Activation of the transcription factor NF- κ B by *Campylobacter jejuni*

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***Campylobacter jejuni* is a food-borne pathogen responsible for infectious enterocolitis. The early-response transcription factor NF- κ B triggers the expression of genes associated with cellular immune and inflammatory responses. Co-incubation of HeLa cells with viable *C. jejuni* leads to the activation of the transcription factor NF- κ B as determined by specific induction of a cellular luciferase-based reporter. Boiled cell-free extracts of *C. jejuni* are also potent dose-dependent stimulators of NF- κ B-dependent transcription, the levels of which can reach up to 1000-fold as compared with independent controls. Using both cultured HeLa cells and human colonic epithelial (HCA-7) cells, the activation of NF- κ B by *C. jejuni* boiled extract has been monitored through the degradation of I κ B α and DNA binding of the nuclear translocated p50/p65 heterodimer of NF- κ B. These events are co-ordinated with elaboration of the pro-inflammatory cytokine interleukin-8. Fractionation of the boiled *C. jejuni* extract suggests that the majority of the bioactive component has a molecular mass of 3 kDa or less, which is insensitive to proteinase K treatment.**

Keywords: transcription, innate immunity, interleukin-8, inflammation, food safety

INTRODUCTION

The bacterium *Campylobacter jejuni* is one of the most common causes of diarrhoea worldwide, and the most common cause of infectious enterocolitis in the Western world (Tauxe, 1992). *C. jejuni* is a Gram-negative microaerophile that is often found as a commensal organism in the intestinal tracts of wild and domestic animals. The organism is particularly common in avian species, and it is this association with domestic poultry that is often a source of human infection (Saleha *et al.*, 1998). Food-borne infection can occur through the direct consumption of undercooked chicken and turkey, or via the cross-contamination of other foods with the raw product before cooking. Other sources of human infection include red meats, unpasteurized milk and untreated drinking water supplies.

Patients suffering campylobacteriosis present a range of clinical symptoms, from mild watery diarrhoea to severe bloody diarrhoea accompanied with fever and abdomi-

nal cramps. A recent history of *Campylobacter* infection is also frequently associated with the neurological disorder Guillain-Barré syndrome (Rees *et al.*, 1993; Nachamkin *et al.*, 1998). Guillain-Barré syndrome can result in paralysis and, occasionally, impaired respiratory function. The pathogenic mechanisms responsible for the acute intestinal infection of humans are poorly understood but are thought to involve the processes of colonization, adherence, cellular invasion and toxin production (Ketley, 1997). There are clearly some variations in these processes, as not all clinical isolates of *C. jejuni* are demonstrably able to invade cultured human cells or produce defined toxins. However, a common feature of *Campylobacter* infectious enterocolitis is a localized acute inflammatory response that can lead to tissue damage and may be responsible for many of the clinical symptoms (Ketley, 1997).

The NF- κ B/rel family of transcription factors comprises a structurally related series of DNA binding and transactivation proteins, which have been shown to play an early response role in a large number of cellular processes, including the host inflammatory response to microbial infection. NF- κ B/rel members function as part of the innate immune response to microbial

Abbreviations: BCE, boiled-cell extract; EMS, electrophoretic mobility shift; IL, interleukin; LOS, lipo-oligosaccharide; TNF- α , tumour necrosis factor- α .

pathogens, acting to stimulate the transcription of the genes for cytokines and chemokines (Silverman & Maniatis, 2001). The resulting secretion of cytokines/chemokines and other mediators leads to the activation of macrophages and the recruitment of polymorphonuclear leukocytes in the inflammatory response. Continued stimulation of these response mechanisms can result in chronic inflammatory states and fibrogenesis of the intestinal tract (Schmid & Adler, 2000).

NF- κ B/rel members are composed of DNA-binding proteins (NF- κ B1p50 and NF- κ B2p52) in association with rel proteins (RelA p65, RelB and c-Rel) which bear the transactivation domain. NF- κ B/rel complexes are held in the cytoplasm in non-induced cells by inhibitor proteins called I κ B (I κ B α , I κ B β , I κ B γ and I κ B ϵ). These proteins bind to NF- κ B/rel and mask the nuclear localization domain in order to prevent nuclear translocation. Activation of NF- κ B involves the phosphorylation and subsequent ubiquitin-mediated proteosomal degradation of I κ B (Mellits *et al.*, 1993; Silverman & Maniatis, 2001). I κ B is phosphorylated by I κ B kinase (IKK α , IKK β catalytic subunits and the regulatory subunit IKK γ). IKK is subject to activation by the receptor pathway-dependent kinases MAP/ERK kinase kinase 1 (MEKK1) and NF- κ B-inducing kinase (NIK) (Silverman & Maniatis, 2001).

Here we show that *C. jejuni*, like other gastrointestinal pathogens (*Salmonella typhimurium*, *Shigella flexneri*, *Helicobacter pylori*, enterovirulent *Escherichia coli* and *Yersinia enterocolitica*), can activate NF- κ B in epithelial cells and thereby elicit a pro-inflammatory response. Moreover, we show that a cell-free, heat-stable extract of *C. jejuni* can activate NF- κ B through the degradation of I κ B α and the subsequent binding of NF- κ B subunits to the NF- κ B target DNA sequence. We also demonstrate that the NF- κ B activation is co-ordinated with the production of the pro-inflammatory cytokine interleukin-8 (IL-8). As IL-8 is a chemotactic factor of immune-active cells and a mediator of local immune responses, these data therefore predict a mechanism by which *C. jejuni* can bring about the intestinal inflammation commonly associated with campylobacteriosis.

