade 2 or worse (grade 0, normal; grade 1, erythema and loss of vascular pattern; grade 2, contact bleeding; grade 3, spontaneous bleeding; and grade 4, ulceration)<sup>11</sup> and was confirmed by histological examination. Patients with IBD were all taking 5-aminosalicylic acid preparations, either alone (n=5) or with steroids (n=3), steroids and azathioprine (n=2), cyclosporin (n=1), or metronidazole (n=1). The study was approved by the Nottingham University Hospital Ethics Committee and all patients gave written, informed consent.

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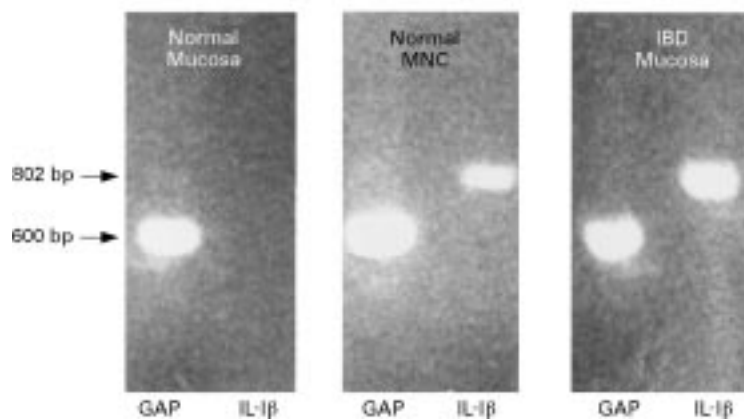


Figure 1 Expression of mRNA transcripts for IL-1 $\beta$  and glyceraldehyde-6-phosphate dehydrogenase (GAP) in a normal colonic biopsy specimen; lamina propria mononuclear cells (MNC) isolated from samples of normal colonic mucosa; and a biopsy specimen with active IBD. The figure shows an example of 10/13 normal colonic biopsy samples which did not express transcripts for IL-1 $\beta$ .

#### ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells were separated by centrifugation of human blood (from healthy volunteers) through lymphocyte separation medium (ICN Biomedicals Inc., Costa Mesa, California). The mononuclear cell interface was collected and washed three times in Hanks buffered salt solution (Gibco BRL). After the final wash the cells were resuspended in RPMI 1640 (Gibco BRL) containing 5% fetal calf serum (FCS), the cells were counted, and viability was assessed by ability to exclude trypan blue (used at a final concentration of 4  $\mu$ g/ml; Sigma).

#### ISOLATION OF LAMINA PROPRIA CELLS

Normal (at least 10 cm from tumour) and active IBD (seven UC, four CD) mucosal tissue was obtained from fresh colonic resection specimens. The median age of eight patients from whom normal tissue was ob-

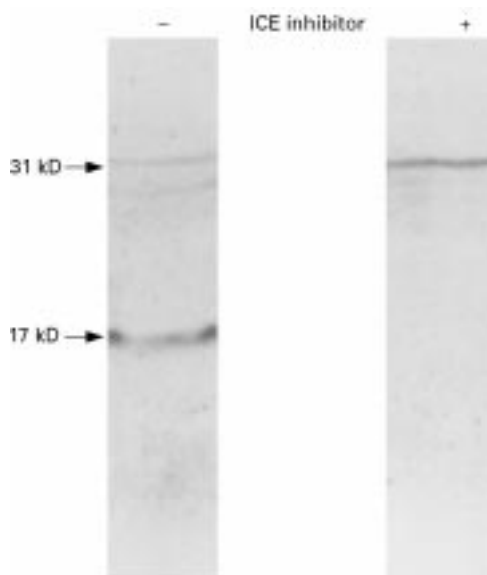


Figure 2 Expression of IL-1 $\beta$  protein in LPS stimulated peripheral blood mononuclear cell culture supernatants in the absence (-) and presence (+) of the ICE inhibitor, Ac-Tyr-Val-Ala-Asp-CHO.

