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Campylobacter-induced interleukin-8 responses in human intestinal epithelial cells and primary intestinal chick cells

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Abstract

Campylobacter (C.) jejuni and C. coli can cause gastrointestinal disorders in humans characterized by acute inflammation. Inflammatory signals are initiated during interaction between these pathogens and human intestinal cells, but nothing is known about the stimulation of avian intestinal cells by Campylobacter. Interleukin-8 (IL-8) as a proinflammatory chemokine plays an important role in mobilizing cellular defence mechanism. IL-8 mRNA expression in both human intestinal cells (INT 407) and primary intestinal chick cells (PIC) was determined by quantitative real-time RT-PCR. The secretion of IL-8 protein by INT407 was measured using ELISA. Although C. jejuni and C. coli are considered to be harmless commensals in the gut of birds, the avian Campylobacter isolates investigated were able to induce the proinflammatory IL-8 in PIC as well as in INT407. In an in vitro system, C. jejuni as well as C. coli were able to induce IL-8 mRNA in PIC. Relation between the virulence properties like toxin production, the ability to invade and to survive in Caco-2 cells and the level of IL-8 mRNA produced by INT 407 and PIC after infection with Campylobacter strains was also investigated.

Key words: Campylobacter jejuni; Campylobacter coli; Interleukin-8; Human intestinal epithelial cells; Primary intestinal chick cells; Real-time quantitative PCR
1. Introduction

Campylobacter (C.) jejuni and its close relative C. coli are important human pathogens. They can cause diseases such as gastroenteritis characterized by severe inflammation of the intestinal mucosa with an influx of professional phagocytes (Ketley, 1997; Altekruse et al., 1999; Jones et al., 2003). Campylobacteriosis is described as a multifactorial process involving the intake of the Campylobacter strains in the gastrointestinal tract, followed by adherence to intestinal epithelial cells, secretion of virulence proteins and cell invasion (Raphael et al., 2005).

Epithelial cells are able to secrete chemotactic mediators after contact with pathogenic bacteria as described for Salmonella typhimurium, Helicobacter pylori and others (Thorpe et al., 1999; Aubert et al., 2000; Gewirtz et al., 2000; Bäckhed et al., 2003). They deliver the initial signals for the immune response of the host (Eckmann et al., 1995). Chemotactic mediators belonging to the family of C-X-C chemokines, such as interleukin 8 (IL-8), play a major role in mobilizing cellular defence mechanisms to eliminate bacteria by recruiting and activating neutrophils and T cells (Kagnoff et al., 1997). It has been shown on a range of human-derived epithelial cell lines that C. jejuni is able to induce proinflammatory chemokine release, especially IL-8 (Hickey et al., 2000; Mellitis et al., 2002; Bakhiet et al., 2004; Watson et al., 2005). IL-8 as a potent stimulator of neutrophil activation and chemotaxis within the intestinal mucosa is associated with numerous acute and chronic inflammatory reactions (Sturm et al., 2005).

It is undisputed that one of the main risk factors for human diseases is eating or handling of poultry meat contaminated by Campylobacter strains. Many reports describe that Campylobacter strains vary in their ability to resist environmental stressors during poultry processing (Alter et al. 2005; Newell et al. 2001). The relative proportion of Campylobacter subtypes changes during this processing, hence the surviving subtypes can reach the food chain. Normally, C. jejuni and C. coli colonize the gastrointestinal tract of many birds including chicken and turkeys and other animals as harmless commensals with little or no pathology (Newell et al., 2003; Hendrixson et al., 2004). Some strains, however, were
described to be invasive and/or toxigenic and may cause distension of the intestine, liver abnormalities and diarrhoea in chicken (Saleha et al., 1998). Nothing is known about the stimulation of avian intestinal cells by Campylobacter. Recently, the expression of proinflammatory cytokines in response to Campylobacter infection in avian primary chick kidney cells and in an avian macrophage cell line has been reported (Smith et al., 2005). However, the effect of Campylobacter strains on intestinal chick cells has not been reported until now.