METHODS

Bacterial strains and growth conditions. The type strain *C. jejuni* NCTC 11168 (Penner serotype 2) was selected for the majority of these experiments as it has been well characterized and the complete genome sequence is available (Parkhill *et al.*, 2000). *C. jejuni* strain FB1 is a low-passage clinical isolate from a patient suffering from bloody diarrhoea and fever. *C. jejuni* strain G1 was isolated from a patient suffering from Guillain-Barré syndrome (Karlyshev & Wren, 2001). Campylobacters were propagated on blood-agar plates (Oxoid blood-agar base CM 271 with 5%, v/v, defibrinated horse blood) at 42 °C under microaerobic conditions for 24 h. *E. coli* strain BL21 was cultured on Luria agar plates at 37 °C for 16 h.

Preparation of Campylobacter extracts. A 24 h culture of *C. jejuni* NCTC 11168^T was used to inoculate 150 ml nutrient broth no. 2 (CM 67; Oxoid) dispensed in 250 ml conical flasks; the flasks were then shaken under microaerobic conditions for 24 h at 42 °C. The bacteria were collected by

centrifugation at 10000 g for 15 min. The bacterial cell pellet was then resuspended in PBS and washed by centrifugation for a total of three times. The cell pellet was weighed then resuspended in PBS to 10% (w/v). This suspension was then boiled for 10 min and cooled on ice. The suspension was then centrifuged at 13000 g and the supernatant collected. This extract was then filtered through a 0.2 μ m filter, to remove any residual bacteria, and stored at -20 °C until required.

The extract was fractionated by ultrafiltration using molecular mass cut-off filters of 30, 10, 5 and 3 kDa applied in PBS according to the manufacturers' instructions (Millipore and Gelman Laboratories). The fractions were treated with proteinase K (100 μ g ml⁻¹) for 1 h at 55 °C and reboiled before use in reporter cell activation assays. Fractions pre- and post-proteinase K treatment were electrophoresed in 12.5% SDS-polyacrylamide gels and visualized with Coomassie blue and silver stain to ensure the correct functioning of the molecular mass cut-off filters and the complete digestion of the protein component of the extract.

Cell culture and induction. HeLa 57A cervical epithelial cells (Rodriguez *et al.*, 1999) and HCA-7 colonic epithelial cells (Kirkland, 1985) were grown in monolayer cultures of approximately 5×10^6 in Dulbecco's Modified Eagle's Medium supplemented with penicillin at 100 μ g ml⁻¹, streptomycin at 100 μ g ml⁻¹ and fetal calf serum at 10% (v/v). To select for the transcriptional markers present in HeLa 57A cells, these cultures were supplemented with G418 (Gibco) at 0.5 μ g ml⁻¹. Three hours prior to induction, cells were starved of serum, and inductions were carried out by adding tumour necrosis factor- α (TNF- α) at 50 ng ml⁻¹ (obtained from the EU Programme EVA/MRC Centralized Facility for AIDS Reagents, NIBSC, UK; grant nos QLK2-CT-1999-00609 and GP828102) or *C. jejuni* extract at the concentration indicated. Live *C. jejuni* infections were performed using fresh overnight cultures of *C. jejuni*, which were allowed to equilibrate in Dulbecco's Modified Eagle's Medium before introduction to tissue culture cells at an m.o.i. of 100.

Reporter cell assays. HeLa 57A cells carry an NF- κ B-dependent promoter driving *luc* transcription, and an independent Rous sarcoma virus promoter driving the expression of *lacZ* (Rodriguez *et al.*, 1999). Replicate luciferase and β -galactosidase reporter assays (four to six independent determinations) were performed with cytoplasmic protein extracts. Luciferase activity was measured using a Turner bioluminometer with luciferin as substrate, as recommended by the manufacturer (Promega). β -Galactosidase activity was measured using a colorimetric assay with the substrate ONPG, as recommended by the manufacturer (Clontech). To calculate the degree of NF- κ B induction, all luciferase activities were normalized against internal β -galactosidase activities, so as to provide a constitutive control of the basal expression levels, and expressed as a multiple of the uninduced control (fold-activation).

Electrophoretic mobility shift (EMS) assay. Nuclear and cytoplasmic protein extracts were prepared from tissue-culture cells as described by Mellits *et al.* (1993), using 6 cm dishes (containing 5×10^5 cells). Protein concentrations were estimated by the Bradford assay (Bio-Rad). For EMS assays, nuclear protein extracts (10 μ g) were incubated together with a ³³P-labelled oligonucleotide probe containing the NF- κ B binding site as characterized for the human β -interferon promoter (positive regulatory domain II; Visvanathan & Goodbourn, 1989) in binding buffer [10 mM HEPES/KOH (pH 7.9), 50 mM KCl, 5 mM DTT, 1 mM EDTA containing 2 μ g poly(dI-dC)]. DNA-protein complexes were fractionated on 5% native polyacrylamide gels, dried and visualized using

a phosphor-imager (Bio-Rad). Supershift assays were performed with polyclonal antibodies against the p50 and p65 subunits of NF- κ B (gifts from Ronald T. Hay, University of St Andrews, UK).