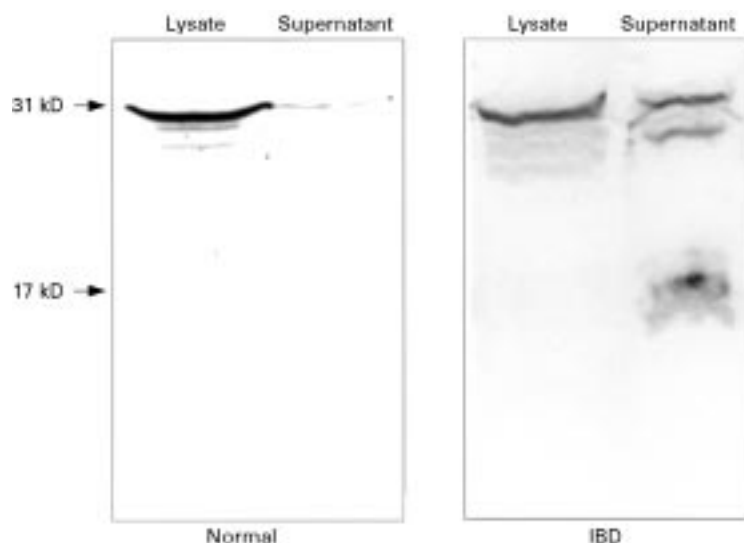
tained was 63 years (range 50–75), of whom five were male. The median age of the IBD patients was 44 years (range 20–62), of whom seven were male and all but one were being medically treated (with steroids alone (n=1), 5-aminosalicylic acid preparations alone (n=4), both therapies alone (n=1) or with azathioprine (n=1), cyclosporin (n=1), or metronidazole (n=1)). In all IBD cases the indication for surgery was active disease refractory to medical therapy. Lamina propria cells were isolated using a new method.<sup>12</sup> Briefly, mucosal strips were shaken at 37°C for three 30 minute periods in calcium and magnesium free Hanks balanced salt solution (Gibco BRL) containing 1 mmol/l EDTA (Sigma) to remove epithelial cells. The de-epithelialised mucosal pieces were subsequently cultured at 37°C in RPMI containing 10% FCS. During incubation, lamina propria cells emerged into the medium. After 24 hours the de-epithelialised mucosa was removed and cells in the culture dish collected following incubation at 4°C for one hour and vigorous washing to detach adherent cells.

#### CELL CULTURE STUDIES

Isolated cells ( $5 \times 10^6$  cells/ml) were cultured at 37°C in 5% FCS/RPMI alone or with added bacterial LPS (*Escherichia coli* 055:B5, 10  $\mu$ g/ml; Sigma). In some experiments, cells were cultured with both LPS and Ac-Tyr-Val-Ala-Asp-CHO (final concentration 100  $\mu$ M; Neosystem Laboratoire, Strasbourg, France), a specific peptide aldehyde inhibitor of ICE.<sup>4</sup> After 20 hours, the cells and supernatants were collected for reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis. For the latter, the cells were lysed by rapid freeze thawing three times and the lysates and supernatants stored (at -70°C) in the presence of protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 mg/ml pepstatin, and 1 mg/ml leupeptin; Sigma) and 1 mM EDTA. In the studies with the ICE inhibitor, the supernatants were also used for measurement of mature IL-1 $\beta$  by ELISA.

#### RT-PCR

RNA was isolated from mucosal biopsy specimens and isolated lamina propria cells using a guanidinium isothiocyanate-phenol:chloroform extraction method (Micro RNA Isolation Kit, Stratagene). The RNA was reverse transcribed to complementary DNA (cDNA) using oligo(dt) primers (Pharmacia Biotech Inc.) and moloney murine leukaemia virus reverse transcriptase (Stratagene). The cDNA was amplified by PCR using the following primer pairs: 5'-CCACCCATGGCAAATTCATGGCA-3' (sense) and 5'-TCTAGACGGCAGGTCA GGTCCACC-3' (antisense; both from Stratagene) to amplify a 600bp glyceraldehyde-6-phosphate dehydrogenase (GAPDH) product; 5'-ATGGCAGAAGTACCTAAGCTCGC-3' (sense) and 5'-TTGACTGAAGTGGTACG TTAACACA-3' (antisense; both from Clontech Laboratories, Palo Alto, California) to amplify an 802bp IL-1 $\beta$  product; and 5'-AACCCAGCCTATGCCAC-3' (sense) and

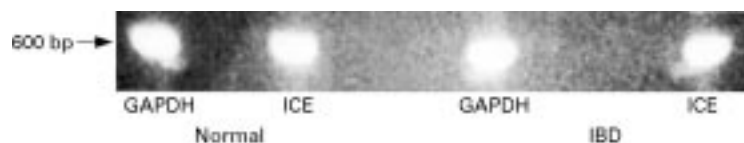


**Figure 3** Expression of IL-1 $\beta$  protein by LPS stimulated lamina propria cells isolated from normal and IBD colonic mucosa. Cell lysates and supernatants were studied by western blot analysis. In cells obtained from IBD colon, both 31 kD and 17 kD forms of the cytokine were detected in the supernatants. The figure shows representative experiments performed on lamina propria cells obtained from four normal and five IBD colons.