The aim of our work was to investigate whether Campylobacter isolates from the end of poultry processing and characterized by specific virulence properties are able to induce IL-8 in primary intestinal chick cells (PIC cells) following in vitro incubation. The reactions of the chick cells were compared with responses of human intestinal epithelial cells (INT407) after stimulation with these strains.

2. Material and methods

2.1. Bacteria strains

Six C. jejuni and two C. coli isolates (Table 1) from turkey carcasses at the end of the slaughter process were kindly provided by T. Alter (Institute of Food Hygiene, University of Leipzig, Germany). The isolates were routinely grown on Mueller-Hinton (MH) agar plates (Institut für Immunpräparate und Nährmedien, Germany) for 24 h at 37 °C under microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂). The bacteria were harvested in phosphate buffered saline (PBS), pH 7.2 and adjusted to an optical density of 0.45 at 588 nm (Photometer CADAS 30, Lange GmbH, Germany) corresponding to about 5 x 10⁸ bacteria per ml. Before the Campylobacter challenge, the bacterial suspensions were diluted 1:10 with cell culture medium containing 1% foetal calf serum (FCS, Gibco, Germany). The number of bacteria was determined by plate counting.

The putative virulence properties like survival in Caco-2 cells over 48 h as well as production of cytolethal distending toxin (CDT) and cytolethal rounding toxin (CRT) have been reported for these strains (Tab. 1, Hänel et al., 2007).
The C. jejuni strain 81-176 from human origin and its CDT-deficient mutant were kindly provided by C.L. Picket, University of Kentucky.

2.2. Cells

2.2.1. Human intestinal cell line INT407

Human intestinal epithelial cells (INT407; ECACC No.: 85051004) were maintained in minimum essential medium (MEM, Sigma-Aldrich, Germany) with non-essential amino acids (NEAA, Sigma-Aldrich) supplemented with 10% FCS and 2 mM L-glutamine.

2.2.2. Primary intestinal chick cells (PIC)

Specific pathogen free (SPF) chickens (White Leghorn) were hatched at the facilities of the institute from eggs received from Charles River Deutschland GmbH (Extertal, Germany). The isolation of the intestinal cells from these chickens was performed as described by Athmann et al. (2002). Briefly, the intestines from one-day-old chicks were removed and put in PBS with gentamicin (150 µg/ml) and amphotericin B (25 µg/ml). The intestines were slit open and cut into small fragments which were intensively washed with Hanks balanced salt solution, pH 7.4 (HBSS, Sigma-Aldrich) and then sliced into smaller pieces. These pieces were digested for 4 h at 37 °C using 300 Units/ml collagenase (Sigma-Aldrich) in HBSS. Afterwards, the cell suspension was pipetted vigorously and left to sediment under gravity for several times. Finally, the supernatant was transferred in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 2.5% FCS and 2% sorbitol (Sigma-Aldrich). After centrifugation at 100 x g for 3 min the cell pellet was resuspended in MEM with NEAA containing 2 mM L-glutamine, 5% FCS, 5% chick serum (Sigma-Aldrich), 0.5% chick embryo extract (MP Biomedicals, Germany) and 120 mg/ml sodium pyruvate. Cells were counted with a haemocytometer and viability was assessed by trypan blue exclusion. Cells (6 x 10^4 cells/cm²) were seeded in culture flasks coated with collagen type II (Sigma-Aldrich). The cell morphology was assessed by light microscopy.
2.2.3. Immunohistochemistry

For immunohistochemical investigations, cells (INT407, PIC) grown in chamber slides (Becton Dickinson, United States) were prepared as described previously (Berndt et al. 2001). Briefly, monolayers were fixed with acetone and subsequently incubated with a chick cross-reactive monoclonal mouse anti human cytokeratin type II antibody (clone: MCA888H; Serotec, Germany), secondary goat-anti mouse immunoglobulin (Sigma-Aldrich) and peroxidase-anti peroxidase complex (Sigma-Aldrich). The enzyme-linked antibody was visualised by reaction with 3,3'-diaminobenzidine (Merck, Germany) and hydrogen peroxide. As a negative control, slides were incubated with normal mouse serum instead of the primary monoclonal antibody. The cells were counterstained with haematoxylin and mounted with Canada balsam (Riedel de Haen, Germany). The analysis of cell staining was performed by light microscopy. The percentage of cytokeratin positively stained cells was calculated by counting positive and negative stained cells of at least 100 cells per well.