Western blots. Protein samples (10 μ g) were heated in SDS loading buffer at (90 °C for 3 min) before fractionation on 12.5% SDS-polyacrylamide mini-gels and transfer to 0.45 μ m PVDF membranes (Pierce). Membranes were blocked using milk protein and probed with primary antibodies directed against I κ B α (monoclonal 10B, a gift from Ronald T. Hay, University of St Andrews), I κ B β (polyclonal C-20, sc-945; Santa Cruz Biotech) and I κ B ϵ (polyclonal M-364, sc-7155; Santa Cruz Biotech) or as an independent control 14-3-3 protein (polyclonal K19, sc-629; Santa Cruz Biotech); bound antibodies were detected using species-specific secondary antibodies conjugated to alkaline phosphatase (Sigma) and visualized with nitroblue tetrazolium (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

IL-8 determination. IL-8 released into cell-culture supernatants was determined for induced and mock-induced HeLa 57A and HCA-7 cells over a 9 h time-course. The induction factor for NF- κ B-dependent gene expression was determined in parallel for the HeLa 57A reporter cell line. IL-8 was measured using a sandwich ELISA. IL-8 was captured with murine anti-human IL-8 and detected with biotinylated goat anti-human IL-8 using streptavidin-coupled horseradish peroxidase according to the manufacturer's instructions (R&D Systems).

RESULTS

Activation of NF- κ B by *C. jejuni*

To investigate if *C. jejuni* could activate the transcription factor NF- κ B, we utilized the NF- κ B reporter cell line HeLa 57A (Rodriguez *et al.*, 1999). HeLa 57A

Fig. 1. NF- κ B-dependent gene expression in HeLa 57A cells co-incubated with *C. jejuni* (open bars) or non-pathogenic *E. coli* (solid bar), or treated with TNF- α (50 ng ml⁻¹; hatched bar). HeLa 57A cells were co-incubated with *C. jejuni* or *E. coli* (BL21) at an m.o.i of 100:1 and harvested 16 h post-infection. NF- κ B gene expression was determined by measuring the induction of luciferase activity relative to that from mock treatment and presented as fold activation. The data are recorded as the means of three determinations $\pm$ SD.

Fig. 2. NF- κ B-dependent gene expression stimulated by *C. jejuni* BCE in HeLa 57A cells. (a) Dose-dependent stimulation of NF- κ B-dependent gene expression by *C. jejuni* BCE (15–150 $\times 10^3$ ng ml⁻¹). Cells were harvested at 6 h post stimulation. (b) Time-course of NF- κ B induction of HeLa 57A cells stimulated with *C. jejuni* BCE (15 μ g ml⁻¹; open bars) or TNF- α (50 ng ml⁻¹; hatched bars). Cells were harvested at 30 min intervals up to 4 h post-stimulation. NF- κ B-dependent gene expression was determined by measuring the induction of luciferase activity relative to that from mock treatment and presented as fold activation. The data are recorded as the means of five determinations $\pm$ SD. *C.j.*, *C. jejuni*.

cells contain stable transfected copies of NF- κ B-dependent *luc* (luciferase) and NF- κ B-independent (Rous sarcoma virus enhancer-driven) *lacZ* (β -galactosidase) reporter genes that together provide a sensitive, but internally controlled, measure of NF- κ B-dependent gene expression. Live *C. jejuni* or the non-pathogenic *E. coli* strain BL21 were incubated with HeLa 57A cells at an m.o.i. of 100:1, and the reporter gene activities were determined at 16 h post-infection. The *C. jejuni* type strain NCTC 11168 was shown to induce NF- κ B-dependent gene expression up to 100-fold compared to basal expression from mock-infected cells (Fig. 1). Co-incubation of the clinical *C. jejuni* isolates from either a typical sporadic enterocolitis case (FB1) or a patient suffering from Guillain-Barré syndrome (G1) could also induce NF- κ B-dependent gene expression in HeLa 57A cells (Fig. 1). However, under these conditions, *E. coli* BL21 did not appreciably induce NF- κ B-dependent gene

Fig. 3. Time-course of $\text{I}\kappa\text{B}\alpha$ degradation stimulated by *C. jejuni* BCE in HeLa 57A and HCA-7 cells. Western blots of cytoplasmic protein extracts probed with antibodies directed against $\text{I}\kappa\text{B}\alpha$ or 14-3-3 proteins are shown. Cells were harvested at 30 min intervals post-stimulation with *C. jejuni* BCE ($15 \mu\text{g ml}^{-1}$) or $\text{TNF-}\alpha$ (50 ng ml^{-1}) and compared with an untreated control. (a) HeLa 57A cells treated with $\text{TNF-}\alpha$; (b) HeLa 57A cells treated with *C. jejuni* BCE; (c) HCA-7 cells treated with $\text{TNF-}\alpha$; (d) HCA-7 cells treated with *C. jejuni* BCE.

expression relative to that from mock-infected cells. By comparison, $\text{TNF-}\alpha$ (50 ng ml^{-1}) treatment produced 220-fold induction of luciferase activity at 3 h under the same conditions. No induction of luciferase activity was observed when cell-free filtrates of *C. jejuni* spent growth medium were applied to HeLa 57A cells. Similarly, cell-free filtrates of *C. jejuni* previously incubated in tissue-culture medium did not induce luciferase activity. These results imply that the activation of NF- κB is not due to a soluble secreted component.