5'-TGGGCTTTCTTAATAGC-3' (antisense) to amplify a 602bp ICE product. A total of 30 PCR cycles was used and the products were visualised on ethidium bromide stained 2% agarose gels. RNA, cDNA, and PCR products were stored at  $-70^{\circ}\text{C}$ . The ICE primer pairs were synthesised in our institution, based on the published nucleotide sequence.<sup>4</sup> Initial experiments showed that PCR products of the expected size were obtained using these primers from RNA isolated from the human monocytic cell line, THP.1, and peripheral blood mononuclear cells (unpublished data), which are known to express ICE.<sup>3,4</sup> Finally specificity for ICE was confirmed by sequencing the PCR product obtained from an IBD cell isolate.

#### WESTERN BLOT ANALYSIS

Protein in cell lysates and supernatants was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 15%/5% gel (by the method of Laemmli<sup>13</sup>) and transferred onto nylon (Hybond-N; Amersham International plc). Immunostaining was performed using rabbit antihuman IL-1 $\beta$  antibody which detects both pro- and mature peptide K4.7,<sup>14</sup> 1  $\mu\text{g}/\text{ml}$  (a gift from Dr Chaplin, Howard Hughes Medical Institute, St Louis, Missouri, USA), or ICE antiserum which recognises the p45 and p20 (but not p10; Dr H Allen, personal communication) subunits (BBC4, 1  $\mu\text{g}/\text{ml}$ ; a gift from BASF Bioresearch Corporation, Worcester, Massachusetts, USA). Immunoblots for IL-1 $\beta$  were incubated with an antirabbit secondary



**Figure 4** Expression of ICE and GAPDH mRNA in biopsy specimens from normal and IBD mucosa.

antibody conjugated with alkaline phosphatase (Sigma) and developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (from Promega). For detection of ICE protein, the Vectastain Elite ABC kit (Vector Laboratories) was used according to the manufacturer's instructions.

#### ELISA FOR IL-1 $\beta$

An enzyme linked immunosorbent assay (ELISA) that measures predominantly mature IL-1 $\beta$  (Quantikine IL-1 $\beta$  Immunoassay Kit, R&D Systems Inc.) was used. Pro-IL-1 $\beta$  shows cross reactivity of up to 13% (20% on a molar basis) in this ELISA.

#### STATISTICS

Analysis was performed by Fisher's exact test or paired Student's *t* test. Data are expressed as mean (SEM) unless otherwise specified.

## Results

#### ISOLATED PERIPHERAL BLOOD AND LAMINA PROPRIA CELLS

Mean cell viability (assessed by trypan blue exclusion) of isolated peripheral blood mononuclear cells was 94.0 (3.4)%.

Mean lamina propria cell viabilities from normal and inflamed mucosa were 92.6 (1.5)% and 93.0 (3.3)% respectively. There was no significant difference in the proportion of macrophages (CD68 positive) present in normal and IBD isolated cell preparations (10.5 (1.3)% versus 8.4(0.9)% respectively). Previous studies have shown that IL-1 $\beta$  is produced almost exclusively by intestinal macrophages<sup>8-10</sup> and therefore cell separation was not performed.