2.3. Campylobacter challenge

Both cell types (INT407 and PIC) were grown to a confluent monolayer in cell culture flasks, washed and released using trypsin-EDTA (Sigma-Aldrich). The cells were resuspended in the respective cell culture medium and seeded at 0.5 ml per well in 24-well tissue culture plates. For PIC cells plates were coated with collagen type II. The cell numbers were 2 x 10^5 cells per well for INT407 and 3 x 10^5 cells per well for PIC cells. The cell monolayer was allowed to reform during 24 h incubation in a humidified atmosphere at 37 °C and 5% CO₂. After washing with PBS, the bacterial suspensions were added to the cells at a MOI of about 1:100. Cell culture media and phorbol myristate acetate (PMA, 10 ng/ml, Sigma-Aldrich) were used as negative and positive controls, respectively. The 24-well plates were incubated for 3 h at 37 °C and 5% CO₂ and afterwards the supernatants were removed. Cells were washed two times with PBS and incubated in fresh cell culture medium with 1% FCS. Finally, the supernatants were harvested after total incubation times of 4 h (3 h incubation of the cells with bacteria and 1 h incubation with cell culture medium), 8 h (3 h+5 h) and 24 h (3 h+21 h),...
filtered through 0.22-µm pore-size syringe filters and stored at -80 °C until analyzed for cytokine. The cell pellets were harvested by addition of trypsin-EDTA. After resuspension in cell culture medium, centrifugation at 300 x g for 5 min and washing two times with PBS, the cells were stored at -80 °C until RNA isolation.

In preliminary tests the ability of PIC to produce IL-8 mRNA was checked using lipopolysaccharide (LPS) from E. coli 026:B6 (Sigma-Aldrich) in the concentration range from 0.1 µg/ml to 100 µg/ml. The isolated RNA from cell pellets was used as positive control in further investigations. Since INT407 did not react to LPS up to a concentration of 400 µg/ml we used PMA as positive control for the production of IL-8 mRNA.

2.4. RNA extraction and cDNA production

Total cell RNA was extracted using the RNeasy mini kit (Qiagen, Germany) according to the manufacturer’s instructions. The method was slightly modified by inclusion of a DNA digestion step with RNase-Free DNase (Qiagen, Germany). The quantity and purity of the RNA samples were determined spectrophotometrically by measuring the absorbance at 260 nm and the 260/280 nm absorbance ratio (Biophotometer, Eppendorf, Germany). The isolated RNA was stored immediately at -80 °C. Before starting the reverse transcription (RT), the isolated RNA was adjusted to 0.05 µg/µl and finally denatured for 5 min at 65 °C.

RT was performed in 20 µl final volume with Omniscript® reverse transcriptase (Qiagen, Germany) in the presence of RNase inhibitor (Promega, Germany) using random hexamer primer pd(N)₆ (Roche Diagnostics, Germany).

The cDNA was analyzed immediately or stored at -20 °C until use.

2.5. Qualitative PCR

The qualitative PCR was performed in a 50 µl final volume containing 25 µl Hot Star Taq master mix (Qiagen, Germany) with 2.5 U Hot Star Taq Polymerase (Qiagen), 200 mM of each dNTP, 0.45 µM Primer and cDNA equivalent to 15 ng starting RNA. Primers used for human IL-8 and for chick IL-8 were described in Table 2. The chicken primer pair was
designed using the Primers express software (Applied Biosystems, Germany). To avoid amplification of cellular DNA of each primer pair, at least one primer spans an intron-exon boundary. Additionally, the primers designed were checked by BLAST search (www.ncbi.nlm.nih.gov) Amplification was performed in a Thermocycler (BIOMETRA, Germany) under conditions as described by Sharma et al. (1995) for human IL-8: 30 cycles, each including denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. For chick IL-8, 35 cycles were used, each including denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min. PCR products were analyzed by electrophoresis on ethidium bromide stained 2% agarose gels (Peqlab, Germany) and visualized by UV-light.