A heat-stable cell-free extract of *C. jejuni* can activate NF- κB

As a first step to investigate the factor(s) responsible for NF- κB activation, we prepared a boiled cell-free extract of *C. jejuni* enriched in cell-surface components. This simple procedure was adopted because it was reproducible and avoided the use of exogenous extraction reagents, which might interfere with the cell-culture-based assay of NF- κB -dependent gene expression. The extracts were prepared from *C. jejuni* cells collected from overnight liquid culture; the cells were boiled, clarified by centrifugation and passed through $0.2 \mu\text{m}$ filters to remove any remaining bacteria. Typically, these extracts contain protein at a concentration of 0.5 mg ml^{-1} . Boiled cell-free extracts were tested for their ability to induce NF- κB -dependent gene expression using HeLa 57A cells over a range of protein concentrations (15 ng ml^{-1} to $150 \mu\text{g ml}^{-1}$). *C. jejuni* boiled-cell extract (BCE) induced NF- κB -dependent expression of luciferase in HeLa 57A cells (Fig. 2). The luciferase induction factors measured 4 h post-treatment can be seen to increase in proportion to the dose of *C. jejuni* BCE applied (Fig. 2a). A *C. jejuni* BCE protein concentration of $15 \mu\text{g ml}^{-1}$ was adopted for use in subsequent experiments to characterize the activation of

NF- κB . This value was selected since it lies within the linear range of those tested and produces a measurable induction factor of 422-fold, which is comparable with the factor (440-fold) determined for the standard positive experimental control of $\text{TNF-}\alpha$ at 50 ng ml^{-1} .

To determine the time-frame in which NF- κB can bring about gene expression in response to the *C. jejuni* BCE, we monitored the NF- κB -dependent induction of luciferase activity in HeLa 57A cells during the first 4 h after treatment, and compared these with $\text{TNF-}\alpha$ over the same period (Fig. 2b). Significant induction of luciferase activity (30-fold) was first detected at 120 min post-induction with *C. jejuni* BCE. Similarly, $\text{TNF-}\alpha$ -induced luciferase activity was first detected at 120 min, although at a greater induction factor of 180-fold. However, by 240 min, the induction factors of *C. jejuni* BCE and $\text{TNF-}\alpha$ were comparable: 280-fold and 320-fold, respectively.

C. jejuni extract activates NF- κB via $\text{I}\kappa\text{B}\alpha$ degradation and DNA binding

NF- κB is activated following the degradation of a cytoplasmic inhibitor protein, $\text{I}\kappa\text{B}$. Degradation of $\text{I}\kappa\text{B}$ releases a heterodimer composed of NF- κB and rel proteins, which relocate to the nucleus to bind promoter DNA elements and exert transcriptional activation. To correlate the *C. jejuni* BCE-induced NF- κB -dependent gene expression with the degradation of a specific member of the $\text{I}\kappa\text{B}$ family, we performed a series of Western blots and probed these with antibodies against $\text{I}\kappa\text{B}$ proteins. Cytoplasmic protein extracts were prepared from HeLa 57A cells induced with either *C. jejuni* BCE or $\text{TNF-}\alpha$. Western blots of cytoplasmic protein extracts harvested at 30 min intervals post-induction were probed with antibodies directed against $\text{I}\kappa\text{B}\alpha$ and 14-3-3 proteins (Fig. 3). The 14-3-3 protein levels are

Fig. 4. *C. jejuni* BCE activates NF- κ B DNA-binding complexes from HeLa 57A and HCA-7 cells. EMS assays were performed with nuclear protein extracts prepared over a 4 h time-course after treatment with either *C. jejuni* BCE (15 μ g ml⁻¹) or TNF- α (50 ng ml⁻¹). The oligonucleotide probe contains the positive regulatory domain II region of the human β -interferon promoter that detects NF- κ B and a β -interferon-specific complex marked 'B'. The 'B' complex constitutes an internal control for the formation of protein-DNA complexes and is present in the untreated control (lane 1). Supershift assays (lanes 8–13) were performed on the 30 and 120 min post-induction nuclear protein extracts for HeLa 57A and HCA-7 cells, respectively, with either pre-immune serum (p.i.) or serum antibodies directed against the NF- κ B subunits p50 (α p50) or p65 (α p65). (a) NF- κ B activation of HeLa 57A cells; (b) NF- κ B activation of HCA-7 cells.

invariant with these treatments and act as a control of protein loading in these experiments. I κ B α levels in 57A HeLa cells are reduced as compared with untreated cells at the 30 and 60 min time-points in either TNF- α - or *C. jejuni* BCE-treated cells (Fig. 3a, b; lanes 2 and 3). Later time-points show I κ B α levels returning as a consequence of the resynthesis of I κ B α (Fig. 3a, b; lanes 4 and 5).

To ensure that the observed response was not limited to cervical epithelial cells, cytoplasmic protein extracts were prepared from human colonic epithelial (HCA-7) cells treated in the same way. I κ B α levels were also markedly reduced in response to TNF- α or *C. jejuni* BCE treatment in HCA-7 cells. However, in contrast to that in HeLa 57A cells, the degradation of I κ B α in response to TNF- α in HCA-7 cells is delayed until 90 min after treatment (Fig. 3c; lanes 2–4). *C. jejuni* BCE treatment, however, results in a reduction of the I κ B α signal at 30 min, which returns to untreated levels by 120 min (Fig. 3d; lanes 2–5). Parallel Western blots showed no degradation of the alternative inhibitor proteins I κ B β and I κ B ϵ in either cell line over the time-courses indicated.