#### EXPRESSION OF IL-1 $\beta$ mRNA

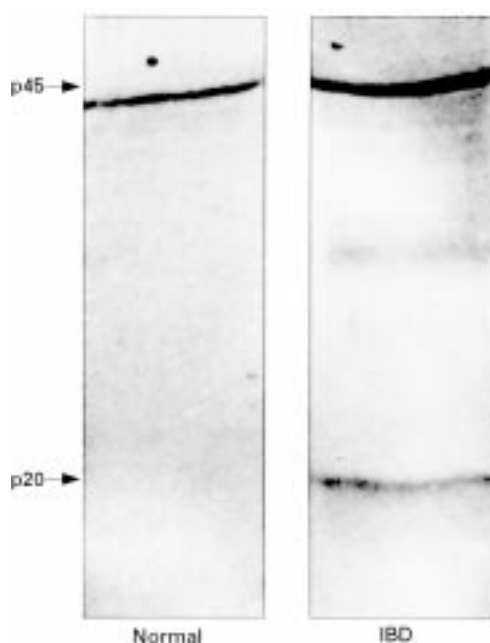
GAPDH mRNA (a constitutive marker) was expressed by all the samples studied. Of mucosal biopsy specimens from 13 normal colons, three expressed IL-1 $\beta$  mRNA, compared to all nine biopsy specimens from actively inflamed colons (seven with UC and two with CD;  $p < 0.001$ ; fig 1). All isolated lamina propria cells from six normal and four IBD colons expressed IL-1 $\beta$  mRNA.

#### EXPRESSION OF IL-1 $\beta$ PROTEIN

To investigate the capacity of cells to translate IL-1 $\beta$  transcripts, western blot analysis of cultured cells was performed using an antibody that detects both pro-IL-1 $\beta$  and mature IL-1 $\beta$ .<sup>14</sup> In supernatants of LPS stimulated peripheral blood mononuclear cells, both pro- and mature forms of IL-1 $\beta$  were present (fig 2).

Of four normal colonic lamina propria cell cultures, pro-IL-1 $\beta$  was identified in all cell lysates, three unstimulated culture supernatants and all four LPS stimulated cell culture supernatants (fig 3). However, the mature 17 kD form of the cytokine was not seen in any of these samples.

Western blot analysis was also performed following culture (in the presence or absence of added LPS) of lamina propria cells isolated from active IBD mucosal samples obtained from five separate intestinal resection speci-

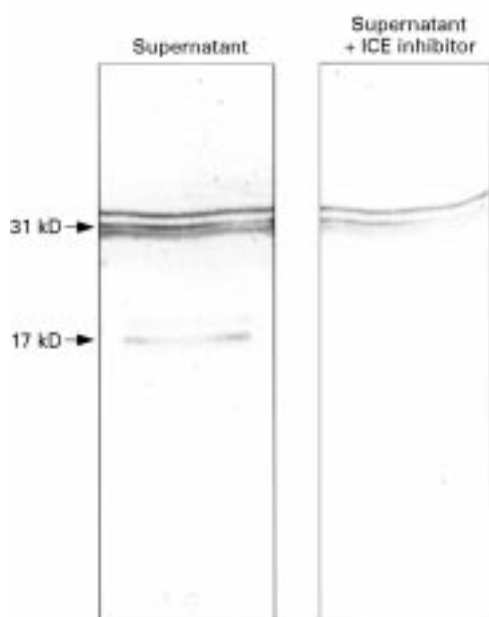


**Figure 5** Expression of ICE protein by LPS stimulated lamina propria cells isolated from normal and IBD colonic mucosa. In contrast to IBD, the p20 form of ICE was not present in the lysate of cells from normal colonic mucosa. The lighter band between p45 and p20 is likely to represent a 36 kD intermediate cleavage product of p45 (and which is further processed down to p20).<sup>38</sup>

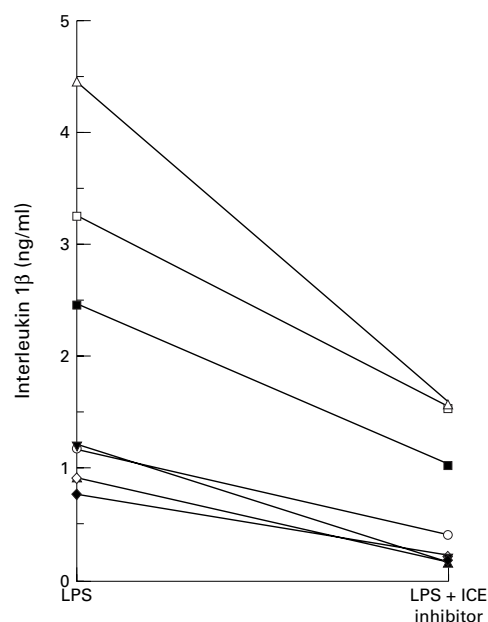
mens. In all five LPS stimulated and four unstimulated cultures, both the 31 kD and 17 kD forms of IL-1 $\beta$  were present in the supernatant (fig 3). The cell lysates expressed pro-IL-1 $\beta$  only.