2.6. Quantitative real-time PCR

Quantification of human and chicken IL-8 mRNA expression was performed by the Taqman real time PCR system (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Germany) in a 96-well microtiter format using SYBR green PCR Mastermix according to the instructions of the manufacturer (Quiagen). cDNA’s prepared as described above (2.4.) were used as targets for the quantitative PCR. Description of primers for human IL-8 and chick IL-8 as well as primers used for the endogenous control genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and chicken beta-actin are given in Table 2. All primers used were synthesized by Jena Bioscience (Jena, Germany).

IL-8 and the respective endogenous control genes GAPDH or beta-actin were amplified in separate wells of 96-well plates. The PCR reactions contained either 300 nM of each primer for human IL-8 and human GAPDH or 450 nM for chick IL-8 and chick beta-actin and commercially available PCR Mastermix (QuantiTect™ SYBR green PCR, Qiagen, Germany) which includes SYBR Green I as a fluorescent reporter and Rox as internal reference for normalization of the fluorescence signal. 7.5 ng cDNA template was analyzed per reaction. In every run, three no-template controls (DEPC-water) were included. Amplification conditions were 2 min at 50 °C, 15 min at 95 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at 60 °C.
and 30 s at 72 °C. For the chick reactions 40 cycles were carried out and the annealing
temperature was 61 °C. Subsequently, melting curve analysis was performed to measure the
specificity of amplification. Final quantification was done using the comparative Ct-method
and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA,
here non-infected cells (Leutenegger et al., 2000; Sarkar et al., 2004). The threshold cycle
number (Ct) of triplicate reactions was calculated using the ABI Prism software 1.23 (relative
quantification). The levels of IL-8 expression were normalized to GAPDH and beta-actin
respectively using the formula $2^{\Delta\Delta Ct}$ in which $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$ with $\Delta Ct$
as difference between $Ct$ of target gene (IL-8) and $Ct$ of housekeeping gene (GAPDH or beta-
actin). For the comparative Ct-method the amplification efficiencies of the target and the
endogenous control were estimated. For this, dilution steps of cDNA preparations in triplicate
were amplified to obtain standard curves and the slopes of curves for IL-8 and the
housekeeping gene were compared. An amount of 0.0075 ng cDNA was detectable for both
human and chick IL-8.

2.7. Determination of human IL-8 protein by ELISA

The determination of IL-8 protein in cell supernatants was performed using a commercially
available ELISA following the manufacturer’s instructions (Biosource, CA, USA). Briefly, the
supernatants and the standard in serial dilution were pipetted in wells of anti-human-IL-8
monoclonal antibody coated plates and a biotinylated antibody was immediately added to all
wells. After incubation of 2 h and intensive washing steps streptavidin-horseradish
peroxidase conjugate was added. Tetramethyl benzidine served as substrate reagent. The
absorbance was measured at 450 nm using an ELISA reader (Tecan, Germany) and the
amount of IL-8 protein was calculated in comparison to the standard by the reader software.
The results from at least three independent tests are presented as means ± standard
deviation (SD). Statistical analyses of the comparison between control and samples as well
as between C. coli and C. jejuni were performed by the Kruskal-Wallis- and Mann-Whitney-
test. A P value of < 0.05 was considered statistically significant.
3. Results

3.1. Comparison of the growth of PIC and INT407

The cultured PIC grew in a fibroblast-like manner characterized by their elongated and spindle shape. Compared to the INT407, the PIC showed a lower tendency to confluent growth. The PIC could be cultured without loss of viability or morphological organization up to 10 passages.