The DNA-binding activities of the NF- κ B complexes from HeLa 57A and HCA-7 cells in response to treatments with either *C. jejuni* BCE or TNF- α were examined using an EMS assay. Nuclear protein extracts were prepared over a 4 h time-course post-treatment

and incubated with a labelled oligonucleotide probe containing the NF- κ B binding site derived from the positive regulatory domain II region of the human β -interferon promoter. The probe detects NF- κ B and a probe-specific complex marked 'B' (Fig. 4). The 'B' complex provides a useful internal control of the formation of protein-DNA complexes in these assays, and is present in the untreated control. Treatment of HeLa 57A cells with either *C. jejuni* BCE or TNF- α produces an NF- κ B-specific retarded band, after 30 min, which is absent with the untreated control extract (Fig. 4a; lanes 1, 2 and 5). The NF- κ B-DNA complex produced by either treatment persists through to 240 min, but, by this time, the intensities of the corresponding bands are diminished (Fig. 4a; lanes 3, 4, 6 and 7). To confirm the composition of the DNA-protein complexes observed in the EMS assay, supershift experiments were performed in which DNA-binding reactions were incubated with antibodies directed against the NF- κ B component subunits p50 (NF- κ B1) and p65 (RelA). Incubation with pre-immune serum does not further retard the treatment-specific band. However, incubation with either anti-p50 or anti-p65 produces a characteristic shift in the NF- κ B protein-DNA complexes formed by either *C. jejuni* BCE or TNF- α treatment (Fig. 4a; lanes 8–13). EMS assays and supershift experiments with HCA-7 cell nuclear extracts also indicate the formation of NF- κ B protein-DNA

complexes in response to either *C. jejuni* BCE or TNF- α treatment (Fig. 4b). In corroboration of the I κ B α degradation data, the DNA-binding activity observed for HCA-7 cells in response to TNF- α is delayed from 30 until 120 min post-treatment (Fig. 4b, lanes 2 and 3) when compared with the response of HeLa 57A cells to TNF- α (Fig. 4a, lane 3). In contrast, HCA-7 cells suffer no such delay in response to *C. jejuni* BCE treatment. *C. jejuni* BCE treatment leads to the formation of NF- κ B protein-DNA complexes within 30 min, the levels of which remain unabated through to the 240 min time-point (Fig. 4b, lanes 5–7). The NF- κ B DNA-binding activity induced in HCA-7 cells by TNF- α , however, does not persist and is noticeably diminished by 240 min.

C. jejuni BCE triggers IL-8 release

A consequence of the activation of NF- κ B is likely to be the up-regulation of a set of immune response genes, a key member of which is the pro-inflammatory cytokine IL-8. To examine the release of IL-8 from epithelial cells in response to *C. jejuni* BCE treatment, we measured the concentrations of IL-8 in cell-culture supernatants at 3 h intervals over a 9 h time-course. Fig. 5(a) and Fig. 5(b) show the respective concentrations of IL-8 secreted from HCA-7 colonic epithelial cells and HeLa 57A cells in response to treatment with *C. jejuni* BCE or TNF- α . Both HCA-7 and HeLa 57A cells accumulate IL-8 in response to *C. jejuni* BCE over the 9 h period. Indeed, IL-8 accumulation in response to *C. jejuni* BCE treatment was found to be considerably higher than those recorded for control TNF- α treatment, irrespective of the cell type (Fig. 5a, b). HCA-7 cells produce significantly more IL-8 than HeLa 57A cells in response to either *C. jejuni* BCE or TNF- α treatment. HCA-7 cells accumulated 5268 ± 575 pg ml $^{-1}$ IL-8 9 h post-induction by TNF- α , which is more than 10 times that secreted by HeLa 57A cells (483 ± 24 pg ml $^{-1}$) treated in the same way. As a consequence, a dramatic response can be observed from HCA-7 cells treated with *C. jejuni* BCE: 6665 ± 2332 pg IL-8 ml $^{-1}$ is secreted at 3 h post-induction, which increases further to 24269 ± 3393 pg ml $^{-1}$ IL-8 at 9 h (Fig. 5a). Basal levels of IL-8 from mock-treated HCA-7 cells were relatively low (393 ± 6 pg ml $^{-1}$) and undetectable in the culture supernatants of mock-treated HeLa 57A cells. *C. jejuni* BCE is clearly a potent elicitor of the cytokine IL-8.

To determine if the greater yields of IL-8 triggered by *C. jejuni* BCE induction as compared with TNF- α in this experiment were relative to the activation of NF- κ B, we measured the induction of NF- κ B-dependent luciferase activity from protein extracts of HeLa 57A cells. Fig. 5(c) shows that the induction of NF- κ B by TNF- α remains constant (420–470-fold) over the time-course, whereas the *C. jejuni* BCE-treated cells experience a peak in NF- κ B-dependent gene expression (1250-fold) 6 h post-induction. These data are consistent with the relatively modest accumulation of IL-8 following TNF- α induction as compared with the significantly greater

Fig. 5. IL-8 secretion from HCA-7 (a) and HeLa 57A cells (b) stimulated with *C. jejuni* BCE (open bars) or TNF- α (hatched bars). Cell cultures were harvested at 3 h time-points up to 9 h after treatment. Total IL-8 secretion was determined using sandwich ELISA. IL-8 was undetectable in mock-treated HeLa 57A cells and did not exceed 393 pg ml $^{-1}$. The data are recorded as the means of 10 determinations $\pm$ SD. NF- κ B-dependent gene expression was also determined for HeLa 57A cells treated with *C. jejuni* BCE (15 μ g ml $^{-1}$) or TNF- α (50 ng ml $^{-1}$) (c) NF- κ B-dependent gene expression was determined by measuring the induction of luciferase activity relative to that from mock treatment and presented as fold activation. The data are recorded as the means of three determinations $\pm$ SD. *C.j.*, *C. jejuni*.