**EXPRESSION OF ICE mRNA AND PROTEIN**

The production of pro-IL-1 $\beta$ , but not mature IL-1 $\beta$ , by normal colonic macrophages could be explained by the lack of expression of ICE or



**Figure 6** Release of IL-1 $\beta$  protein by IBD lamina propria cells cultured in the presence and absence of ICE inhibitor.



**Figure 7** ICE inhibitor (Ac-Tyr-Val-Ala-Asp-CHO) reduces release of mature IL-1 $\beta$  by lamina propria cells isolated from mucosa with active IBD. Paired samples ( $n=7$ ) of isolated lamina propria cells were cultured with LPS, in the presence or absence of Ac-Tyr-Val-Ala-Asp-CHO.

the presence of ICE in biologically inactive form only. The expression of ICE transcripts and protein was therefore investigated.

Analysis by RT-PCR showed the presence of ICE transcripts in all mucosal biopsy specimens (12 normal and 11 active IBD; fig 4) and all isolated lamina propria cell preparations (six normal and three IBD). Western blot analysis of isolated lamina propria cell lysates (from four normal and four IBD colons) showed the presence of the p45 precursor form of ICE in all lysates. In all IBD, but not normal cell lysates, a large amount of the biologically active p20 ICE subunit was also present (fig 5).

**EFFECT OF ICE INHIBITION ON RELEASE OF MATURE IL-1 $\beta$**

Ac-Tyr-Val-Ala-Asp-CHO, a specific peptide aldehyde inhibitor of ICE,<sup>4</sup> inhibited the release of mature IL-1 $\beta$  by LPS stimulated peripheral blood monocytes (fig 2). Similarly, western blot analysis showed that the mature 17 kD form of IL-1 $\beta$  was not detected in supernatants of IBD lamina propria cells cultured in the presence of the ICE inhibitor (fig 6). The effect of the ICE inhibitor was also investigated by ELISA on seven paired samples of isolated lamina propria cells from mucosa with active IBD (fig 7). Cells cultured with LPS for 20 hours released a median of 1.20 (range 0.78–4.42) ng/ml IL-1 $\beta$ , which was significantly reduced in the presence of Ac-Tyr-Val-Ala-Asp-CHO to a median of 0.43 (range 0.20–1.60) ng/ml IL-1 $\beta$  ( $p<0.01$ ; fig 7). Ac-Tyr-Val-Ala-Asp-CHO did not affect the viability of the isolated lamina propria cells (88.1 (4.2)% preculture, and 89.8 (3.4)% postculture).

## Discussion

In the lumen of the normal human colon, there is a large and complex resident population of microorganisms (approximately  $10^{12}$ /g contents), Gram negative bacteria being the predominant species.<sup>15</sup> LPS, which is the outer membrane glycolipid of Gram negative bacteria, is a potent stimulator of IL-1 $\beta$  production by monocytes. Various other agents can induce the processing of pro-IL-1 $\beta$  by mononuclear phagocytes, but these are generally experimental stimulants which are not found in the normal colonic lumen.<sup>1-4, 16</sup> LPS, however, is a physiological factor which is present in high concentration in the colon in close proximity to the mucosal cells; it has been implicated in the pathogenesis of active inflammatory bowel disease,<sup>17-19</sup> and has been used in this and other studies<sup>14, 20</sup> because of the exquisite sensitivity of mononuclear phagocytes to its effects.<sup>1</sup>