The structure of the cells was determined using a chick cross-reactive monoclonal mouse anti human cytokeratin type II antibody and compared with the human epithelial cells of INT407 (Fig. 1). About 90% of PIC-cells and about 70% of INT 407 cells were positively stained for cytokeratin. Light microscopy of PIC cells revealed a moderate staining intensity and a characteristic cytoplasmic network of cytokeratin fibres indicating epithelial properties of the primary cells. The reason for the moderate staining intensity could be the use of a chick cross-reactive anti human antibody. In comparison, the cytokeratin staining of INT407 cells was more intensive and showed a more diffuse character. The primary cells can be considered as a culture with epithelial properties.

3.2. TaqMan amplification efficiencies

The $\Delta\Delta C_t$ method for relative quantification requires that efficiencies of target and endogenous control amplified in different wells are approximately equal. Therefore, 10-fold dilution steps of human and chicken IL-8 cDNA were amplified in triplicate. The standard curves calculated run approximately parallel and the differences of the slopes between curves obtained from GAPDH and human IL-8 was 0.04 and for beta-actin and chick IL-8 was 0.05.

3.3. IL-8 mRNA expression in PIC and INT407 after infection with Campylobacter isolates and PMA

The ability of PIC cells to produce IL-8 mRNA was checked using lipopolysaccharide (LPS) from Escherichia coli 026:B6 in different concentrations. Since the expression of IL-8 mRNA could be
induced by LPS, it was possible to compare the effects of avian Campylobacter isolates on human and chick intestinal cells with regard to IL-8 mRNA expression.

All Campylobacter isolates tested were able to induce IL-8 mRNA in PIC as well as in INT 407. Representative results of IL-8 mRNA expression after 8 h total incubation time are shown in Fig. 2. Campylobacter strains adhered and invaded both human INT 407 and PIC to the same extent after 3-hours-postinfection (data not shown). Both cell types produced significantly higher IL-8 mRNA levels in comparison to non-infected cells at a total incubation time of 4 h (Fig. 3 and 4). The C. jejuni av 322 B induced the highest level of mRNA from all C. jejuni isolates investigated in INT407 at 8 h and in PIC at 4 h. In PIC a time-independent production for all isolates apart from av 322 B was determined. Six from eight isolates produced the highest amount of IL-8 mRNA after 4 h. Marked differences between C. jejuni and C. coli in the time-dependent course and the amount of IL-8 mRNA were not determined. In INT407, however, a time-dependent production of IL-8 mRNA was detected for both C. coli strains (av 352 and av 321 A). The infection of INT407 with these strains resulted in an increase in the amount of IL-8 mRNA from 4 h to 24 h, while the C. jejuni strains caused only a slight increase with highest levels at 4 h or 8 h. PMA (10 ng/ml) as mitogen induced a 55-fold increase of IL-8 mRNA in INT407 after a 4-hour incubation time, a 78-fold increase after 8 h and a 6.3-fold increase after 24 h. In PIC, however, significantly higher amounts of IL-8 mRNA were not measured after stimulation with PMA.

3.4. Detection of IL-8 protein in cell culture supernatants of INT407

In order to characterize the level of effective IL-8 production, cell supernatants were analyzed using ELISA. Secretion of IL-8 protein could be detected only in samples taken at 24 h (Table 3). In samples collected at 4 h and 8 h, IL-8 was not detectable with the ELISA assay used. At 24 h, all strains produced significantly higher amounts of IL-8 than the cell control (P < 0.05). Significant differences between C. coli and C. jejuni were not found (P = 0.2) but the C. coli strain av 352 produced the highest amount of IL-8 protein. Both human strains
produced amounts of IL-8 protein comparable with them of chicken strains, in which the
CDT-deficient mutant produced significant less IL-8 protein than the strain 81-176 (Tab. 3).
The production of IL-8 protein in PIC cells could not be determined because a specific
chicken ELISA was not available.