Fig. 6. NF- κ B-dependent gene expression in HeLa 57A cells stimulated with *C. jejuni* BCE (15 μ g ml⁻¹; open bars), a <3 kDa fraction of the initial BCE (shaded bars) or TNF- α (50 ng ml⁻¹; hatched bars). Samples treated with proteinase K (100 μ g ml⁻¹ for 1 h at 55 °C) are as indicated. Cells were harvested at 6 h post-stimulation, and NF- κ B gene expression was determined by measuring the induction of luciferase activity relative to that from mock treatment and presented as fold activation. The data are recorded as the means of three determinations $\pm$ SD. C.j., *C. jejuni*.

values measured in response to *C. jejuni* BCE in HeLa 57A cells. Moreover, the increased rate of IL-8 accumulation evident at 9 h is consistent with the peak stimulation of NF- κ B-dependent gene expression determined 3 h earlier.

Fractionation and proteinase K sensitivity of the *C. jejuni* BCE

As a first step to identify the bioactive components present in *C. jejuni* BCE we fractionated the extract according to molecular mass by ultrafiltration. Soluble fractions containing molecules in the mass ranges >30, 10–30, 3–10 and <3 kDa were assayed in HeLa 57A cells for their ability to induce NF- κ B-dependent gene expression. These fractions were electrophoresed in 12.5% SDS-polyacrylamide gels and visualized with Coomassie blue and silver stain to monitor the performance of the molecular mass cut-off filters. The majority of the activity was found in a low-molecular-mass <3 kDa fraction (70%); most of the remaining activity was retained in the >30 kDa fraction (20%). Although the relative proportions of the total NF- κ B stimulatory activities found in the <3 and >30 kDa fractions were observed to be fairly constant over several experiments, the luciferase induction values were never completely additive to that determined for the initial *C. jejuni* BCE. The extant activity was undetectable in the intermediate fractions.

Fig. 6 shows the effect of prior digestion with proteinase K on the abilities of the initial *C. jejuni* BCE and the <3 kDa fraction to stimulate NF- κ B-dependent gene expression in HeLa 57A cells. Samples collected pre- and post-proteinase K digestion were electrophoresed in

12.5% SDS-polyacrylamide gels and visualized by Coomassie blue and silver stain to ensure the complete digestion of the protein components of the extract fractions. Proteinase K digestion will eliminate the TNF- α response but will only reduce the *C. jejuni* BCE response by 32%. The <3 kDa fraction is insensitive to proteinase K activity. These data imply that the majority of the NF- κ B stimulatory activity is a low-molecular-mass non-protein component. However, it is probable that the initial *C. jejuni* BCE also contains a protein component with the ability to activate NF- κ B, though constituting only a minor contribution.

DISCUSSION

Epithelial cells in general and intestinal epithelial cells in particular are constantly exposed to bacteria and bacterial products. Epithelial cells are therefore largely insensitive to the presence of bacterial flora but must maintain the ability to respond to potentially injurious pathogenic species. An early cellular response to bacterial infection is the activation of the transcription factor NF- κ B. Once activated, NF- κ B can turn on the transcription of over 100 target genes, many of which are involved in the immune response (Baeuerle & Henkle, 1994). We have demonstrated that live cells of *C. jejuni* can activate the transcription factor NF- κ B in epithelial cells and that this response is not immediate but requires 16 h co-incubation of the bacteria and cells before it becomes measurable. Cell-free filtrates of *C. jejuni* previously incubated in tissue-culture medium did not activate NF- κ B. We therefore considered that cell-surface components were probably involved in the activation of NF- κ B by *C. jejuni*, and, furthermore, we reasoned that a boiled-cell extract (BCE) could enrich for these surface components without our having to resort to the use of cell extraction reagents which might confound the results. Indeed, the BCE of *C. jejuni* could induce NF- κ B-dependent gene expression in a dose-dependent manner (Fig. 2a). To confirm these findings and broaden the scope of the study, we used human colonic epithelial cells in parallel with cervical epithelial cells to examine the process of activation of NF- κ B by *C. jejuni* BCE. Consistent with the detection of NF- κ B-dependent gene expression at 2 h post-treatment with *C. jejuni* BCE, we found that the NF- κ B inhibitor protein I κ B α is subject to degradation within 30 min in either cell line. I κ B subunits mask the NF- κ B nuclear localization signal and thereby hold the complex in the cell cytoplasm. Upon induction, I κ B is degraded and releases NF- κ B to migrate to the nucleus and activate the transcription of target genes. Transcriptional activation is mediated through the recognition of a specific DNA sequence, the κ B-site, present in the promoter DNAs of target genes. As part of the same experiment, we monitored NF- κ B-dependent DNA-binding activities in nuclear protein extracts from either epithelial cell line by EMS assay and supershift experiments. EMS assay results confirm that the degradation of I κ B α corresponds with the release and DNA-binding of NF- κ B. The supershift experiments expand this result to confirm the

interaction of the p50 and p65 subunits of NF- κ B with their target DNA sequence. Taken together, these data clearly indicate that *C. jejuni* can activate the transcription factor NF- κ B in epithelial cells and is likely to provoke an inflammatory response via this mechanism.