Previous studies have shown that IL-1 $\beta$  in the colon is expressed almost exclusively in the lamina propria macrophages<sup>8-10</sup> and we have recently shown this to be the case for ICE.<sup>21</sup> Our studies of IL-1 $\beta$  transcription have shown that normal colonic macrophages are capable of responding to isolation from the mucosa with activation of the IL-1 $\beta$  gene and that, in agreement with a previous report,<sup>22</sup> a minority of normal colonic biopsy samples expressed IL-1 $\beta$  mRNA. In our cell isolation model, epithelial cells are first detached using EDTA, which will expose pores in the basement membrane,<sup>12</sup> thus allowing access of LPS from residual luminal contents into the lamina propria in which macrophages are prominent.<sup>23, 24</sup> We postulate, therefore, that it is the exposure to LPS which induces the expression of IL-1 $\beta$  in macrophages being isolated from normal mucosa. The findings of occasional IL-1 $\beta$  expression in apparently normal colonic mucosal biopsy specimens might be due to a similar process. LPS exposure could occur *in vivo* when injured or effete epithelial cells detach, temporarily allowing LPS to penetrate the lamina propria through basement membrane pores and stimulate the underlying macrophages. Our studies suggest, however, that on exposure to LPS, normal resident colonic macrophages are incapable of activating ICE and therefore cannot process pro-IL-1 $\beta$  to the biologically active form. This limitation of the macrophage response is likely to be important in avoiding inflammatory tissue injury provoked by penetration of the epithelium by LPS in the normal colon.

IL-1 $\beta$  mRNA was expressed in all biopsy specimens from colons affected by active IBD, a finding that might be explained by the increased LPS penetration of the epithelial barrier that occurs in active inflammatory bowel disease.<sup>17-19</sup> In contrast to normal macrophages, however, IBD cells expressed active ICE and released both pro-IL-1 $\beta$  and mature IL-1 $\beta$ . This is unlikely to be due to the treatment given to the patients as the drugs that may influence IL-1 $\beta$  production (prednisolone and 5-aminosalicylic acid) are likely to have opposite effects<sup>6, 7</sup> to those observed. Thus IBD colonic macrophages differ from normal co-

lonic macrophages in being able to process pro-IL-1 $\beta$  in a manner similar to circulating monocytes. Our data would therefore also be consistent with previous studies suggesting that IBD macrophages are recently recruited peripheral blood monocytes,<sup>25-27, 35</sup> which are known to express ICE and readily process pro-IL-1 $\beta$  to the mature peptide following stimulation with LPS.<sup>1-5</sup>

ICE activity has been reported to be associated with apoptosis.<sup>28</sup> However, more recent studies suggest that ICE is not essential for programmed cell death but that it is the sole enzyme responsible for processing of pro-IL-1 $\beta$  to the mature peptide in macrophages.<sup>29-31</sup> Similarly, we have not found evidence of apoptosis in isolated and LPS stimulated IBD intestinal macrophages (unpublished data), consistent with a primarily proinflammatory role for active ICE expression in IBD.

Migration of circulating monocytes and polymorphonuclear cells into injured tissue is a characteristic feature of an inflammatory response. Recent studies suggest that in the colon, inflammatory responses may be initiated by epithelial cell secretion of chemokines, such as monocyte chemoattractant protein 1 (MCP-1) and IL-8, following invasion by pathogenic bacteria<sup>32, 33</sup> or injury induced by bacterial toxins.<sup>34</sup> As yet uncharacterised injury to epithelial cells, and subsequent release of chemoattractants, may also be responsible for initiating relapse in IBD. Our studies suggest that in the absence of epithelial cell injury, the resident colonic macrophages do not initiate an inflammatory response following exposure to LPS. Thus epithelial cell injury and secretion of chemokines and/or penetration of microorganisms into the lamina propria may be a prerequisite for the development of an inflammatory reaction in the intestinal mucosa. Monocytes recruited during this process would have the capacity, on exposure to luminally derived LPS, to release mature IL-1 $\beta$ , which together with other cytokines and mediators (such as oxygen radicals<sup>35</sup>) could cause a cascade of tissue damaging inflammatory responses.

IL-1 $\beta$  itself is likely to be of major importance in the pathogenesis of IBD. Inhibition of its activity with a receptor antagonist has previously been shown to reduce the inflammatory response in an animal model of colitis.<sup>36</sup> Our studies have shown that specific inhibition of ICE with a peptide aldehyde inhibitor leads to a significant reduction in the amount of mature IL-1 $\beta$  released. The magnitude of this reduction may be greater than our data suggest since the ELISA used to detect mature IL-1 $\beta$  cross reacts with pro-IL-1 $\beta$  (approximately 20% on a molar basis; information from R & D Systems Inc.), the release of which increases following ICE inhibition.<sup>4, 37</sup> Agents that specifically inhibit ICE activity could therefore be of therapeutic value in patients with active inflammatory bowel disease.

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