4. Discussion
Campylobacteriosis is a multifactorial process characterized by gastrointestinal symptoms
such as diarrhoea, cramping, abdominal pain, and fever. A hallmark of this human disorder
cau sed by C. jejuni or C. coli is the inflammation of the intestinal mucosa. These pathogens
are able to initiate inflammatory signals by interaction (adhesion and/or invasion) with host
cells and activate signalling pathways that induce the production of inflammatory cytokines
and recruitment of phagocytes, especially neutrophils, into infected tissue (Jones et al. 2003;
Everest, 2005). The function of chemotactic cytokines (chemokines) such as IL-8 produced
by human cells after contact with pathogens is described as crucial for acute inflammatory
diseases. IL-8 and other proinflammatory mediators are important in initiating the host
mucosal inflammatory response, which is critical for both the induction of diarrhoea and the
clearance of infection (Watson et al. 2005).

However, in the vast majority of animals colonised with C. jejuni or C. coli intestinal
inflammation or diseases are absent. Little is known about the interaction between avian
intestinal cells and Campylobacter strains. The biological role of avian IL-8 has not been
defined clearly yet (Smith et al., 2005). Any differences in innate responses to this pathogen
between the human and avian hosts should lead to a greater understanding of the disease
process in humans. Therefore, we used the human intestinal cell line INT 407 and cells
isolated from the avian intestine (PIC) to study the IL-8 mRNA production after infection of
the cells with different Campylobacter strains. The primary intestinal cell type (PIC) from one-
day-old chicks prepared for our investigations reacted to LPS already at a concentration of
0.1 µg/ml with the production of IL-8 mRNA and showed epithelial properties. On this reason,
these cells were assumed to be a suitable model to check the interaction between avian isolates and avian intestinal cells.

The results of the study clearly show that in vitro C. jejuni as well as C. coli were able to induce IL-8 mRNA in avian intestinal cells to a level comparable to human intestinal cells. The strains investigated were characterized by the putative virulence properties toxin production and survival in Caco-2 cells (Table 1, Hänel et al. 2007).

PIC cell type reacted in a similar manner like the human intestinal cell type although some differences between the reactions of both cell types following infection with Campylobacter strains, PMA and LPS were detected. PMA as mitogen induced a high level of IL-8 mRNA in INT407, whereas PIC did not react to PMA. The reason for this is unclear, because the interaction between PMA and PIC has not yet been studied in detail. Otherwise, INT407 did not react to LPS up to a concentration of 400 µg/ml. Investigation by flow cytometry using the monoclonal antibody anti-human CD 14 showed that CD 14 was not detectable on INT407. The stimulation with C. jejuni strains of both PIC and INT407 resulted in an increase in the IL-8 mRNA level at 4h, which, in general, began to decrease after this time. The two C. coli strains, however, showed another time course of IL-8 induction in human intestinal cells than the C. jejuni strains. For these strains mRNA level increased up to 24 h, but this effect could not be confirmed in PIC.

In both cell types, PIC and INT407, the strain av 322 B induced the highest level of mRNA from all C. jejuni isolates investigated. This strain is characterized by several putative virulence properties like production of CDT, the ability to invade and to survive in Caco-2 cells. Bakhiet et al. (2004) showed that the induction of chemokines by intestinal cells is most probably mediated by the action of CDT. However, Hickey et al. (1999, 2000) suggested that two independent mechanisms, one of which requires adherence and/or invasion and the second of which requires CDT production, are necessary for the IL-8 release from INT407 cells. This thesis is supported by the high IL-8 mRNA response to strain av 322 B producing CDT and surviving in Caco-2 cells. Otherwise, the C. jejuni strains which invade Caco-2 cells but do not have the ability to survive in cells induced amounts of mRNA in INT407 as well as
in PIC cells which were only slightly higher than the values of non-infected cells (Tab.1, Fig.3 and Fig. 4). The ability of both C. coli strains to survive in Caco-2 cells and possibly in INT 407 could explain the strong IL-8 mRNA induction in human intestinal cells up to 24 h. PIC cell types could react more sensitive to CDT/CRT. C. jejuni strains, which produced toxin, induced higher mRNA amounts in PIC than in INT407, but both C. coli strains without toxin production induced lower IL-8 mRNA levels in PIC than in INT 407.