NF- κ B regulates the transcription of a series of pro-inflammatory proteins. In intestinal epithelial cells the responses to NF- κ B activation include the production of cytokines and chemokines (IL-1 β , IL-6, IL-8, macrophage inflammatory protein-2, growth-related oncogenes α and β), cell-surface receptors (IL-2R), adhesion molecules (ICAM-1), inflammatory enzymes (inducible nitric oxide synthase and cyclo-oxygenase-2), stress proteins (complement factors B, C3, C4) and immunoregulatory molecules (major histocompatibility complexes I and II) (Jobin & Sartor, 2000). Amongst these, IL-8 is an important chemokine of epithelial cells: elaboration of IL-8 is associated with the recruitment of inflammatory cells such as polymorphonuclear leukocytes to sites of infection or tissue damage (Eckmann *et al.*, 1993a, b, 1995; Jung *et al.*, 1995). The promoter of the human IL-8 gene contains several consensus DNA-binding sites for transactivating proteins; these include κ B sites which constitute the target DNA sequence of NF- κ B (Mukaida *et al.*, 1990, 1994; Kunsch & Rosen, 1993). NF- κ B has emerged as the critical element for transcription of the IL-8 gene, for although it may participate in cooperative activation with other transcription factors such as NF-IL-6 or AP-1, NF- κ B remains a necessity (Mukaida *et al.*, 1990; Yasumoto *et al.*, 1992; Matsusaka *et al.*, 1993). Our experiments clearly correlate the elaboration of IL-8 with the activation of NF- κ B by *C. jejuni* BCE. *C. jejuni*, in common with several enteric bacterial pathogens, has been shown to elicit IL-8 secretion from epithelial cells (Hickey *et al.*, 1999). Two mechanisms have been proposed by which *C. jejuni* can interact with intestinal epithelial cells and bring about the release of IL-8: the first requires the adherence and/or invasion of cells by *C. jejuni* (Hickey *et al.*, 1999); the second is through the direct action of the cytolethal distending toxin that is present in most strains of *C. jejuni* (Hickey *et al.*, 2000). Our initial data, in which we demonstrate that live *C. jejuni* cells can activate NF- κ B and thereby provide the transactivation required to induce IL-8 gene expression, is consistent with the first of these proposals. We would suggest that the surface-active components associated with IL-8 production actually function by stimulating NF- κ B, and that these components are represented in *C. jejuni* BCE. However, it is unclear whether the tripartite structure that constitutes the cytolethal distending toxin would survive the boiling process to form an active component of the *C. jejuni* BCE. If the cytolethal distending toxin were to be present in *C. jejuni* BCE then its contribution to the total NF- κ B activation potential is likely to be minor, since the majority of the activity is proteinase K insensitive and falls into a low-molecular-mass fraction.

In these studies, we have used the cellular response to TNF- α as an experimental control and benchmark of the

ability of *C. jejuni* BCE to activate NF- κ B. The signal transduction pathway by which cells respond to TNF- α has been the subject of considerable study, and probably represents one of the best examples of receptor-mediated induction of NF- κ B (Jobin & Sartor, 2000; Silverman & Maniatis, 2001). TNF- α is a pro-inflammatory cytokine associated with inflammation and immune response. In these roles, TNF- α serves as an activator of NF- κ B, and is itself subject to activation by NF- κ B. In our experiments, *C. jejuni* BCE activates NF- κ B within 2 h in a comparable time-frame to TNF- α . Given that TNF- α induces a rapid and orchestrated series of events, we suggest that the activation by *C. jejuni* BCE is also a direct response, independent of the production of any secondary inducers such as cytokines.

How *C. jejuni* BCE brings about this response is not clear at this time. However, we have demonstrated that the activation of NF- κ B by *C. jejuni* BCE, like TNF- α , is mediated through the transient degradation of I κ B α . In cervical epithelial cells, the degradation of I κ B α occurs within 30 min of induction by *C. jejuni* BCE but returns to untreated levels by 90 min. This occurs because newly synthesized I κ B α is translocated to the nucleus, where it serves to sequester NF- κ B and down-regulate the activation. It is quite noticeable that in human colonic epithelial cells the degradation of I κ B α in response to TNF- α is delayed until 90 min. This was not the case when these cells were induced by *C. jejuni* BCE. This difference was also manifest in the DNA-binding assay, in which NF- κ B-dependent gel retardation was not observed until 120 min after TNF- α treatment but was evident at 30 min after *C. jejuni* BCE treatment. These data are consistent with a previous report in which the kinetics of I κ B α degradation in human colonic epithelial cells have been found to be delayed and incomplete in comparison with other cell types in response to cytokines such as TNF- α (Jobin *et al.*, 1997). Differences in the timing of the response of colonic epithelial cells between *C. jejuni* BCE and TNF- α probably reflect operational changes in the TNF- α signal pathway of these cells, since the *C. jejuni* BCE response is similar to that found for HeLa 57A cells. It would seem likely that the bioactive components of *C. jejuni* BCE operate through an alternative to the TNF- α signal pathway leading to I κ B α degradation, and that this pathway is not subject to delay as observed for TNF- α -treated colonic epithelial cells.

In common with other gastric and enteric pathogens (*H. pylori*, *Salmonella typhimurium*, *Shigella flexneri*, *Y. enterocolitica* and enterovirulent *E. coli*), *C. jejuni* can activate NF- κ B. What the presentation of these pathogens to epithelial cells will have in common are elements of the surface carbohydrates, lipopolysaccharide (LPS) or lipo-oligosaccharide (LOS) linked to lipid A. LPS is a potent stimulator of NF- κ B in endothelial cells, but epithelial cells are largely insensitive to LPS (Pugin *et al.*, 1993). Similarly, we have found that *C. jejuni* LOS preparations do not activate NF- κ B (our unpublished data). In the interests of maintaining the homeostasis of intestinal epithelial cells in an environment awash with

microbial flora, the threshold concentration at which the primary recognition of LPS/LOS might occur through a Toll-like receptor would have to be set high. It is therefore possible that at high concentrations of LPS, as experienced with invasive or intimately adhered bacteria, LPS recognition through an internal receptor may have a role to play. This possibility has been argued at least for *Shigella flexneri* on the basis that the microinjection of normally non-stimulatory LPS will activate NF- κ B, and that LPS immunodepletion of bacteria-free supernatants will reduce their ability to activate NF- κ B (Philpott *et al.*, 2000). The need to discriminate pathogens and their products from non-pathogens is exemplified by *E. coli*, which is normally a commensal in the human gut but is distinguishable by intestinal epithelial cells from enteropathogenic *E. coli* (Savkovic *et al.*, 1997). It is becoming evident that the mechanisms by which intestinal epithelial cells activate NF- κ B in response to these pathogens are multifaceted. For example, the activation NF- κ B to trigger IL-8 production in *Y. enterocolitica* has been correlated with the specific internalization of the carboxy-terminal region of the protein invasins (Schulte *et al.*, 2000). Invasion of *Y. enterocolitica* is dependent on the surface interaction of invasins with host β 1-integrins. By comparison, most strains of *C. jejuni* are capable of adhesion to host cells, but not all are observed to invade. Several cell-surface-associated proteins from *C. jejuni* have been reported to act as adhesins, including the major cell-binding factor (Fauchère *et al.*, 1989; Pei & Blaser, 1993), which is also found to be a major antigenic component (Kervella *et al.*, 1993) and has been identified as a member of the ABC transporter family (Pei *et al.*, 1998). Disruption of the corresponding gene reduced, but did not abolish, epithelial cell adhesion, implying the presence of alternative adhesins. Similarly, disruption of the gene for the fibronectin-binding protein CadF reduced, but did not abolish, adhesion (Konkel *et al.*, 1997). Two proteins have been reported to bind the plasma membranes of epithelial cells, i.e. a 59 kDa outer-membrane protein and the 43 kDa major outer-membrane protein (Moser *et al.*, 1997; Schroder & Moser, 1997). Finally, a surface-exposed lipoprotein, JlpA, has also been identified as functioning as an adhesion factor for *C. jejuni* (Jin *et al.*, 2001). All these various surface adhesion proteins are, at face value, candidates for the minor, but direct, proteinase-K-sensitive NF- κ B stimulatory activity we find in *C. jejuni* BCE. However, the overall contribution of adhesin proteins to the stimulation of NF- κ B by live *C. jejuni* may be more important, as they may be the basis for intimate cell binding that enables non-protein bacterial surface components to cross the host cell membrane. The nature of the non-protein low-molecular-mass molecules that constitute the bulk of the NF- κ B stimulatory activity is not clear at this time. What can be said is that the low molecular mass of the active fraction would preclude intact versions of the obvious surface carbohydrates present in *C. jejuni*, i.e. the LOS and capsular polysaccharide (Karlyshev and Wren, 2001), although it is implicit that fragments of these molecules

might serve to stimulate NF- κ B, whereas the complete molecules may not.

The activation of NF- κ B shows a positive correlation with several intestinal inflammatory diseases (Crohn's disease, ulcerative colitis, self-limited colitis and inflammatory bowel disease); moreover, the degree of activation can be correlated with the severity of the mucosal inflammation (Barnes & Karin, 1997; Jobin & Sartor, 2000). Several anti-inflammatory drugs used in the treatment of inflammatory bowel disease act directly or indirectly to suppress NF- κ B activation (Jobin *et al.*, 1996, 1999; Ardite *et al.*, 1998; Egan *et al.*, 1999). It is therefore likely that inappropriate activation of NF- κ B in gut tissues will lead to bouts of acute inflammation, and it is possible that repeated gratuitous activation could lead to chronic intestinal inflammatory conditions. The heat-dissociated components we find in *C. jejuni* BCE are likely to provoke such a response. The level of *Campylobacter* contamination entering the human food chain is high: this is evident from the fact that *C. jejuni* has, in recent times, become the most common form of bacterial food poisoning in the developed countries, but its impact may even be greater. Domestic poultry can carry up to 1000 million campylobacters in the gut, and is not abnormal for carcasses produced for retail to harbour up to 1 million campylobacters (Saleha *et al.*, 1998). Governments and retailers have, quite correctly, stressed the importance of cooking to prevent *Campylobacter* food poisoning, but the question arises as to whether, in so doing, we are in fact facilitating the extraction of potent, heat-stable, NF- κ B-activating components from what could be substantial *Campylobacter* populations.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Mrs W. Fielder.

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Received 1 March 2002; revised 1 May 2002; accepted 15 May 2002.