After challenge of INT 407 as well as PIC with the human strains, the levels of IL-8 mRNA were comparable with those obtained with the chicken strains. Any differences between the level of IL-8 mRNA of strain 81-176 and its mutant was not determined on both cells at 4h. The amount of IL-8 mRNA induced by strain 81-176, however, was significantly higher than the amount of the CDT-deficient mutant in PIC at 8h. Additionally, the CDT-deficient mutant produced significant less IL-8 protein than the strain 81-176 (Tab. 3). This result agreed with the results of Hickey (1999, 2000).

In addition to the quantitative determination of mRNA induction, the secretion of IL-8 protein by INT407 was measured in order to characterize the level of effective IL-8 production after 24 h. Our results of human IL-8 protein measured by ELISA are comparable to published values (Hickey et al., 2000). Since the expression of mRNA does not necessarily result in translation to protein, it would be of interest to determine the amounts of IL-8 protein secreted by PIC. Differences in the ability to produce biologically active IL-8 may dictate the outcome of the balance between colonization and disease. Unfortunately methods for the detection of chicken IL-8 protein are not available at present.

To sum up, this work presents the first results of the comparison between the effects of avian and human Campylobacter strains on intestinal primary chick cells and a human intestinal cell line. It could be shown that the primary intestinal cell type isolated from one-day-old chicks were able to express IL-8 mRNA after stimulation with avian Campylobacter strains. Our data confirm the assumption that Campylobacter strains can stimulate the avian host in a proinflammatory manner. The lack of inflammatory symptoms in chicks could be possibly attributed either to the protective role of anti-Campylobacter maternal antibodies (Sahin et al.
or to the induction of an exclusively local response (Smith et al., 2005). The putative virulence properties of the strains could influence the strength of the interaction between Campylobacter and the chick immune system.

5. Acknowledgements

We thank C. Muselmänn, S. Drexler and A. Hinsching for the excellent technical assistance. Furthermore, we would like to thank T. Alter for providing the Campylobacter strains and F. Schulze for helpful discussions.
6. References


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7. Legends of Figures

Figure 1: Immunohistochemical staining of INT407 (A) and PIC (B) cells for cytokeratin II (brown).

A typical pattern of keratin fiber staining can be seen in the cytoplasm of PIC cells. INT407 cells show a more diffuse staining pattern.

Figure 2: A representative agarose gel visualized by ethidium bromide. The gel shows the expression of human- IL-8 (upper band) and the control (GAPDH) genes (lower band) in INT407 and the expression of chick IL-8 (lower band) and the control (beta-actin) genes (upper band) in PIC cells after incubation with C. coli av 352 at 8 h total incubation time as determined by gene-specific co-amplification of the genes by RT-PCR.

Figure 3: Estimation of human IL-8 mRNA induction in INT407 by Campylobacter isolates. The IL-8 mRNA production was determined by quantitative real-time RT-PCR. The IL-8 mRNA expression as shown in histogram was calculated as fold change relative to non-infected cells (cell control) and normalized against GAPDH (control) expression.

All data are from three independent experiments and represent the averages and standard deviations between of the fold changes between experiments.

Figure 4: Estimation of chick IL-8 mRNA induction in PIC cells by Campylobacter isolates. The IL-8 mRNA production was determined by quantitative real-time RT-PCR. The IL-8 mRNA expression as shown in histogram was calculated as fold change relative to non-infected cells (cell control) and normalized against beta-actin (control) expression.

All data are from three independent experiments and represent the averages and standard deviations between of the fold changes between experiments.
8. Legends of Tables

Table 1: Characterization of Campylobacter isolates

Table 2: Primer sequences for qualitative PCR and real-time RT-PCR

Table 3: Production of IL-8 protein in INT407 detected by ELISA after 24 h

All data shown are presented as mean IL-8 amounts [pg/ml] ± standard deviation (SD) from at least three independent tests.
Table 1. Characterization of *Campylobacter* isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>Reference Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival in Caco-2 cells after 48 h</th>
<th>Toxin titer&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>av 245</td>
<td>+</td>
<td>CDT 1:16</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>av 322 B</td>
<td>+</td>
<td>CDT 1:16</td>
</tr>
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<td><em>C. jejuni</em></td>
<td>av 67/3</td>
<td>-</td>
<td>CDT 1:8</td>
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<tr>
<td><em>C. jejuni</em></td>
<td>av 356</td>
<td>-</td>
<td>CRT 1:64</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>av 347</td>
<td>-</td>
<td>CRT 1:32</td>
</tr>
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<td>av 64/3</td>
<td>-</td>
<td>CDT 1:8</td>
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<tr>
<td><em>C. coli</em></td>
<td>av 352</td>
<td>+</td>
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</tr>
<tr>
<td><em>C. coli</em></td>
<td>av 321 A</td>
<td>+</td>
<td>none</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>81-176</td>
<td>n.d.</td>
<td>CDT 1:64</td>
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<tr>
<td><em>C. jejuni</em></td>
<td>81-176 cdt mutant</td>
<td>n.d.</td>
<td>none</td>
</tr>
</tbody>
</table>

<sup>a</sup> Abbreviation: (Alter et al. 2005)

<sup>b</sup> (Hänel et al. 2007)

CDT = Cytolethal distending toxin

CRT = Cytolethal rounding toxin

n.d. = not done
<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Length</th>
<th>Accession No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8, human</td>
<td>Forward</td>
<td>4</td>
<td>NM00058</td>
<td>Sarkar et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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<td></td>
<td></td>
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<tr>
<td>GAPDH, human</td>
<td>Forward</td>
<td>76</td>
<td>BC02934</td>
<td>Brink et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8, chicken</td>
<td>Forward</td>
<td>281</td>
<td>AJ009800</td>
<td>Berndt et al. 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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<td></td>
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</tr>
<tr>
<td>Beta-actin, chicken</td>
<td>Forward</td>
<td>354</td>
<td>X00182</td>
<td>Berndt et al. 2007</td>
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</tbody>
</table>
Table 3. Production of IL-8 protein in INT 407 detected by ELISA after 24 h

<table>
<thead>
<tr>
<th>Isolates</th>
<th>IL-8 [pg/ml]</th>
<th>Standard deviation [SD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell control</td>
<td>91.5</td>
<td>37.8</td>
</tr>
<tr>
<td>av 245</td>
<td>287.5*</td>
<td>16.1</td>
</tr>
<tr>
<td>av 322 B</td>
<td>219.4*</td>
<td>25.1</td>
</tr>
<tr>
<td>av 67/3</td>
<td>226.0*</td>
<td>35.6</td>
</tr>
<tr>
<td>av 356</td>
<td>269.0*</td>
<td>30.0</td>
</tr>
<tr>
<td>av 347</td>
<td>205.3*</td>
<td>52.4</td>
</tr>
<tr>
<td>av 64/3</td>
<td>263.3*</td>
<td>26.3</td>
</tr>
<tr>
<td>av 352</td>
<td>309.7*</td>
<td>41.8</td>
</tr>
<tr>
<td>av 321 A</td>
<td>223.0*</td>
<td>25.0</td>
</tr>
<tr>
<td>81-176 (WS)</td>
<td>272.8*</td>
<td>20.3</td>
</tr>
<tr>
<td>81-176 (Mu)</td>
<td>241.6*</td>
<td>12.5</td>
</tr>
</tbody>
</table>

*) significant differences between IL-8 protein values of cell control and cells infected with Campylobacter strains (P < 0.05)
Figure 1

INT 407

PIC
Figure 2

- Human IL-8 (289 bp)
- GAPDH (76 bp)
- Beta-actin (354 bp)
- Chick IL-8 (281 bp)

St, INT 407, PIC, St - Standard
Figure 3

INT 407

Fold change in IL-8 mRNA

- cell control
- Av 245
- Av 322B
- Av 67/73
- Av 356
- Av 347
- Av 64/3
- Av 352
- Av 321A
- 81-176 (VS)
- 81-176 (Ma)

C. jejuni
C. coli
C. jejuni (human)

- 4h
- 8h
- 24h